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Understanding the Antimicrobial Mechanism for Applications of Polyaniline and Functionalised Polyanilines

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Medicine, the University of Auckland, 2016.
Abstract

The discovery that the conducting polymer polyaniline (PANI) and its functionalised derivative poly(3-aminobenzoic acid), P3ABA, are antimicrobial has presented new possibilities for their application. An understanding of the antimicrobial mechanism will support the pursuit of these new applications. The mode of action of PANI and P3ABA was first investigated using *E. coli* single gene deletion mutants. This investigation was based on the idea that bacteria carrying mutations in genes encoding antimicrobial targets will have reduced sensitivity to treatment, while the opposite occurs for bacteria carrying mutations in genes encoding antimicrobial stress-response mediators. Following this, the involvement of reactive oxygen species, and targeting of metabolic and respiratory machinery, in the antimicrobial action of PANI and P3ABA were explored by determining activity against target bacteria in aerobic and anaerobic conditions, and in rich and minimal media.

The antimicrobial mechanism of polyaniline is hypothesised to involve production of hydrogen peroxide and dysregulation of iron homeostasis, a notion that is supported by the supersensitivity of a hydrogen peroxide scavenger mutant to PANI treatment; the insensitivity of an iron import mutant to PANI treatment; and protection against PANI killing of *E. coli* and *S. aureus* in anaerobic conditions. The antimicrobial activity of P3ABA is different and is postulated to involve targeting of ATP synthase, uncoupling electron transport from ATP synthesis, resulting in a futile proton cycle and loss of proton motive force. The insensitivity of an ATP synthase deletion mutant to P3ABA treatment supported this hypothesis, along with the greater activity of P3ABA against *E. coli* in rich media (with increased respiration rates) compared to minimal media. P3ABA antimicrobial action is also hypothesised to involve perturbation of iron homeostasis, supported by the increased sensitivity of a deletion mutant missing a gene involved in regulation of iron levels, and acid stress, supported by the increased sensitivity of a deletion mutant missing an acid stress response mediator.

The protection of surfaces from microbial contamination as part of measures to reduce transmission of pathogens in hospitals and during food processing is one potential application. The incorporation of PANI and P3ABA into model absorbent and non-absorbent materials gave a profile whereby PANI was effective at protecting absorbent surfaces from *E. coli, S. aureus* and *M. smegmatis*, while non-absorbent surfaces offered no protection against these
organisms. P3ABA was able to reduce contamination of *E. coli*, *S. aureus*, *M. smegmatis* and *M. tuberculosis* on absorbent surfaces while non-absorbent surfaces containing P3ABA were effective against *E. coli*, *M. smegmatis* and *M. tuberculosis*. The bactericidal activity of PANI and P3ABA containing non-absorbent surfaces was most active against low bacterial loads and in the absence of organic matter.
Acknowledgments
Firstly, I would like to express my gratitude to my supervisor, Associate Professor Simon Swift, for the support, guidance and instruction during the research and writing of this thesis. I would also like to acknowledge my co-supervisor Dr Marija Gizdavic Nikolaidis for her insightful comments and for providing the conducting polymers used in this work. Special thanks go to Dr James Dalton, your advice, patience, input and considerable knowledge, enabled me to reach this point. I would also like to thank the Swift and Wiles groups for their encouragement and company over the last five years. Finally, I would especially like to acknowledge my friends and family for their unwavering support, encouragement and care, which helped me persevere in the face of setbacks and enabled completion of this thesis.

“There is no error in biology, only variation”
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Glossary

2ABAPANI  Poly(aniline-co-2-aminobenzoic acid)
2-way RM  Two-way repeated measures analysis of variation
ANOVA
3ABA  3-aminobenzoic acid
3ABAPANI  Poly(aniline-co-3-aminobenzoic acid)
ABA  Aminobenzoic acid
ADP  Adenosine diphosphate
AMPs  Antimicrobial peptides
APS  Ammonium persulfate
ATP  Adenosine triphosphate
BAC  Benzalkonium chloride
CFU/ml  Colony forming units per millilitre
CP  Conducting polymer
DHAP  Dihydroxyacetone phosphate
DMSO  Dimethyl sulfoxide
EPR  Electron paramagnetic resonance
ETC  Electron transport chain
FDA  Food and Drug Administration
fPANI  Functionalised polyaniline
GSH  Reduced glutathione
HCWs  Healthcare workers
HIV  Human immunodeficiency virus
HOCl  Hypochlorous acid
HPF  Hydroxyphenyl fluorescein
MBC  Minimum bactericidal concentration
MDR-TB  Multidrug resistant TB
MIC  Minimum inhibitory concentration
MRSA  Methicillin-resistant S. aureus
NaDCC  Sodium dichloroisocyanurate
-OCI  
Hypochlorite ion

OD<sub>600</sub>  
Optical density at 600nm

P3ABA  
Poly(3-aminobenzoic acid)

PMF  
Proton motive force

PSO<sub>3</sub>H  
Polysulfanilic acid

QAC  
Quaternary ammonium compound

qPCR  
Quantitative polymerase chain reaction

RH  
Relative humidity

ROS  
Reactive oxygen species

RPE  
Ribulose-5-phosphate 3-epimerase

SDH  
Succinate dehydrogenase

SEBS  
Styrene ethylene butylene styrene

TCA  
Tricarboxylic acid

VRE  
Vancomycin-resistant Enterococcus spp.

WHO  
World Health Organisation

XDR-TB  
Extensively drug-resistant TB
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of the thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

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**Certification by Co-Authors**

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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Chapter 1: Introduction

1.1 Antimicrobial surfaces are needed in many settings

Infectious disease caused by viruses, fungi, parasites and bacteria can be treated with antimicrobial agents. Microbial resistance to antimicrobial agents is increasing worldwide and represents a major threat to proper treatment of infectious disease resulting in increased morbidity, mortality and potentially treatment failure (1). Antimicrobial resistance is a burden to both the individual and society in terms of well-being and the significant healthcare costs associated with infectious disease. Development of antimicrobial resistance is an inescapable consequence of natural selection and is associated with exposure to antimicrobial agents (1). The paucity of novel antimicrobial agents being developed has also exacerbated the problem. The emergence and spread of antimicrobial resistant microorganisms needs to be curtailed to counteract this threat; this requires a multifaceted approach including the restriction of the use of antimicrobial agents; the development of vaccines and improved infection control to prevent the spread of infectious agents (1).

Antibiotic resistant bacteria are a prominent threat to public health in terms of infectious disease. Antibiotic resistance is widespread in bacteria that cause many common infections (associated with morbidity and mortality) in both hospitals and the community (1). The outcome of this resistance being bacterial infections that are recalcitrant to antibiotic treatments. Efforts need to be made to decrease the unnecessary exposure of bacteria to antibiotics to reduce the selective pressure driving the development of resistance so that existing antibiotics retain their efficacy for as long as possible (1). In part, this can be achieved by controlling the spread of pathogenic bacteria and therefore reducing the number of infections that require antibiotic treatment. Bacteria can spread to cause new infections by contact between an infected individual and another person or through surfaces that are contaminated with pathogenic bacteria (2). Utilisation of antibacterial surfaces is a promising means of preventing bacterial transmission through contaminated surfaces and so reducing the associated antibiotic use (2). It is probable that the same principle can be applied to fungi and viruses.
1.1.1 Bacterial contamination of surfaces

1.1.1.1 Bacteria can be transmitted through contact between people and surfaces

Bacteria can be transferred to surfaces where they can persist or actively colonise to form a biofilm. Contaminated surfaces are associated with the passive spread of bacteria to other surfaces or new hosts, which is mediated by physical contact between people or other organisms (Fig. 1.1)(3–6). The likelihood of transmission occurring is dependent on many factors such as the frequency a surface is touched, the length of time bacteria is present on a surface and dynamic environmental conditions including organic soiling, humidity and temperature(7). During contact, bacteria can move between a hand and a surface, the degree of which is dependent on the frequency of contact(5, 7). Surfaces in hospitals that come into contact with hands are regularly contaminated with nosocomial pathogens(4, 7). A single hand contact event has been shown to be sufficient to transmit *Escherichia coli, Staphylococcus aureus, Serratia rubidaea* and *Salmonella* spp. from a contaminated surface(3, 5, 8). Bacterial spread through a toilet facility has been modelled using the following contact pathway: a hand contaminated with *E. coli* contacts a toilet brush handle (1) which is then touched by a new hand (2) before contacting a door handle (3); the door handle is touched by another new hand (4), which contacts a tap (5); finally a third new hand (6) touches the tap(6). All objects and hands were sampled to determine the level of contamination(6). *E. coli* was isolated from all objects and hands sampled, decreasing in number after each contact event, which demonstrates how bacteria can spread between hands and surfaces.

A systematic review was conducted examining publications focused on in vitro pathogen persistence on surfaces, particularly the duration of persistence, and evaluated parameters that can influence persistence(7). The longer a bacterium persists on a surface, the greater the opportunity to spread and be a source of transmission(7). Overall, Gram-positive bacteria are able to survive on surfaces longer than their Gram-negative counterparts(9, 10). Most Gram-positive bacteria (including *Enterococcus* spp. and *S. aureus*), many Gram-negative species (including *E. coli, Pseudomonas aeruginosa* and *Acinetobacter* spp.), mycobacteria (such as *Mycobacterium tuberculosis*) and endospore-forming bacteria can persist on dry surfaces for months(7). One study demonstrated that vancomycin-resistant *Enterococcus faecium* can survive on surfaces for up to four years(11). Some Gram-negative bacterial species, such as *Bordetella pertussis* and *Proteus vulgaris* can only survive for a few days(7).
Relative humidity (RH) and temperature can have a significant impact on bacterial survival on surfaces (7, 12, 13). Bacteria act as hygroscopic bodies as the rate of desiccation is dependent on the RH (14). High humidity (more than 70% RH) and low temperatures (4°C-6°C) can positively influence persistence duration, particularly for Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium* (7, 12, 13). An intermediate range humidity (~ 50% RH) is associated with the poorest levels of survival measured for most bacterial species (15, 16). Some bacteria, notably *S. aureus* and *Acinetobacter* spp., can also persist in conditions of low humidity (less than 50% RH), which can increase bacterial attachment strength (17, 18). The recent discovery of bacterial biofilms on dry hospital surfaces demonstrates the importance of these factors (19). There is some evidence that suggests that high inocula and the presence of organic matter, such as from sputum, protein and serum, contribute to increased persistence too (7).

### 1.1.1.2 Bacteria can actively colonise surfaces to form biofilms

Bacteria can actively attach to and colonise surfaces resulting in the formation of biofilms. Biofilms are communities of microorganisms surrounded by a protective matrix of self-derived extra polymeric substances (20). Bacteria can form biofilms on almost any surface in various industrial and clinical settings (21). Bacterial attachment to surfaces is influenced by surface properties, such as porosity, roughness and hydrophobicity (12). Bacteria growing in biofilms have different physiological states to their planktonic counterparts, especially relating to growth, gene transcription and protein production. The altered state of biofilm derived bacterial cells confers diminished sensitivities to hostile environmental conditions, including desiccation, acid exposure and antimicrobial treatments (20, 21). For example, standard hospital disinfectants – benzalkonium chloride, chlorhexidine gluconate and triclosan – at recommended concentrations are unable to kill *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) grown as biofilms on common hospital surface materials while their planktonic equivalents are susceptible (22).

Bacteria growing in biofilms can detach from the surface structure to seed further biofilms and establish infections (23). Biofilms also facilitate the persistence of bacteria on surfaces resulting in surface associated pathogen reservoirs, which increase the risk of transmission (24). Due to their recalcitrant nature, biocidal treatments are ineffective at removing biofilms from surfaces necessitating the use of physical removal methods. However, this approach is not always
practical or feasible for surfaces that are not readily accessible, such as the inner surfaces of pipes[21, 24]. Given the difficult nature of treating established biofilms, biofilm control strategies aimed at preventing bacterial colonisation and subsequent biofilm formation are more efficacious[21, 24]. One such strategy is creating surfaces that contain antimicrobial agent which can kill the offending bacteria while they are still in their susceptible planktonic state[21].

1.1.1.3 Bacterial contamination of surfaces can have negative consequences in a variety of settings
Bacteria can be passively spread through surface contamination and surface-hand contact events in a variety of settings including hospitals, food processing plants and in farming[7]. Bacterial surface contamination and subsequent transmission can have a negative impact in these settings with health and/or financial implications[25]. Surfaces contaminated with human pathogens can facilitate the spread of infectious disease resulting in morbidity and mortality. This most commonly occurs in hospitals but can also occur in other areas with high densities of people[26, 27]. Management of the transmitted disease and the associated increased duration of hospital stays both contribute to the financial burden of surface derived infections[25]. Similarly, infectious disease can be transmitted to livestock animals through contamination of surfaces with pathogens, which can also have a negative financial impact. Transmission of infectious disease in hospital and farming settings can contribute to increasing rates of antibiotic resistance as a consequence of treating the infections[25]. Contamination of surfaces in food processing plants can have medical and financial implications if the contaminant is a human pathogen leading to both sickness and loss of profits. The consequences of bacterial surface contamination necessitate the development and use of antimicrobial surfaces for improved transmission control.

1.1.2 Common settings that would benefit from utilisation of antimicrobial surfaces
Bacterial contamination of surfaces can be problematic in a variety of settings including in hospitals, on farms and in food processing plants. The basis of problems in each setting is dependent on particular microbiology and influenced by environmental factors, although, there are some commonalities in terms of the type of pathogen and transmission pathways[21, 28]. Therefore, while some specific solutions may be needed, one solution may be applicable
to multiple settings. Control of surface contamination and the associated spread can be achieved using surfaces impregnated with an antimicrobial agent that are able to kill surface bound bacteria.

1.1.2.1 Hospitals
Healthcare-associated infections are a major contributor to patient morbidity and mortality (29). In hospitals and other healthcare facilities, bacterial contamination of surfaces and the subsequent associated transmission is a recently recognised contributor to the burden of nosocomial infections. It should also be noted that bacterial contamination of implanted medical devices, such as catheters, can contribute to infectious disease burden in hospitals due to biofilm formation and subsequent seeding of infection; however, this is not the focus of this work. Infected patients shed bacteria, including MRSA and vancomycin-resistant Enterococcus spp. (VRE), into their immediate environment (30). Surfaces near shedding patients such as walls, door handles, bed frames and light switches, tend to be touched frequently and therefore are more likely to be contaminated (29–31). The frequency of contamination also depends on which body sites on the patient are infected (32). A recent study found that gastrointestinal colonisation with MRSA results in the higher amount of surface contamination (59%) compared to patients who had MRSA at other body sites (contaminating 23% of surfaces) (32). A concentration of less than 250 aerobic CFU per 100 cm² of surface area has been proposed as a standard for effective cleaning (33, 34). Nosocomial pathogens isolated from hospital surfaces are typically in the range of 1-100 CFU/cm² (4, 30). For a microbial burden exceeding 250 CFU/100 cm², transmission from the surfaces to health care workers and/or patients increases and there is still a significant risk of transmission occurring, despite the relatively low inocula present, as most environmentally associated nosocomial pathogens have a low infectious dose (15, 30, 34). Therefore, any contamination of a hospital surface by a pathogen should be considered a transmission risk.

Pathogenic bacteria that are deposited on surfaces by shedding patients or from the hands of healthcare workers treating the patients can be transmitted to new hosts resulting in nosocomial infections. A generalised scheme of bacterial transmission through surface contamination is outlined in Figure 1.1. The level of bacterial transfer that occurs between a contaminated surface and a hand following contact has been demonstrated to occur at a comparable level to direct contact with an infectious patient, which is a well-established
transmission route(7, 35, 36). Bacterial contamination of healthcare workers’ hands can happen in the absence of direct patient contact, with this being attributed to contact with environmental surfaces(31, 37). Uncolonised patients have an increased risk of becoming infected with a pathogen when admitted to a room previously occupied by an infected patient(29). Hand washing can help control the spread of infection in hospitals; however, without decontamination of surfaces, the reservoirs of pathogens will be able to engage in further spread. Healthcare-associated infections are costly to manage – it was estimated in 2012 that the annual cost of healthcare associated infections in the United States of America was US$9.8 billion(38). A 2003 estimate for New Zealand was more than NZD $130 million with the cost increasing each year(39, 40). Utilisation of contamination resistant surfaces in the hospital environment has potential to reduce pathogen transmission and hence the burden of nosocomial infections.

Figure 1.1. Infectious disease transmission is mediated by surface contamination and bacterial spread. Contact between an infected person and an environmental surface can result in bacterial transfer (1) to the surface (contamination). Bacteria from the contaminated surface can be spread to an uninfected person (2) resulting in disease transmission (3) or can be spread to a new surface (4). The newly infected person may also spread bacteria to a new surface (5). The bacteria from the recently contaminated surface (6) can be further spread to other surfaces (7) or may form a biofilm reservoir (8). The biofilm reservoir may mediate further bacterial spread (9) and is resistant to disinfection cleaning.
The increasing global incidence of antibiotic resistance in bacteria is a threat to infection control and public health. The emergence of antibiotic resistance in bacterial pathogens is inevitable, with higher rates of resistance being associated with elevated antibiotic use (25). Hospitals are characterised by high use of antibiotics, resistance to which is reflected in the local resistance profiles of environmental pathogens, such as VRE and MRSA (25, 41). The transmission of these pathogens necessitates superfluous antibiotic treatments, increasing the exposure of bacteria to antibiotics and therefore potentially contributing to increases in the incidence of resistance (25). Preventing the spread of resistant pathogens through hospitals would decrease the use of antibiotics and the amount of environmental contaminants exposed to these treatments. The involvement of contaminated surfaces in pathogen transmission pathways in hospitals necessitates improved control of surface microbiology through utilisation of antimicrobial surfaces. The potential impact of an antimicrobial surface on surface contamination mediated infectious disease transmission is outlined in Figure 1.2.

**Figure 1.2. Antimicrobial surfaces may interrupt infectious disease transmission and bacterial spread mediated by surface contamination.** Contact between an infected person and an environmental surface can result in bacterial transfer (1) to the surface (contamination). A certain period of time is required for surface bactericidal activity, following which the surface will no longer be contaminated (2). Elimination of surface contamination would prevent spread of bacteria to an uninfected person (3) and to a new surface (4) thereby disrupting the transmission pathways outlined in Fig. 1.1.

### 1.1.2.2 Farms
Bacteria can readily spread through farms, which poses problems in terms of the welfare of the animals and farm workers as well as potentially the consumers of the animal products and carcasses (42). Farm animals infected with or carriers of human gastrointestinal pathogens shed
bacteria in their faeces, which can contaminate the surrounding environment including water supplies, food sources and surfaces (42). Surfaces on farms, including gates and fencing, can come into contact with infected animal faeces through several different routes (12). These routes include agricultural practises, such as manure spreading, direct excretion by infected animals or passive contamination through footwear while climbing over a fence (12, 42). Transmission to humans can occur through direct hand contact with the contaminated surface resulting in infection of the human host and potential further spread around the farm (12, 43). Transmission to farm animals can also happen when surfaces in animal housing units come into contact with contaminated work boots or from contaminated equipment (12, 42, 43).

Transmission pathways through farms have been observed for *E. coli* O157:H7, a prevalent pathogenic *E. coli* serotype that can infect humans as well as livestock, particularly cattle (12). The high rate of faecal shedding by cattle (10^3–10^5 CFU/g faeces) combined with the substantial persistence time (more than 28 days) of *E. coli* O157:H7 under a variety of environmental conditions means that this bacteria can be readily dispersed around the farm, in part due to contamination of surfaces (12, 15). It has been demonstrated that contact between a hand and a gate contaminated with faecal matter containing ~10^5 CFU/g *E. coli* O157:H7 can result in transfer of 5%-8% of the faecal matter to the hand (12). This corresponds to ~10^3 CFU transmitted, which far exceeds the infectious dose of this bacteria (1-100 CFU) (12). A similar situation has been described for *Salmonella enterica* contamination of pig farms (43). Bacterial spread through farms could be reduced through utilisation of surfaces that resist contamination, such as for work boots or fences. Illness derived from the transmitted bacteria can have a negative financial impact due to the cost of managing the infectious disease and the loss of profits associated with unsafe animals. Managing transmission of pathogens through farms may in part be aided through the application of antimicrobial surfaces.

### 1.1.2.3 Food processing plants

Pathogenic bacteria can be spread through food processing plants in a similar manner to that in hospitals and on farms. This has been well characterised for *E. coli* O157:H7, which has been isolated from a number of meat processing surfaces in plants (44, 45). Faecal shedding from farm animals infected with *E. coli* O157:H7 can result in contamination of the hide, which can facilitate the introduction of the *E. coli* into food processing plants (44, 46). Once *E. coli* O157:H7 has been introduced to a plant, it can be spread through surface contact transmission
pathways and form biofilms on the surface of equipment used during different stages of slaughtering and processing (44, 46). Genotypic analysis of *E. coli* isolated from carcasses, ground beef and meat processing surfaces at a cattle processing plant revealed that the source of some of the *E. coli* meat contamination is the plant environment (45). For example, 50% of conveyers were found to be contaminated with *E. coli* (45).

The ability of *E. coli* O157:H7 to form biofilms on a range of surfaces present in meat processing plants, such as stainless steel, facilitates transmission of *E. coli* O157:H7 to naïve carcasses and fresh meat products due to contact with the contaminated equipment (44, 46). This biofilm formation also promotes persistence of *E. coli* O157:H7 in meat processing plants due to the recalcitrant nature of biofilm-associated bacterial cells to biocidal treatments and harsh environmental conditions (46). Therefore, *E. coli* O157:H7 growing as biofilms on meat processing surfaces may be an ongoing source of contamination. Meat contaminated with *E. coli* O157:H7 (and other human pathogens) is a major concern for food and public safety as consumption of improperly cooked contaminated meat can result in severe enteric and systemic diseases (45). Utilisation of antimicrobial food processing surfaces could help prevent both bacterial spread and biofilm formation in food processing plants.

1.2 Characteristics of antimicrobial surfaces

There are many different approaches being undertaken which are working towards the common goal of development of efficacious antimicrobial surfaces. Consideration needs to be given during development of these surfaces to the properties of the final product as this can affect its applicability to settings that require antimicrobial surfaces. Properties of contamination resistant surfaces include the spectrum of activity, the efficacy of the surface (especially in relevant conditions), environmental stability, whether the antimicrobial agent is immobilised or leaches and the commercial viability of the surface (in terms of cost, ease of production and regulatory approval).

1.2.1 Spectrum of activity

Antimicrobial surfaces may be broad spectrum in action or more targeted towards controlling the spread of a particular organism. Hospitals would benefit most from the development of self-sanitising surfaces with a broad spectrum antimicrobial agent. Contaminating bacteria on
hospital surfaces may be Gram-positive such as VRE and MRSA, Gram-negative such as *E. coli*, or mycobacteria such as *M. tuberculosis* (7). For a self-sanitising surface to be effective in this setting, it would need to be active against all potential challenges. One suitable candidate for creation of hospital contamination resistant surfaces is copper and its alloys (47). Copper has been shown to knockdown a range of pathogenic clinical isolates including *P. aeruginosa*, VRE, MRSA and *E. coli* within 40-100 min of treatment (48). Strikingly, even high doses (~ $10^7$ CFU) of *E. coli* O157:H7 and MRSA can be killed following a 1 h exposure to copper surfaces (49–51). In contrast to the situation in hospitals, farms and food processing plants may be concerned with a narrower range of pathogens and spoilage organisms depending on the type of farm or the food being processed. For example, farms and food processing plants that work with cattle that are carriers of *E. coli* O157:H7, a surface that is active against this contaminating strain (possibly extending to other potential enteric pathogens) would be most beneficial (44, 46). Broad spectrum activity of an antimicrobial surface would extend the potential applications of the surface as it can mitigate a range of pathogenic challenges whereas narrow spectrum surfaces are more limited in their applications.

### 1.2.2 Efficacy of antimicrobial surfaces

Establishing the efficacy of an antimicrobial surface in terms of absolute activity and duration of activity is important. The initial step in developing contamination resistant surfaces is identification of potential antimicrobial agents to be incorporated in the surface. The activities of potential agents are first verified in solution or suspension against the species of interest. However, this activity is not always translated when the antimicrobial agent is incorporated into a surface (52). Bactericidal agents that require contact with (and possibly entry into) bacterial cells will be less active when incorporated into a matrix (52, 53). Any antimicrobial agents that are immobilised onto surfaces through chemical means may lose their activity through this process. This effect is notable for antimicrobial peptides (AMPs) when the functional group that exerts bactericidal activity is used to link them to a surface rendering the peptides inactive (52). Antimicrobial agents with potential to create contamination resistant surfaces need to be verified that they are active whilst incorporated into a surface.

The duration of time that an antimicrobial surface is active for is another important consideration. Some contamination resistant surfaces may not require antimicrobial activity for an extended period of time as the surface may be able to be replaced frequently. For
example, hospital privacy curtains are frequently contaminated with pathogens including VRE and MRSA and are good candidates for contamination resistant surfaces(54). Depending on the healthcare facility, these curtains tend to be changed after several weeks(54). Therefore, potential contamination resistant curtains only need to retain their antimicrobial activity for a few weeks, which is a more achievable goal than long-term activity; however, it does require more tracking and maintenance. Some settings require antimicrobial activity of surfaces to continue over extended periods of time, such as for food processing equipment surfaces. Ideally, the activity of the surface would last as long as the equipment needs to be used as it may be expensive or difficult to replace(52). Each setting that could benefit from a contamination resistant surface will require efficacious activity within a particular time-frame, which must be satisfied by the potential antimicrobial surface.

1.2.3 Efficacy in appropriate environmental conditions
Contamination resistant surfaces need to be active against target organisms in the environmental conditions relating to the potential applications, particularly in terms of temperature and RH(14). Typically, indoor environments, such as hospitals, have ~ 24% RH and are ~ 20°C(55). A standard method for testing the antimicrobial activity of surfaces, Japanese Industrial Standard JIS Z 2801, is characterised by high humidity (more than 90% RH) and high temperature (35°C)(55). Silver ions can be incorporated into surfaces to impart resistance to bacterial contamination(55). However, the activity of these surfaces is greatly affected by both RH and temperature. Silver ion containing coupons mediated a greater than 5 log reduction in MRSA numbers following a 24 h treatment at more than 90% RH and ~ 35°C, which decreased to a less than 0.3 log reduction at 22% RH and ~ 20°C(55). A similar effect has been found for copper alloy coupons challenged with notable bacterial pathogens causing foodborne infections, S. enterica and Campylobacter jejuni(56). These coupons mediated a significant knockdown in a shorter timeframe at 25°C compared to at 10°C, a common temperature found in meat processing plants(56). A reduced activity at lower temperatures for copper coupons has also been noted against E. coli O157:H7(57). The effect of RH and temperature needs to be reflected in the verification of antimicrobial activity of contamination resistant surfaces.

Surfaces in all settings described above are often contaminated with organic matter, such as blood and faeces in hospitals, and complex food matrices in food processing plants. The presence of organic matter can affect the activity of antimicrobial surfaces and needs to be
addressed when developing an antimicrobial surface for a particular application. Copper alloy coupons demonstrated significant loss of activity against *E. coli* O157:H7 when coated in liquid beef extract, which represents soiling of food processing surfaces(57). Electrostatically charged surfaces are particularly vulnerable to being coated in charged organic matter, including protein in food processing plants(52). This organic coating may neutralise the surface charge and would limit their applicability to utilisation in food processing surfaces(52). Contamination resistant surfaces need to be sufficiently active in the appropriate environmental conditions of the desired application.

### 1.2.4 Environmental stability

For antimicrobial surfaces to be a viable option they will need to be able to withstand any adverse environmental conditions relating to their application. For example, food processing equipment may be exposed to extreme temperatures, acidic and alkaline conditions, and mechanical abrasion(52). Contamination resistant surfaces based on AMPs may not be suitable for use on food processing equipment as the peptides could hydrolyse and denature under such conditions resulting in loss of antimicrobial activity(52). Both hospitals and food processing plants undertake regular cleaning of surfaces using cleaning and sanitisation agents. Contamination resistant surfaces in these settings would need to be resistant to damage from these agents. Copper-based surfaces show great potential; however, they are susceptible to loss of activity following cleaning with common sanitisation agents(58). Repeated disinfection of a copper surface with 1% sodium hypochlorite and 70% industrial methylated spirit resulted in ‘surface conditioning’, which was associated with decreased killing of *S. aureus*(58). Antimicrobial surfaces for outdoor settings, such as farm surfaces, would need to be resistant to the damaging effects of UV light(52). Thus, UV sensitive antimicrobial agents, including chlorinated N-halamines, would not be suitable for an outdoor surface application(52). Surfaces that have lost activity due to extreme environmental conditions and/or cleaning would need to be frequently replaced, which may result in the cost outweighing the benefit. Potential antimicrobial surfaces need to maintain their activity in relevant adverse environmental conditions.

### 1.2.5 Leaching

Antimicrobial agents used in creating contamination resistant surfaces may be immobilised in the surface or released over time, the latter of which is necessary for when the agent needs to
enter the cell to exert bactericidal activity. Antimicrobial surfaces based on metal ions and nanoparticles, such as copper and silver, exert their activity by releasing the antimicrobial agent, which is then able to penetrate and kill the bacterial cell(47, 52). There is limited application of these surfaces in food processing environments due to public health and environmental concerns based on leaching(52). Loss of the antimicrobial agent over time means that the concentration of the agent will fall below the threshold needed to exert antimicrobial activity. Contamination resistant surfaces that leach the antimicrobial agent will only retain activity over short time periods and for a limited number of challenges. The 4 h antibacterial activity of silver nanoparticle based surface decreased from a more than 90% reduction in *E. coli* numbers for the first challenge to a 20% reduction for the 11th challenge(59). Thus, surfaces that leach are not suitable for applications that require long term activity (such as for food processing equipment) or activity against repeated challenges (such as for high-touch hospital surfaces). Once leaching-based antimicrobial surfaces have exhausted their supply of the antimicrobial agent they must be replaced(52). Antimicrobial surfaces that can exert their activity without leaching will not experience diminished activity due to loss of the antimicrobial agent and may potentially be better suited to settings characterised by long-term, repeated challenges, such as food processing equipment(52).

### 1.2.6 Commercial viability
Successful development of antimicrobial surfaces necessitates that they are suited to commercialisation. Antimicrobial surfaces need regulatory approval for their intended use, which is particularly important for food contact surfaces(60). In the United States of America, antimicrobial agents incorporated into surfaces require US Food and Drug Administration (FDA) approval, especially if the agent is leached from the surface, making it an indirect food additive(60). This requires the agent to be well characterised in terms of human and environmental toxicological implications, particularly in the food industry(52). Silver nanoparticles must be leached from a surface to exert antimicrobial activity; however, these nanoparticles have been demonstrated to be cytotoxic towards several mammalian cell lines(61, 62). The potential for FDA approval must be considered when developing a contamination resistant surface.

Consideration also needs to be given to the cost and ease of production of contamination resistant surfaces(63). Creation of such surfaces necessitates that the antimicrobial agent and
the supporting matrix are not expensive materials, and large-scale production of the surface is affordable (63). If the cost far exceeds the benefit then the antimicrobial surface may not worth developing as it will never be commercially translatable (52, 63). In international markets the price of copper is increasing (64); copper cost less than 100 US cents per pound (1 pound = 0.454 Kg) in 2000, which escalated to 350 US cents per pound in 2010 (64). If the cost-to-benefit ratio is skewed by increasing copper prices it may no longer be a viable option for creation of contamination resistant surfaces.

1.3 Current strategies for preventing surface contamination
Antimicrobial surfaces that are resistant to contamination by bacteria may be beneficial in a variety of settings, including hospitals and food processing plants. Reduction of microbial contamination on a surface in these settings could help disrupt transmission pathways and thereby potentially could reduce infectious disease rates and the associated antibiotic usage (25). An ideal antimicrobial surface would be active against relevant bacteria at appropriate bacterial loads (as determined by the application) and active in environmental conditions relating to potential applications in terms of temperature, RH, pH, exposure to cleaning products and contaminating organic matter (table 1.1) (14, 55, 65). Surfaces in hospitals tend to be contaminated with blood or faeces while food processing plant surfaces are contacted by complex food matrices (65–67). The time required for decontamination would need to be sufficiently short to effective in breaking transmission pathways. Activity needs to be retained for sufficiently long periods of time to be cost effective, especially for equipment in food processing plants, and after repeated bacterial challenges (68). Surfaces that leach antimicrobial agents will lose activity over time (52). An ideal antimicrobial surface would also need to be cheap and easy to make, suitable for large-scale production and have regulatory approval for their intended use, which is important for food contact surfaces (table 1.1) (60, 68).
There are many approaches being undertaken to develop antimicrobial surfaces including use of bactericidal metals (notably silver and copper), attachment of antimicrobial peptides, incorporation of N-halamines, impregnation with triclosan and use of quaternary ammonium compounds, with a range of efficacies and suitability to the potential applications(83, 84). A small selection of these approaches is reviewed below to cover important concepts in lieu of a comprehensive review of antimicrobial surfaces, in the interest of brevity.
1.3.1 Decontamination of surfaces by treating with disinfectants and cleaning agents

In settings where microbial contamination of surfaces is problematic, such as in hospitals and in food processing plants, there are usually established cleaning schedules (85–87). The aim of cleaning environmental surfaces is to reduce both organic and microbial contamination of surfaces. High risk areas, including rooms of patients infected with a pathogen, may have targeted cleaning of surfaces (85). Routine disinfection of environmental surfaces is carried out by a human operator and typically involves both cleaning with a detergent and a disinfectant (85, 86). The function of a detergent is to remove microorganisms and organic matter from surfaces while the purpose of disinfection is to reduce bacterial numbers on a surface (88). Cleaning of surfaces with only a detergent can result in spread of bacteria (86).

Seven detergent wipes were examined for their propensity to transfer S. aureus, A. baumannii and Clostridium difficile endospores using the 3-stage wipe protocol (86). All wipes tested consistently transferred bacteria and endospores over 3 consecutive surfaces (86). For this reason, it is necessary for surfaces to be cleaned with a disinfectant too.

Common disinfectants used to decontaminate surfaces in hospitals and food processing plants are quaternary ammonium compounds (QACs) and chlorine-releasing agents such as sodium hypochlorite and sodium dichloroisocyanurate (NaDCC) (46, 89–91). QACs consist of a nitrogen atom linked directly to four groups, which leaves the nitrogen positively charged (88, 92). One of these groups is an alkyl residue that can vary in length, forming the basis of QAC classification (88, 92). A commonly used QAC is benzalkonium chloride (BAC) (88, 91). In general, the antimicrobial action of QACs occurs following binding to the cell surface and involves inactivation of respiratory enzymes, denaturation of essential proteins, and disruption of cell membrane integrity (88). QACs have variable microbicidal activity against Gram-negative and Gram-positive bacteria (42, 89, 93, 94). One study found that the MBC of BAC against E. coli PHL 628 was 24 mg/ml, Bacillus subtilis 6633 was 9 mg/ml and S. aureus 6538 was 70 mg/ml (93). The reduced susceptibility of S. aureus to BAC may be a reflection of expression of efflux pumps, such as those encoded by the plasmid-carried qac genes (92).

Chlorine-releasing agents are widely used disinfectants in hospitals and in food processing plants (89, 95). Sodium hypochlorite can exist in two forms depending on the pH of the solution, as hypochlorous acid (HOCl) or as a hypochlorite ion (-OCl). Both HOCl and -OCl can
act as strong oxidising agents targeting a range of biomolecules including proteins, amino acids and DNA(95). Commonly used solutions of sodium hypochlorite range from 50 to 200 mg available chlorine/l, which corresponds to a pH of 8.5-10(95). At this pH, sodium hypochlorite exists mostly in -OCl form(95). Sodium hypochlorite is strongly bactericidal, has a broad spectrum of activity and is active in unsoiled conditions within short contact times(89, 95). A 1% sodium hypochlorite solution reduced $10^9$ CFU/ml of *E. faecalis*, *E. coli* and *S. aureus* to below the limit of detection in less than 5 min(96).

Pathogenic bacteria are spread through hospitals and food processing plants despite regular cleaning of environmental surfaces. Indeed, a systematic review of studies examining an environmental disinfection intervention in hospitals found that infection rates were not influenced by surface disinfection(85). This may reflect recontamination of a surface shortly after cleaning that would render the cleaning ineffective, which would be particularly likely in high-touch areas. It may also reflect a failure of disinfection to reduce bacterial numbers. For example, 26.6% of patient rooms remained contaminated with *A. baumannii* or MRSA after 4 rounds of 5,000 ppm (5 mg/ml) bleach disinfection(97). A similar study found that 16% of patient rooms were contaminated with VRE following disinfection with QACs(98). A lack of benefit from environmental surface disinfection may also be due to use of an ineffective disinfectant, use of a narrow spectrum disinfectant not active against target bacteria, bacterial resistance to the disinfectant, use of an incorrect concentration of the disinfectant, an insufficient contact time or interference by organic matter(88, 99).

Effective disinfection requires the human operator to carry out the cleaning procedures correctly, which involves selection of the appropriate disinfectant and accurate dilution to the correct concentration and pH(88, 100, 101). The degree to which disinfectants are affected by concentration varies depending on the nature of the disinfectant(99, 101). The change in activity of a disinfectant for a given change in concentration can be represented by the concentration exponent (η)(99, 101). The antimicrobial activity of disinfectants with high concentration exponents, such as alcohols and triclosan, is greatly affected by concentration (and therefore dilution of the product) while disinfectants with low concentration exponents are influenced to a lesser extent(99). For example, DDDMAC is a QAC whose bactericidal activity is affected by its concentration(102). A 5 min exposure of DDDMAC reduced $10^8$ CFU/ml of *E. coli* and *S. aureus* by 5 log at a concentration of 20 mg/ml and showed no
microbicidal activity at 0.5 mg/ml (102). Following preparation, the disinfectant solution needs to be spread over the surface ensuring that there is sufficient distribution of the active agent for the required contact time (28, 103). Failure of the human operator to select an appropriate disinfectant, properly formulate the disinfection solution or allow sufficient contact for the required time on surfaces results in a failure to reduce bacterial numbers on the surface (42, 101).

Disinfectants require particular environmental conditions to exert effective activity against target bacteria. The efficacy of disinfectants is determined in laboratories in ideal conditions; however, when disinfectants are used in real world settings they may encounter different environments, such as contamination with organic matter (42). The presence of organic matter on a surface may reduce the efficacy of a disinfection treatment (42). Loss of activity can be due to organic particles interfering with binding of the antimicrobial agent to the bacterial cell, which may be particularly relevant for agents that use charged-based interactions, such as QACs (53, 88). Loss of activity in the presence of organic soiling can also be due to the antimicrobial agent reacting non-specifically with biomolecules, such as what occurs with chlorine-releasing agents, which would reduce the amount of the antimicrobial agent available to inactivate bacteria (95). For example, a 5 min exposure of NaDCC in clean conditions reduced levels of *C. difficile* by more than 5 log (89). In dirty conditions, simulated with 0.3% bovine serum albumin and 0.3% erythrocytes, NaDCC reduced *C. difficile* numbers by less than 1 log following a 60 min treatment (89). Sodium hypochlorite also has decreased efficacy in the presence of organic matter (95). A reduction in disinfectant activity in the presence of organic matter has been observed in boot dips on farms (42). Farm workers dip their boots into a disinfectant solution to reduce spread of pathogens in animal houses (42). Boot dip samples from several farms were collected, permitting a range of disinfectants to be tested (42). All samples demonstrated decreased antimicrobial activity in the presence of organic matter (as assessed by turbidity) (42). The negative influence of organic soiling on disinfectant action necessitates the cleaning of environmental surfaces, first with detergent followed by cleaning with a disinfectant (88, 89, 95).

The impact of the human operator on effective disinfection of environmental surfaces is significant. Inappropriate selection, formulation and use of a disinfectant can lead to a decontamination failure (101). Mishandling and misuse of disinfectants has been observed in
farms and healthcare facilities(42, 88). Common errors include not accurately measuring disinfectants when making solutions, use of solutions that are too dilute and use of out of date disinfectants(42, 88). Facilities attempt to address the problems associated with human operators by training, monitoring cleaning and giving feedback to staff(101, 103). Improvement of these conventional methods depends on modification of human behaviour, which is often difficult(101, 103). Monitoring of cleaning in hospitals can be achieved by marking surfaces with fluorescent dye spots and examining surfaces for the spots after cleaning(101). One study found that changing the location of fluorescent dye spots resulted in a reduction of objects that were cleaned from 90% to 60%, which highlights the difficulties experienced in attempting to change staff behaviours to reduce the frequency of disinfection failures(103, 104).

A failure of surface disinfection can facilitate bacterial spread as further cleaning can move bacteria to other surfaces(30). Cleaning with only a detergent soaked cloth has been demonstrated to spread E. faecalis from one contamination point to many environmental surfaces(105). Viable bacteria left on a surface following an ineffective disinfection procedure can result in an accumulation of bacteria on the surface over time, which will increase the risk of transmission(28). Bacteria not removed by disinfection cleaning can form biofilms, which promote survival in adverse environmental conditions, including low humidity and future surface disinfection(88). Cleaning of surfaces with an unsuitable concentration of the disinfectant may even promote biofilm formation(106). Exposure of E. coli and P. aeruginosa biofilms to sublethal levels of BAC resulted in formation of thicker and denser biofilms relative to the control due to over-secretion of matrix components during biofilm development(106).

Bacterial spread that occurs through hospitals, food processing plants and farms despite cleaning efforts necessitates implementation of a different approach. The difficulties associated with human operator involvement with disinfection of surfaces can be avoided by using antimicrobial surfaces that can reduce surface contamination without human input. These surfaces would act to reduce contamination as soon as it occurred, reducing the time viable bacteria are present on the surface compared to surfaces that are cleaned as part of a roster. Therefore, there would be a decreased opportunity for bacteria to be spread or to colonise the surface preventing biofilm formation. Routine cleaning of surfaces is an essential component of infection control focused on disrupting transmission pathways as organic soiling
could reduce the antimicrobial activity of a surface. Detergent cleaning products need to be compatible with the antimicrobial surface to ensure activity is not lost following cleaning.

1.3.2 Triclosan impregnated surfaces
Triclosan is a phenolic compound, 5-chloro-2-(2,4-dichlorophenoxy)phenol, with antimicrobial activity at concentrations of 0.2% to 2%(2, 81, 83, 107). It is widely used as an antiseptic, a disinfectant and as an additive to products, such as soaps, and surfaces, including plastics (83, 108). Triclosan incorporated into plastics and other surfaces is used to reduce bacterial loads in the food processing industry (108). Use of triclosan in hospitals is more limited (2).

Triclosan is bacteriostatic at low concentrations and has bactericidal activity at higher levels, which involves different mechanisms of action (81). Bacteriostatic activity is mediated through specific inhibition of the NADH-dependent enoyl-acyl carrier protein reductase, FabI (100, 108). Triclosan is a potent irreversible inhibitor that competes with the natural substrate for binding (107). Lethal activity is associated with non-specific membrane damage resulting in loss of membrane integrity (108, 109). The mechanism of action is complex and has not been fully elucidated as bactericidal activity cannot be completely explained by inhibition of metabolic pathways (81, 107, 109). However, inhibition of such pathways could possibly contribute as part of the lethal mechanism at a low bactericidal concentration (107).

Triclosan has a broad spectrum of activity against many Gram-negative and Gram-positive bacteria; however, some bacteria are resistant to triclosan, such as P. aeruginosa (81). In general, Gram-positive organisms have greater susceptibility to triclosan compared to Gram-negative rods (83, 99). This antimicrobial agent is also active against mycobacteria but is not endosporicidal (81). Low concentrations of triclosan (0.05-3 µg/ml) are able to inhibit the growth of target bacteria including E. coli and S. aureus (99). In contrast to this, much higher concentrations are required for lethal activity with the MBC of triclosan against E. coli and S. aureus being 25-100 µg/ml (99). For this reason, the activity of triclosan is often bacteriostatic (83, 88). The activity of triclosan is affected by its concentration and by the growth phase of the cells (99, 108). Phenolics, such as triclosan, have a high concentration exponent (4-8.8) meaning that activity is rapidly lost as the concentration decreases (99). Triclosan is more active against exponentially growing cells compared to stationary phase cells (108). 1 mg/ml of triclosan mediated an 8 log knockdown of log phase S. typhimurium following a 10 min treatment while the same treatment of stationary phase S. typhimurium
only resulted in a 3.6 log reduction (108). The influence of the bacterial phase of growth is less apparent at higher concentrations of triclosan (107).

Triclosan can be used as an additive to create contamination resistant surfaces (110). Triclosan incorporated surfaces have limited efficacy due to insufficient bioavailability of triclosan and/or due to the metabolic state of the target bacterial cells (99, 108). Triclosan impregnated surfaces may be ineffective at reducing bacterial load due to the reliance of activity on bacterial growth rate, especially at low triclosan concentrations commonly found on surfaces (99). Stationary phase and biofilm cells are the most commonly found states of bacteria isolated from environmental surfaces (108). Plastic containing 1500 ppm (1.5 mg/ml) of triclosan did not significantly decrease bacterial load of S. typhimurium, S. aureus or E. coli, which was hypothesised to be due to a lack of activity against stationary phase cells (111). Indeed, S. typhimurium stationary phase cells have been demonstrated to survive, albeit at reduced numbers, following treatment with triclosan at 2000× MIC, which was lethal for log phase cells (108). Similarly, 35 mg/ml of triclosan mediated a 6 log reduction of log phase cells in 2 min while metabolically inactive cells washed in water were only reduced by 2 log in 20 min (107). This is in contrast to activity against stationary phase S. aureus washed in water, which was reduced by 6 log in 10 min (107). The overall dependence of triclosan activity on bacterial growth phase suggests it may not be sufficiently active in surface applications.

Triclosan can be incorporated into polymers, including polystyrene and polyethyleneimine, through melt-mixing (110). Antimicrobial activity is dependent on diffusion of triclosan out of the surface (53, 112). A surface coating consisting of styrene-acrylate copolymer and triclosan was developed; however, triclosan was only released in efficacious amounts when n-heptane was the solvent and negligible amounts were released when water was the solvent (113). Therefore, in any water based applications this surface would not release sufficient triclosan to mediate a reduction in bacterial numbers. Similarly, a plastic containing a high concentration of triclosan only inhibited growth of E. coli for 5 h and demonstrated no bactericidal activity (110). It was hypothesised that the lack of efficacious activity was due to release of some of the incorporated triclosan (sufficient to transiently prevent growth) while the remainder of the triclosan was trapped in polymer matrix (110). Bacteria isolated from a floor impregnated with triclosan in a poultry processing plant were mostly sensitive to the antimicrobial agent indicating that the lack of antimicrobial activity was due to insufficient concentrations to
achieve knockdown (114). Reliance on release of triclosan from the defined reservoir in the surface limits the applicability of triclosan impregnated surfaces to short-term use only (53, 115). The high concentration exponent of triclosan means that during release of triclosan the activity will be lost rapidly as the reservoir of the antimicrobial agent decreases (99). Conversely, immobilisation of the majority of triclosan in a surface will result in a very low triclosan concentration contacting the bacteria and therefore will exert minimal activity.

The use of triclosan to render surfaces antimicrobial may also be limited due to bacterial resistance. The release of triclosan from a surface over time will eventually result in exposure of adjacent bacteria to sublethal concentrations of triclosan, which may mediate the development of resistance (2). It has been identified that Gram-negative bacteria use several mechanisms to become resistant to triclosan, including mutations in the enoyl reductase enzyme, overexpression of enoyl reductase enzyme, active efflux using pumps and expression of degradative enzymes (81, 100, 108). The most common mechanism observed is overexpression of multidrug efflux pumps, including the AcrAB efflux pump (108). Serial passage of bacteria in increasing triclosan concentrations can produce bacteria with reduced susceptibility to triclosan; however, the MIC of such strains is still typically well below the concentration of triclosan contained in antimicrobial products (81, 83). There is also concern that triclosan use can lead to the development of cross-resistance as expression of multidrug efflux pumps in response to triclosan exposure is known to mediate resistance to many antibiotics (108, 116). For example, overexpression of acrAB in E. coli reduced susceptibility to triclosan, fluoroquinolones, ampicillin and tetracycline (117). Exposure of a triclosan-sensitive mutant of P. aeruginosa to triclosan resulted in expression of an efflux pump and a high level of resistance to ciprofloxacin (118). Similar results were found for E. coli K12, E. coli O55 and E. coli O157:H7 exposed to triclosan (119). Conversely, triclosan tolerant strains of E. coli, S. aureus and Acinetobacter johnsonii were found to have no demonstrable resistance to a range of antibiotics including β-lactams, aminoglycosides, tetracyclines and fluoroquinolones (120). While there is some in vitro evidence that triclosan exposure may mediate cross-resistance to antibiotics, it has been questioned if this is relevant in real world settings, as commonly used concentrations (2-20 mg/ml) of triclosan far exceed the MBC (116). It has also been argued that expression of efflux pumps will make bacteria less able to compete with other bacteria due to the energy demands of producing the pumps (100).
The demonstrable activity of triclosan in solution against actively growing bacteria highlights the potential for incorporation into surfaces to render them antimicrobial. The activity of surface incorporated triclosan is much less effective compared to activity in suspension due to release of insufficient levels of triclosan below the MIC and/or MBC of target bacteria, the relatively inactive metabolic state of contaminating bacteria on surfaces and the potential for resistance to develop. A surface with an immobilised antimicrobial agent that can exert its activity without dissociating from the surface would be able to maintain a necessary concentration to reduce bacterial load. Surface incorporated antimicrobial agents need to have efficacious activity against bacteria that are not actively growing, reflecting the metabolic state of surface derived bacteria(99).

1.3.3 Copper impregnated surfaces
Metals, such as copper, are essential to bacterial growth and survival; however, at elevated concentrations metals can be toxic to bacterial cells(70). Metals have been used for thousands of years as antimicrobial agents(52). Metals can be incorporated into surfaces to impart antimicrobial activity with copper being one of the most studied candidates alongside silver (21, 121). Copper has broad spectrum activity against a wide range of bacteria including *S. enterica*, *C. jejuni*, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii* and *P. aeruginosa* (47, 48, 56, 122, 123). Copper as an antimicrobial agent can exist in different forms including as copper ions in an alloy and as nanoparticles(52). Copper nanoparticles are thought to have enhanced activity derived from the small size (facilitating more efficient penetration of bacterial cells) and a large surface-area-to-volume ratio (increasing the amount of released metal ions)(52, 121). Copper alloy surfaces containing copper ions exert antimicrobial activity as demonstrated by the protective effect of copper ion chelators on bacterial cell viability(124).

Bactericidal action by copper is initiated following contact with bacterial cells and is termed ‘contact killing’(47). Copper must enter and accumulate inside cells to exert action(121). The exact mechanisms of the antimicrobial action of copper have not been fully characterised and are debated in the literature(70). Elucidation of the mode of action of copper is complicated by the multifactorial nature of antimicrobial action combined with different modes of action demonstrated for Gram-negative and Gram-positive bacteria(70, 125, 126). Several uniting factors have been identified to contribute to contact killing, including induction of oxidative
stress, targeting of $[4\text{Fe-4S}]^{2+}$ clusters in enzymes, membrane depolarisation and DNA damage(47, 70, 125).

Copper is able to accept and donate single electrons resulting in a change to oxidation state between Cu$^+$ and Cu$^{2+}$ [Cu(I) and Cu(II)](70, 127). It has been hypothesised that copper mediates antimicrobial action through reaction with hydrogen peroxide (in a similar manner to Fenton reaction that occurs with iron represented by: $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^-$) resulting in production of highly reactive hydroxyl radicals(47, 70, 125, 128). Hydroxyl radicals are known to damage a variety of cellular biomolecules including DNA(47, 128). $S. \text{aureus}$ and $E. \text{coli}$ were protected from copper surface killing by the presence of D-mannitol, a hydroxyl radical scavenger, using a dry surface protocol(125, 127). This, however, has been contested in work, which demonstrated that copper treatment decreased hydrogen peroxide mediated DNA damage, postulated to be due to accumulation of oxidisable copper in the periplasm, far from DNA(127, 129). Copper that penetrated to the cytoplasm was hypothesised to be chelated by intracellular metabolites, including glutathione, preventing redox reactions(129). Indeed, the lethal action of copper surfaces could not be prevented by addition of hydroxyl radical or hydrogen peroxide scavengers, D-mannitol and catalase(124, 126). There is some evidence to suggest that superoxide is produced in copper treated cells and that this ROS mediates the bactericidal action of copper by damaging DNA and respiratory machinery (124, 125).

Copper is thought to target $[4\text{Fe-4S}]^{2+}$ clusters in enzymes that function in a wide range of physiological processes from energy metabolism to DNA repair, which infers that copper toxicity would have an extensive impact on cellular function(130). Targeting of these FeS clusters is hypothesised to be due to inactivation of the clusters and/or inhibition of biogenesis(70, 128, 130). Copper has been demonstrated to inactivate $[4\text{Fe-4S}]^{2+}$ clusters by displacing iron in the catalytic cluster(128). Copper has also been shown to inhibit FeS cluster synthesis by the ISC pathway by completing with iron for binding of IscA, which normally functions to provide iron during cluster assembly, resulting in inactivation of the enzyme(130). Contact killing, especially on dry surfaces, is achieved within minutes of inoculation(131). These fast killing kinetics argue against targeting of FeS cluster containing enzymes being the cause of cell death(131). Additionally, inactivation of metabolic enzymes would have little effect during periods of low or no growth, such as on dry surfaces(131).
Copper is additionally hypothesised to cause lethal DNA damage and/or damage the cell membrane resulting in membrane depolarisation (47, 124, 131–133). The timing and overall contributions of these mechanisms have yet to be fully elucidated (70). It has been argued that copper directly targets membrane proteins or membrane lipids resulting in damage to the cell membrane and loss of integrity, with DNA degradation a downstream effect of this (47, 70, 131, 134). Copper(II) ions have been demonstrated to bind certain cytochromes resulting in a conformational change and loss of electron transfer abilities (124, 135). Copper (II) ions have also been shown to directly damage the adenosine triphosphate (ATP) synthase of Enterococcus hirae leading to conformational changes and inactivation of ATP synthase (136). The postulated induction of oxidative stress (including hydroxyl radical formation) in copper treated cells may also underlie damage to the membrane and respiratory machinery (134). Others postulated that DNA is rapidly degraded following contact with copper and is followed by a reduction in bacterial respiration and depolarisation of the membrane (124, 133). DNA damage is hypothesised to be mediated by superoxide, as addition of Tiron, a superoxide quencher, prevented DNA fragmentation and provided protection against copper surface killing under wet and dry conditions (124, 133). Opposing effects of Tiron on copper-mediated cell death have been observed (127). Despite the many studies investigating the antimicrobial mechanism of copper, the temporal events involved in the bactericidal action of copper remains to be determined (127).

Copper can be incorporated into surfaces as bulk alloys, as nanoparticles in coatings and can be embedded in a polymer matrix (52, 57, 137–140). The efficacy of copper surfaces is determined either through dry or wet inoculation techniques, which attempt to replicate environmental conditions associated with in vivo applications (70, 124–126). The wet inoculation technique involves depositing a small drop of liquid inoculum onto a copper surface, which can take more than 30 min to dry (47, 70). This simulates a wet contamination event, such as a sneeze expelled aerosol (70). The dry inoculation technique consists of application of the inoculum with a cotton swab resulting in a thin film that dries rapidly allowing direct contact of bacterial cells with the coupon surface (47, 70). Dry contamination occurs following a hand contact event (124). For example, one group simulated dry and wet contamination of S. aureus by spreading 1 µl of inoculum over 1 cm² area of a copper coupon that dried in seconds (for dry inoculation) and depositing a 20 µl drop of inoculum containing 10⁷ CFU to the surface (for wet
inoculation)(126). Contact killing on dry copper coupons occurs more rapidly compared to moist coupons(126, 131). Accumulation of copper ions in bacterial cells was faster on dry surfaces than wet surfaces, which reflects the observed killing kinetics and is thought to be due to the absence of buffering medium on dry surfaces(131).

There are many published studies examining the efficacy of copper surfaces both in vitro and in vivo; however, there is wide variation in experimental conditions, including incubation temperatures, RH and copper content of the alloys tested, which can make direct comparisons on the efficacy of copper surfaces difficult(51, 56, 123, 134, 141–143). There are some consistent in vitro findings for copper alloys reported allowing generalised conclusions to be made. Overall, alloys with higher copper contents, higher temperature and higher RH are associated with greater bactericidal activity(47, 70). For example, a 10 min exposure to a coupon with 99.9% copper reduced S. typhimurium by 6 logs while a 60% copper coupon reduced cells only by 3 logs(123). A copper coupon (99.9%) required a 90 min exposure time at 20°C to knockdown E. coli O157:H7 while at 4°C the contact time was extended to 270 min(51). Lethal activity of copper coupons against E. faecalis and S. aureus in 100% RH was abolished at low RH (0% and 22%, respectively)(14, 55). In general, 55%-100% copper in alloys is necessary for biocidal activity(70). Poly(propylene) films containing 1% (v/v) copper nanoparticles prepared by the melt mixed method killed E. coli following treatment for 4 h(139). Overall, copper containing surfaces are found to be active against test bacteria in vitro.

Trials have been undertaken in hospitals to assess the efficacy of copper surfaces with measured outcomes being the amount of microbial contamination and the infection rate in patients(144). In general, copper surfaces were found to have lower levels of contamination compared to control surfaces; however, microbial burden was not completely eliminated(144–149). For example, replacement of high-touch surfaces in an acute medical ward with alloys containing a minimum of 60% copper was demonstrated to reduce microbial burden of surfaces (based on total aerobic CFU counts) more than 90% compared to control surfaces. Another trial in an acute medical ward found that all test surfaces coated with copper alloys had reduced microbial numbers compared to controls; however, indicator microorganisms were recovered from both types of surfaces(147).
The reductions in microbial contamination of surfaces in hospitals through the action of copper supports its application in this setting to reduce transmission of infectious disease (146, 148). Trials examining the impact of use of copper surfaces on hospital associated infection (HAI) rates are limited (70, 150). A randomised control trial assessing the efficacy of copper alloy surfaces in reducing the rate of HAI and patient colonisation with MRSA or VRE was undertaken in an intensive care unit of three hospitals (150). Patients were randomly assigned to rooms that had high-touch copper alloy surfaces (more than 70% copper) or control surfaces (150). The rate of HAI and/or colonisation with MRSA or VRE in rooms with copper alloy surfaces was significantly lower than that in standard ICU rooms (0.071 vs 0.123) (150). Therefore, there is some evidence to suggest that implementation of copper alloy surfaces in hospitals will reduce infectious disease transmission.

The requirement for copper to enter cells to exert lethal activity necessitates that copper is released from the surface (52, 121, 151). Copper release from dry surfaces is thought to be mediated by oxidation of surface copper by oxygen (14). Copper release from wet surfaces is dependent on the nature of the surrounding liquid (151). Copper release from copper coupons in the presence of Tris-Cl buffer (pH 7) and water was determined using inductively coupled plasma atomic emission spectroscopy. The Tris-Cl buffer (pH 7) accumulated 42 mM copper after 90 min while water accumulated only 55 µM. Complete bactericidal activity of the copper coupons in Tris-Cl buffer (pH 7) was achieved in 12 min whereas coupons in water required 6 h for this. It was concluded that dissolved copper concentrations correlated with the rates of killing (151). Therefore, release of ionic copper from solid copper surfaces is an important factor in the killing of surface contaminating bacteria (52, 151).

Copper release from polymer films can also be influenced by the size of the copper particles (137). Polypropylene films were impregnated with copper microparticles or copper nanoparticles (137). The nanocomposites demonstrated rapid release of copper particles during the first day while there was slower, more incremental release from the microcomposites (137). The greater antimicrobial efficacy of nanocomposite films compared to microcomposite films against S. aureus and P. aeruginosa was reflective of the degree of copper ion release (137). The length of time a surface is active will be dependent on the size of the copper ion reservoir in the surface and the rate of release. Surface activity will be lost when the concentration of copper ions released from the surface falls below the threshold.
required for bactericidal action, which may occur from sufficient repeated challenges to reduce
the copper ion pool or from interference by another substance, such as organic matter or
cleaning agents, that chelates or prevents the release of copper ions(124). Thus, copper ion
release is the limiting factor in surface efficacy(124, 151). The disadvantages associated with
relying on an antimicrobial agent that must move out of the surface to exert activity may limit
the usefulness of such a surface.

Copper containing surfaces, as for many antimicrobial surfaces, are susceptible to reductions in
activity following soiling with organic matter(52, 57, 58, 152). Bacteria are able to persist on
copper surfaces for extended times, which is believed to be due to the organic matter forming
a protective layer between the bacteria and the copper surface(14, 57). Loss of activity due to
organic soiling has been demonstrated for copper alloy surfaces challenged with 10^7 CFU of
*E. coli* O157:H7 in a food processing plant setting(57). Soiling with organic matter was
simulated with beef liquid (extracted from minced beef) to reflect the presence of meat
residue during processing(57). Of the 7 cast copper alloys (61%-95% copper) tested, only three
alloys (85%, 93% and 95% copper) were able to kill the whole inoculum during the 6 h
treatment(57). Bacteria inoculated with the beef liquid were able to survive on all alloys tested
except for the alloy containing 95% copper(57). Another study found that the presence of
organic matter in the form of sterile blood or sterile pus was able to extend the survival of
MRSA on copper coins from 24 h to more than 12 days, resulting in only 1 log and 2 log
reductions, respectively(152).

The potential for soiling with organic matter in hospital and food processing plant settings is
high, which necessitates consideration of how the activity of antimicrobial surfaces will be
influenced by soiling as well as the cleaning procedures used to reduce organic contamination
of surfaces(52, 58, 124). The effect of cumulative soiling and cleaning on the antimicrobial
activity of a 99.9% copper coupon was determined using the dry inoculation technique with
*S. aureus* suspended in solutions of 1% bovine serum albumin to reflect protein-based organic
soil(58). Following the 24 h treatment the surfaces were cleaned using a standardised protocol
with two cleaning agents, 1% sodium hypochlorite and 70% industrial methylated spirit(58).
After only two soiling/cleaning cycles it was found that a layer consisting of BSA and bacteria
had formed over the copper surface(58). This layer was not removed by further cleaning cycles
and was associated with increased bacterial survival while the stainless control surface was
highly cleanable (58). The accumulation of organic matter on the copper surface was postulated to be due to the high reactivity of copper, resulting in surface conditioning (58, 124). This phenomenon would reduce the practicality of using copper surfaces in settings, which are associated with soiling with organic matter.

Copper impregnated surfaces have demonstrated some potential for reducing surface contamination and disrupting bacterial transmission pathways. Application of copper surfaces may be limited in settings where soiling with organic matter followed by a cleaning procedure is likely to occur. Contaminating organic matter and/or the cleaning agents may chelate the released copper ions or bind to the surface forming a protective layer. The reliance of copper surfaces on release of copper ions to exert activity may restrict use of copper surfaces to short-term applications and would be dependent on the size of the copper ion reservoir in the surface. A surface with an immobilised antimicrobial agent that can exert its activity without dissociating from the surface, such as PANI and P3ABA, would be able to maintain a necessary concentration to reduce bacterial load over a longer period of time.

1.3.4 Summary of current strategies for preventing surface contamination

There are many approaches relating to the development of antimicrobial surfaces (table 1.1). The efficacy and applicability of these surfaces is influenced by the surface characteristics (section 1.2). Some antimicrobial surfaces lose activity in adverse environmental conditions, such as antimicrobial peptides, which restricts use of the surface to applications that do not encounter such conditions (52, 84, 153). Organic soiling can reduce bactericidal activity of a surface, which is undesirable for settings associated with organic contamination, including food processing plants. For example, QACs lose activity in the presence of organic matter (52, 53, 88). Some contamination resistant surfaces must release the antimicrobial agent to exert bactericidal activity, which means the surface is suitable for short-term applications only in readily accessible locations (to facilitate renewal of the surface after activity is lost). Furthermore, release of the antimicrobial agent restricts application for food contact surfaces due to safety concerns. For example, silver has been shown to be toxic to mammalian cells (62, 71, 154, 155).
N-halamine compounds exert bactericidal activity by releasing free active halogen species into the surrounding environment, which means that surfaces containing N-halamine will lose activity over time(52, 112). Activity can be renewed by application of chlorine or bromine donor compounds, such as sodium hypochlorite, limiting use of N-halamine containing surfaces to accessible locations only(52, 112). Use of surface incorporated N-halamines is further restricted due to the instability of N-halamine in the presence of UV light(52). There is also potential for ongoing halogenation to promote breakage of the chemical bonds that hold N-halamine onto the surface due to the oxidising effect of halogen sources(52). There are concerns that exposure to antimicrobial surfaces, including triclosan and QAC based surfaces, will promote the development of resistance(2, 84). Overall, the current strategies for the development of antimicrobial surfaces have one or more of disadvantages limiting applicability to potential settings (table 1.1).

1.4 Polyaniline and poly(3-aminobenzoic acid) are novel antimicrobial agents

1.4.1 Polyaniline and poly(3-aminobenzoic acid)
Conducting polymers (CPs) are a class of polymeric materials that have electronic and ionic conductivity(156, 157). Conductivity of CPs is attributed to the presence of conjugated double bonds located along the backbone of the polymer, which is otherwise an insulated structure(156). Conjugation describes the alternation of single and double bonds(156). For every bond in the backbone there is a localised ‘sigma’ (s) bond – a strong chemical bond responsible for single bonds – while double bonds also contain a less strongly localised ‘pi’ (p) bond(156). Application of a dopant ion to a conducting polymer imparts conductivity as the dopant ion can carry charge in the form of extra electrons – either as charged polarons (i.e. radical ions) or bipolarons (i.e. dications or dianions) (156). The dopant neutralises the unstable backbone when the polymer is in the oxidised form(156, 157). Electrons from one subunit are attracted to the nuclei in the neighbouring subunit giving rise to charge mobility (called ‘electron hopping’) along and between the polymer chains(156, 158). This organised movement of charge along a polymer chain backbone imparts electrical conductivity(156).

Polyaniline (PANI) and its derivatives comprise an important and widely studied class of conducting polymers(157, 158). Polyanilines are deemed to be derived from a polymer
(Fig. 1.3) consisting of reduced (benzenoid diamine) and/or oxidised (quinoid diamine) aniline subunits (157–159). The relative numbers of these subunits can, in theory, vary continuously from all reduced to all oxidised subunits (157). This determines the average oxidation state 

\( (1 - y) \), which in turn influences other physical characteristics of CPs, such as colour (158, 160, 161). Reduced polymers have an average oxidation state of zero and are termed leucoemeraldine (157, 158, 162). ‘Half-oxidised’ polymers have an overall oxidation state of 0.5 and are called emeraldine (157, 158, 162). Oxidised polymers, known as pernigraniline, have an average oxidation state of 1 (157, 158). The emeraldine state has greater stability at room temperature and the doped form (emeraldine salt) is electrically conducting, which accounts for this being considered the most applicable form of PANI (158, 162). Conductivity can be controlled (from non-doped semiconducting to fully doped highly conducting forms) by adjusting the doping level in a controlled manner (156, 157). By changing dopant concentrations, electrical conductivities can be varied by as much as 15 orders of magnitude (156).

\[
\left[ \left( \text{reduced unit} \right)^y \left( \text{oxidised unit} \right)^{1-y} \right]_n
\]

Legend:
- \( y = 1 \) Leucoemeraldine base (LEB)
- \( y = 0.5 \) Emeraldine base (EB)
- \( y = 0 \) Pernigraniline base (PNB)

**Emeraldine salt (ES)**

Figure 1.3. The structure of polyaniline.
Utilisation of PANI for many different potential applications is restricted because of its insolubility in common solvents, such as dimethyl sulfoxide (DMSO) and water (163, 164). The insolubility of PANI renders it difficult to process, which has led to a number of approaches to improve processability, including doping PANI with functionalised protonic acids and utilisation of functionalised aniline monomers during synthesis (156, 165–167). Aniline monomers can be functionalised through substitution of groups, such as –SO$_3$H or –COOH, into the benzene ring, which results in formation of functionalised anilines, including sulfanilic acid and aminobenzoic acid (ABA), respectively (168). A polymer made using functionalised aniline monomers is a functionalised polyaniline (fPANI) (159, 168). Copolymers are formed following polymerisation of aniline and functionalised aniline monomers while homopolymers consist of only substituted anilines (165–167). In general, fPANIs have improved solubilities but lower electrical conductivities (165–167). The decrease in conductivity is believed to be due to steric effects of the incorporated functional group disrupting the planarity of the conjugated system (156).

One promising fPANI is poly(3-aminobenzoic acid), P3ABA, which is a homopolymer of 3-aminobenzoic acid (3ABA) (163). P3ABA can be synthesised by electro-polymerisation or chemical polymerisation of 3ABA monomers (163, 165, 169). ABAs consist of an aniline benzene ring substituted with a carboxylic acid group (Fig. 1.4) (170). Copolymers of aniline with ABAs, such as poly(aniline-co-3-aminobenzoic acid) and poly(2-aminobenzoic acid), are soluble in common solvents such as N-methyl-2-pyrrolidone and DMSO (165, 168, 171, 172). The increased solubility is derived from the decreased rigidity of the polymer chain leading to weakened interchain hydrogen bonds and greater interaction with the solvents (168, 171). The greater solubility of fPANIs in common solvents is associated with improved processability, facilitating greater potential utilisation of fPANI in industrial and biological applications (163).

![Figure 1.4. Structure of the 3-aminobenzoic acid monomer.](image-url)
1.4.2 Synthesis of PANI and P3ABA

PANI is synthesised via chemical or electrochemical oxidative polymerisation of aniline monomer units(159, 160, 173). The polymerisation process involves removal of electrons from the polymer backbone, which generates areas with positive charges(174). These positive charges are neutralised by application of an anionic dopant, such as aromatic sulphonate variants para-toluenesulphonate and sodium benzenesulphonate, which stabilise the backbone and allow the polymer to form(156, 157). Electrochemical synthesis is achieved by anodic oxidation of aniline monomers on an inert electrode, which is usually made of platinum or gold(161, 164). Chemical synthesis consists of direct monomer oxidation using an oxidising agent, such as ammonium persulfate (APS), in an acidic medium (pH 0-2) (164, 175–177). Monomer oxidation results in formation of dimers, which are immediately oxidised (159, 162, 176). The oxidised dimers react with an aniline monomer via an electrophilic aromatic substitution(162). As this oxidative polymerisation process continues the polymer chains are propagated by addition of an aniline monomer to the chain end eventuating in formation of PANI(162). Electrochemical oxidation is the preferred method as it allows better control and increased flexibility over the polymerisation process enabling better manipulation of properties of the final product(173, 174). The chemical means of synthesis is recommended for production of large quantities of polymer(160).

PANI and P3ABA were synthesised via chemical oxidation of aniline and 3ABA monomers, respectively(163). For PANI synthesis, an aniline/HCl solution and an APS/HCl solution were prepared by dissolving 9.6 ml of aniline and 9.1 g of APS, respectively, in 120 ml of 1.25 M HCl solution(163). The aniline/HCl solution was cooled to 5 °C then the solution of APS/HCl was added dropwise while stirring for 5 h. The reaction mixture was filtered and washed thoroughly with distilled water (∼ 500 ml) and the filtrate was transferred to a flask and stirred for 15 min with 150 ml of acetone and filtered again. Retentate was left to dry in a vacuum oven at 40 °C overnight. For P3ABA synthesis, a 3ABA/HCl solution and an APS/HCl solution were prepared by dissolving 14.45 g 3ABA and 9.1 g of APS, respectively, in 120 ml of 1.25 M HCl solution(163). Every batch was prepared with twice those quantities; using a 1 l round flask in a temperature controlled water-bath set at 5 °C. The solution of APS was added dropwise while stirring, and after 5 h of reaction, the solution was filtered. Only 50 ml of distilled water was used to wash the resulting product before being dried under vacuum at 40 °C(163).
1.4.3 Antimicrobial activity of PANI and P3ABA

PANI and fPANI are novel antimicrobial agents that are active against a broad range of bacteria including *E. coli*, *S. aureus*, *P. aeruginosa*, *E. faecalis*, *C. jejuni* and *Staphylococcus sciuri* (163, 178–180). For example, polyvinyl alcohol films containing 1% PANI cleared *E. coli* and *S. aureus* from the film surface (178). PANI-coated conductive cotton fabric was shown to reduce the load of *S. aureus* by 95% and *E. coli* by 85% (181). The mechanistic basis of antimicrobial activity is hypothesised to be electrical conductivity, which can mediate contact with the negatively charged bacterial cell surface through electrostatic adherence (163, 178, 181). Use of either APS or potassium iodate as an oxidising agent during PANI or fPANI synthesis does not influence the bactericidal activity of the product (140, 163). Copolymers of aniline and ABAs tend to have greater antimicrobial activity compared to PANI (163, 182). The position of the functional group, including –COOH, in the functionalised monomer does not seem to influence the antimicrobial activity of the fPANI (163). The activity of PANI and two fPANIs, poly(aniline-co-2-aminobenzoic acid) (2ABAPANI) and poly(aniline-co-3-aminobenzoic acid (3ABAPANI), in suspension has been investigated (163). A concentration of 10 mg/ml of PANI was required to inhibit the growth of *E. coli*, *P. aeruginosa* and *S. aureus* (163). 2ABAPANI and 3ABAPANI prevented the growth of test strains at a concentration of 1.25-2.5 mg/ml (163).

The length of the polymer chain can influence bactericidal activity with shorter chain lengths associated with reduced activity (182). Low molecular weight oligomers, such as dimers, have been demonstrated to be less active against *P. aeruginosa* than the parent polymer (182).

Determination of the mode of action of an antimicrobial agent is of value (53, 183). Knowledge of mechanism of action aids in understanding and interpreting the antimicrobial activity in experimental assays (53). Rational improvement of an antimicrobial agent, such as functionalisation of PANI, is reliant on elucidation of antimicrobial mechanism (47, 183, 184). The possibility of the emergence and spread of resistant organisms may also be informed from the mode of action of an antimicrobial agent (47, 184).

The antimicrobial mechanisms of an fPANI, a homopolymer of sulfanilic acid (PSO₃H), and P3ABA have been previously examined. Initial hypotheses were generated following analysis of the results of a transcriptomic analysis of *E. coli* MG1655 sublethally treated PSO₃H (163, 185). The PSO₃H exposed *E. coli* demonstrated upregulation of stress response genes involved in defence against oxidative and periplasmic stress as well as genes associated with iron
homeostasis. Expression of some genes in the OxyR regulon, which is activated by hydrogen peroxide, were increased, such as \textit{trxC}, \textit{grxA}, \textit{mntH} and \textit{sufB}(163, 186). The protein products of these genes act to restore redox balance (\textit{trxC}, \textit{grxA}) and reactivate or repair oxidatively damaged enzymes (\textit{mntH} and \textit{sufB})(163, 186). A few genes in the SoxRS regulon, which responds to superoxide-based stress, were also upregulated, such as \textit{soxR}, \textit{soxS} and \textit{fumC} (163, 187). Periplasmic stress response genes included \textit{spy}, \textit{asr} and \textit{cpxP}, which encode proteins that act to reduce protein misfolding and aggregation(163, 188, 189). Upregulated genes involved in iron homeostasis included \textit{sufC} and \textit{fes}(163, 190). The \textit{sufC} gene is a member of the SUF pathway and encodes an ATPase that facilitates transfer of nascent clusters to apo-protein substrates or a carrier protein during repair of oxidised FeS clusters(191, 192). The \textit{fes} gene encodes an esterase enzyme that mediates release of iron from the enterobactin-iron complex during iron import(193, 194).

Downregulation of genes was also identified in the transcriptomic analysis(163). These genes encoded proteins involved in central metabolism and energy generation including tricarboxylic acid (TCA) cycle genes, such as \textit{sdhB} and \textit{aceA}(163). The gene \textit{sdhB} encodes the b subunit of succinate dehydrogenase (SDH)(195, 196). SDH oxidises succinate generating fumarate in the TCA cycle. Electrons from this oxidation are shuttled into the electron transport chain (ETC), providing a functional link between the TCA cycle and the ETC(195, 196). The SdhB subunit contains an [4Fe-4S]^{2+} cluster, which is susceptible to oxidation by hydrogen peroxide resulting in dissociation of an iron atom and loss of enzyme activity(195, 197). Isocitrate lyase, encoded the \textit{aceA} gene, is a glyoxylate shunt enzyme that cleaves isocitrate to generate succinate and glyoxylate(198, 199). Isocitrate lyase expression is induced by oxygen(198). The transcriptional response of \textit{E. coli} MG1655 to the fPANI led to the development of several hypotheses pertaining to potential mechanisms of action including induction of oxidative stress, dysregulation of iron homeostasis, targeting of metabolic and respiratory enzymes and disruption of membrane integrity.

The mode of action of P3ABA has been previously investigated(200). Electron paramagnetic resonance (EPR) spectroscopy was used to detect unpaired electrons in Fe$^{3+}$ permitting quantitation of free iron levels in \textit{E. coli} 25922 sublethally treated with P3ABA(201, 202). The P3ABA exposed \textit{E. coli} cells were found to have 146.48 µM of free iron, which was greater than that of cells treated with 20 mM hydrogen peroxide (90.36 µM) and untreated cells.
An increase in intracellular free iron is known to occur following exposure to hydrogen peroxide and superoxide due to oxidation of the exposed iron atom in [4Fe-4S]^{2+} clusters in metabolic and respiratory enzymes or Fe^{2+} in mononuclear metabolic iron enzymes, which results in iron dissociation (203–205). The increase in free iron detected in P3ABA treated *E. coli* cells corroborates hypotheses generated from the transcriptomic analysis of P3O_{3}H treated *E. coli* cells that relate to induction of oxidative stress, disruption of iron homeostasis and targeting of metabolic and respiratory enzymes.

P3ABA was also demonstrated to disrupt membrane integrity in *E. coli* cells as indicated by the increased sensitivity to P3ABA in a hyperosmotic environment and rapid loss of membrane potential following P3ABA exposure (200). *S. aureus* 6538 treated with 0.5% P3ABA suspension died at a faster rate in a high salinity environment (6% NaCl), which suggested that the combined hurdle of 6% NaCl and 0.5% P3ABA suspension led to disruption of membrane integrity (200, 206). *E. coli* membrane potential was examined using the BacLight Bacterial Membrane Potential Kit and flow cytometry (200, 206, 207). Membrane potential in P3ABA treated cells was 13% of that of untreated cells (200). These results suggested that P3ABA disrupts membrane integrity supporting the hypothesis formed from the transcriptomic analysis of P3O_{3}H treated cells that fPANI treatment disrupts membrane integrity.

In this thesis I sought to build on previous research and characterise the antimicrobial mechanisms of PANI and P3ABA against *E. coli* and *S. aureus*. Elucidation of the mode of action of these antimicrobial agents permitted comparison of the mechanisms, with any differences found potentially derived from the functionalisation of P3ABA.

1.4.4 PANI and P3ABA have potential as non-leaching additives to provide contamination resistant surfaces

PANI and P3ABA are novel antimicrobial agents that have potential as non-leaching additives to provide contamination resistant surfaces (181, 182). These agents have favourable properties supporting the development of surface applications including broad spectrum activity, thermal stability up to 300 °C, environmental stability in the conducting form, and simple and inexpensive synthetic procedures (158, 163, 173, 182). The ease and low cost of production of PANI and P3ABA makes these antimicrobial agents suitable for large scale production. Thermal and environmental stability increases the applicability of PANI and P3ABA.
to potential uses. PANI and fPANI have been demonstrated to be biocompatible with mammalian cells (208–211). It has been found that PANI incorporated into a surface does not leach, which is a desirable property for an antimicrobial surface (163, 181, 185).

In this thesis I sought to investigate the efficacy of PANI and P3ABA when incorporated into different types of surfaces as surface immobilisation may cause a reduction in activity (53). It was hypothesised that the incorporation of PANI or P3ABA into absorbent or non-absorbent surfaces would impart antimicrobial activity that would protect against colonisation.

### 1.5 Aims of the project

The overall aim of this thesis is to investigate and characterise the antimicrobial action of PANI and fPANI against common bacterial targets. Following from this, both the mechanism of action and the activity of the polymers incorporated into different types of surfaces was examined. The aims of the experimental work examining antimicrobial mechanism are based on the previous work discussed in section 1.4.3. These aims encompass further elucidation of the antimicrobial mechanism of P3ABA and determination of the antimicrobial mechanism of PANI to enable a comparison between the two polymers. From these results it may be inferred what part of mechanism results from electrical conductivity and what part of mechanism results from the addition of the functional group. Hypotheses relating to investigation of the antimicrobial mechanism include:

1. **Bacteria carrying mutations in genes encoding targets of the antimicrobial activity will exhibit insensitivity to fPANI and/or PANI.**
2. **Bacteria carrying mutations in genes encoding antimicrobial stress-response mediators will exhibit super-sensitivity to fPANI and/or PANI.**
3. **Antimicrobial action of PANI and P3ABA against model Gram-negative and Gram-positive bacteria is enhanced in the presence of oxygen and decreased in the absence of oxygen reflecting involvement of reactive oxygen species.**

Work presented in this thesis also focussed on characterising the antimicrobial activity of PANI and P3ABA as surface antimicrobials to reduce the transmission of pathogens. It is hypothesised that:
4. PANI and P3ABA exert antimicrobial activity against model Gram-negative, Gram-positive bacteria and acid-fast mycobacteria in aqueous suspension, absorbent and non-absorbent surfaces.

5. The efficacy of PANI and P3ABA at surfaces is influenced by the size of the bacterial challenge and the presence of organic material.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Bacterial strains
The antimicrobial mechanism of PANI and P3ABA was initially investigated using *E. coli* single gene deletion mutants from the Keio collection (212, 213). The parent strain, *E. coli* K-12 strain BW25113 (F- Δ(araD-araB)567, ΔlacZ4787::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514) and a selection of the isogenic *E. coli* deletion mutants were used in this work. These deletion mutants were *E. coli JW3914-1 ΔkatG, E. coli JW1721-1 ΔkatE, E. coli JW0598-2 ΔahpC, E. coli JW3879-1 ΔsodA, E. coli JW1648-1 ΔsodB, E. coli JW1638-1 ΔsodC, E. coli JW0669-2 Δfur, E. coli JW2566-1 ΔtrxC, E. coli JW0833-1 ΔgrxA, E. coli JW5195-1 ΔtonB, E. coli JW2514-4 ΔiscS, E. coli JW0714-1 ΔsdhB, E. coli JW3715-1 ΔatpE, E. coli JW1732-1 Δspy, E. coli JW5826-1 Δasr, E. coli JW2669-1 ΔrecA and E. coli JW1625-1 Δnth.*

*E. coli* ATCC® 25922™ (referred to as *E. coli 25922*) and *S. aureus* subsp. *aureus* ATCC® 6538™ (referred to as *S. aureus 6538*) were selected for further characterisation of the action of PANI and P3ABA because they are standard antibiotic sensitivity testing strains (214–216). They are routinely used as control organisms to verify that antibiotic susceptibility results are accurate (214, 217). *E. coli 25922* was tagged with an integrating plasmid (p16S_lux) containing the bacterial luciferase (lux) operon (designated *E. coli 25922 lux*) (218, 219). *E. coli 25922 lux* was used for testing of surfaces containing PANI and P3ABA (53).

For determination of the antimycobacterial activity of PANI and P3ABA surfaces, *Mycobacterium smegmatis* MC²155 (ATCC 700084) was used as a model for *M. tuberculosis* in studies (220). *M. smegmatis* MC²155 was tagged with an integrating plasmid (pMVhspLuxABG13CDE) containing the bacterial luciferase (lux) operon (designated *M. smegmatis* MC²155 lux) using a standard method (221). *M. tuberculosis* reference strain H37Rv (ATCC 27294) and *M. tuberculosis* clinical isolate Rangipo N were similarly tagged (designated *M. tuberculosis* H37Rv lux and *M. tuberculosis* Rangipo N lux, respectively) (222).
2.1.2 Suppliers
The materials used to complete the experimental work in this thesis were obtained from the following suppliers:

- Ahlstrom (Helsinki, Finland)
- Alphatech (Auckland, New Zealand)
- Amersham Biosciences (through GE Healthcare Life Sciences, Auckland, New Zealand)
- Axygen (through Medi’Ray, Auckland, New Zealand)
- Biotek Instruments, Inc. (through Millennium Science, Auckland, New Zealand)
- BD (Becton, Dickinson and Company; through Fort Richard, Auckland, New Zealand)
- Copan (through Fort Richard, Auckland, New Zealand)
- School of Chemistry, University of Auckland
- Greiner Bio-One (through Medi’Ray, Auckland, New Zealand)
- Lab M Limited (through Fort Richard, Auckland, New Zealand)
- Neptune Scientific (through Medi’Ray, Auckland, New Zealand)
- Pauling Industries (Auckland, New Zealand)
- PerkinElmer Life and Analytical Sciences (through Scimed, Christchurch, New Zealand)
- Sartorius (through Thermo Scientific, Auckland, New Zealand)
- Scharlau, Scharlab S. L. (through Global Science, Auckland, New Zealand)
- SSI (through Alphatech, Auckland, New Zealand)
- Sigma-Aldrich (Sydney, Australia)
- Techno Plas (through Medi’Ray, Auckland, New Zealand)
- Thermo Scientific (Auckland, New Zealand)

2.1.3 Chemicals and cell biology reagents
Table 2.1 outlines the general cell biology reagents and chemicals used in the experiments to test hypotheses. Ethanol was used to establish to a 70% solution for disinfection of experimental film samples (section 2.2.6; section 2.2.7; section 2.2.10; section 2.2.11)(65). Methylene blue and resazurin sodium salt were made up into solutions that were used as indicators of oxygenation (section 2.2.4)(223). Polyaniline and poly(3-aminobenzoic acid) were
the antimicrobial agents that were the focus of this work. Sodium chloride was made into an isotonic solution that was used for dilution of bacterial cells (section 2.2.2.1)(214).

Table 2.1. General cell biology reagents and chemicals used in experiments.

<table>
<thead>
<tr>
<th>Item</th>
<th>Reference number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, general purpose reagent, 100%</td>
<td>SDA-3A</td>
<td>Pauling Industries</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>M9140</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>C_{16}H_{18}ClN_{3}S.xH_{2}O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(3-aminobenzoic acid) P3ABA</td>
<td>N/A</td>
<td>School of Chemistry, Auckland</td>
</tr>
<tr>
<td>Polyaniline PANI</td>
<td>N/A</td>
<td>School of Chemistry, Auckland</td>
</tr>
<tr>
<td>Resazurin sodium salt C_{12}H_{6}NNaO_{4}</td>
<td>R7017</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium chloride NaCl</td>
<td>MC017</td>
<td>Lab M Limited</td>
</tr>
</tbody>
</table>

*N/A indicates not applicable*
Cell biology reagents were made into solutions, which were used to support bacterial growth. The reagents used are outlined in table 2.2.

Table 2.2. Cell biology reagents used to support bacterial growth.

<table>
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<tr>
<th>Item</th>
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<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>7H11 agar, Difco™ Mycobacteria</td>
<td>283818</td>
<td>BD</td>
</tr>
<tr>
<td>7H9 broth, Difco™ Mycobacteria</td>
<td>271310</td>
<td>BD</td>
</tr>
<tr>
<td>ADC Enrichment, BBL™ Middlebrook †</td>
<td>212352</td>
<td>BD</td>
</tr>
<tr>
<td>Agar, Difco™ granulated</td>
<td>214530</td>
<td>BD</td>
</tr>
<tr>
<td>Ammonium sulfate (NH₄)₂SO₄</td>
<td>A4418</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>223050</td>
<td>BD</td>
</tr>
<tr>
<td>Cysteine, L-cysteine HSCH₂CH(NH₂)CO₂H</td>
<td>C7352</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Glycerol, 99.5% reagent grade, ACS</td>
<td>GL00262500</td>
<td>Scharlau, Scharlab S. L.</td>
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<td>Kanamycin sulfate C₁₈H₃₆₋₃₇N₄₅O₁₀₋₁₁.H₂SO₄</td>
<td>60615</td>
<td>Sigma-Aldrich</td>
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<td>LB broth, Difco™, Lennox</td>
<td>240230</td>
<td>BD</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate MgSO₄.7H₂O</td>
<td>M1880</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>OADC Enrichment, BBL™ Middlebrook ††</td>
<td>212240</td>
<td>BD</td>
</tr>
<tr>
<td>Potassium nitrate KNO₃</td>
<td>P8394</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Potassium phosphate dibasic K₂HPO₄</td>
<td>P8281</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Potassium phosphate monobasic KH₂PO₄</td>
<td>P9791</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium citrate tribasic dihydrate HOC(COONa)(CH₃COONa)₂.2H₂O</td>
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<tr>
<td>Sodium succinate dibasic hexahydrate NaOOCCH₂CH₂COONa.6H₂O</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Thiamine hydrochloride</td>
<td>T4625</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tween 80, from non-animal source</td>
<td>P6224</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

† ADC contains per litre of purified water: NaCl 8.5 g, bovine albumin (Fraction V) 50 g, dextrose 20 g, catalase 0.03 g

†† OADC contains per litre of purified water: NaCl 8.5 g, bovine albumin (Fraction V) 50 g, dextrose 20 g, catalase 0.03 g, oleic acid 0.6 ml
2.1.4 Solutions
The cell biology reagents listed in table 2.2 were made into solutions that were used to support bacterial growth. The general growth media solutions generated and the method of sterilisation used are outlined in table 2.3. The minimal media growth solutions used in this experimental work are outlined in table 2.4.

Table 2.3. Growth media solutions used to support bacterial growth.

<table>
<thead>
<tr>
<th>Growth media solution</th>
<th>Composition</th>
<th>Amount in 1 litre of solution (g or ml)</th>
<th>Sterilisation technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>7H9 broth (w/v) †</td>
<td>7H9 broth powder</td>
<td>4.7</td>
<td>Autoclave</td>
</tr>
<tr>
<td>7H9 supplemented broth (v/v)</td>
<td>7H9 broth</td>
<td>900</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>ADC</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>7H11 agar (w/v) †</td>
<td>7H11 agar powder</td>
<td>20.5</td>
<td>Autoclave</td>
</tr>
<tr>
<td>7H11 supplemented agar (v/v)</td>
<td>7H11 agar</td>
<td>900</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>OADC</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Agar, 2× (w/v) stock solution</td>
<td>Agar</td>
<td>30</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Ammonium sulfate, 757 mM (w/v) stock solution</td>
<td>Ammonium sulfate</td>
<td>100</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Casamino acids, 20% (w/v) stock solution</td>
<td>Casamino acids</td>
<td>200</td>
<td>Filter sterilise, 0.2 µm pore size</td>
</tr>
<tr>
<td>Cysteine, 2% (w/v) stock solution</td>
<td>Cysteine</td>
<td>20</td>
<td>Filter sterilise, 0.2 µm pore size</td>
</tr>
<tr>
<td>Glycerol, 50% (v/v) stock solution</td>
<td>Glycerol</td>
<td>500</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Kanamycin, 30 mg/ml stock solution</td>
<td>Kanamycin sulfate</td>
<td>25</td>
<td>Filter sterilise, 0.2 µm pore size</td>
</tr>
<tr>
<td>LB agar (w/v)</td>
<td>LB broth</td>
<td>20</td>
<td>Autoclave</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>LB agar with 30 µg/ml kanamycin (v/v)</td>
<td>LB agar</td>
<td>1,000</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Kanamycin stock solution</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LB broth (w/v)</td>
<td>Tryptone</td>
<td>10</td>
<td>Autoclave</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Solution Description</td>
<td>Component</td>
<td>Concentration</td>
<td>Notes</td>
</tr>
<tr>
<td>--------------------------------------------------------------</td>
<td>----------------------------</td>
<td>---------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>LB broth, 2× (w/v)</td>
<td>NaCl</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tryptone</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>10</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate, 1 M (w/v) stock solution</td>
<td>Magnesium sulphate</td>
<td>24.65</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Potassium phosphate dibasic, 603 mM (w/v) stock solution</td>
<td>Potassium phosphate</td>
<td>105</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Potassium phosphate monobasic, 331 mM (w/v) stock solution</td>
<td>Potassium phosphate</td>
<td>45</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Potassium nitrate, 2 M (w/v) stock solution</td>
<td>KNO₃</td>
<td>202.2</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Sodium citrate, 170 mM (w/v) stock solution</td>
<td>Sodium citrate</td>
<td>50</td>
<td>Autoclave</td>
</tr>
<tr>
<td></td>
<td>tribasic dehydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate, 40% (w/v) stock solution</td>
<td>Sodium succinate</td>
<td>400</td>
<td>Filter sterilise, 0.2 µm</td>
</tr>
<tr>
<td></td>
<td>dibasic hexahydrate</td>
<td></td>
<td>pore size</td>
</tr>
<tr>
<td>Thiamine, 0.5 mg/ml stock solution</td>
<td>Thiamine hydrochloride</td>
<td>0.5</td>
<td>Filter sterilise, 0.2 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pore size</td>
</tr>
<tr>
<td>Tween 80, 25% (v/v) stock solution</td>
<td>Tween 80</td>
<td>250</td>
<td>Autoclave</td>
</tr>
</tbody>
</table>

All solutions are made up to 1 litre using deionised water

w/v indicates weight (g) per unit volume (ml)

v/v indicates volume (ml) per unit volume (ml)

N/A indicates not applicable as solutions do not require sterilisation

† 7H9 broth and 7H11 agar are made up to 900 ml to allow for addition of 100 ml ADC and OADC, respectively
Table 2.4. Minimal media solutions used to support bacterial growth.

<table>
<thead>
<tr>
<th>Growth media solution</th>
<th>Composition</th>
<th>Amount in 1 litre of solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal A salts, 1× solution</td>
<td>Ammonium sulphate stock solution</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulphate heptahydrate stock solution</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Potassium phosphate dibasic stock solution</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Potassium phosphate monobasic stock solution</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sodium citrate stock solution</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Thiamine stock solution</td>
<td>1</td>
</tr>
<tr>
<td>1× Minimal A salts with 0.7% glycerol</td>
<td>50% glycerol stock solution</td>
<td>14</td>
</tr>
<tr>
<td>1× Minimal A salts with 0.4% succinate</td>
<td>40% succinate stock solution</td>
<td>10</td>
</tr>
<tr>
<td>1× Minimal A salts with 0.7% glycerol and potassium nitrate</td>
<td>50% glycerol stock solution</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Potassium nitrate stock solution</td>
<td>10</td>
</tr>
<tr>
<td>1× Minimal A salts with 0.4% succinate and potassium nitrate</td>
<td>40% succinate stock solution</td>
<td>10</td>
</tr>
<tr>
<td>1× Minimal A salts with 0.4% succinate and 0.1% casamino acids</td>
<td>Potassium nitrate stock solution</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>40% succinate stock solution</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Casamino acids stock solution</td>
<td>5</td>
</tr>
</tbody>
</table>

All solutions are made up to 1 litre using deionised water.
General solutions used in this work (table 2.5) were established using the cell biology reagents and chemicals listed in table 2.1.

Table 2.5. General solutions used in experiments.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Amount in 1 litre of solution (g or ml)</th>
<th>Sterilisation technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.85% saline (w/v)</td>
<td>Sodium chloride</td>
<td>8.5</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Ethanol, 70% (v/v)</td>
<td>Ethanol, 100%</td>
<td>700</td>
<td>N/A</td>
</tr>
<tr>
<td>Methylene blue, 0.2% (w/v) stock solution</td>
<td>Methylene blue</td>
<td>2</td>
<td>Filter sterilise, 0.2 µm pore size</td>
</tr>
<tr>
<td>Resazurin, 0.2% (w/v) stock solution</td>
<td>Resazurin sodium</td>
<td>2</td>
<td>Filter sterilise, 0.2 µm pore size</td>
</tr>
</tbody>
</table>

All solutions are made up to 1 litre using deionised water
w/v indicates weight (g) per unit volume (ml)
v/v indicates volume (ml) per unit volume (ml)
N/A indicates not applicable as solution does not require sterilisation

2.1.5 Consumable materials and equipment

The consumable materials utilised in this work with reference number and supplier are outlined in table 2.6. The equipment used to conduct experiments are listed in table 2.7 along with the supplier. The particle size distribution of PANI was determined using the Mastersizer 2000. Optical densities at 600 nm (OD$_{600}$) of bacterial cultures were determined using an Ultrospec 2100 pro UV/Visible spectrophotometer with semi-micro cuvettes having a 1 cm path length (table 2.6). PANI, P3ABA and other reagents used in this work were weighed using a Cubis® Analytical Balance. P3ABA was suspended in liquid media using a QSonica Q700 Sonicator. Bacterial cultures were washed (section 2.2.7; section 2.2.11) by using a Heraeus Biofuge Fresco microcentrifuge to pellet cells before application of fresh media. GasPak™ EZ Small Incubation Containers were used in conjunction with GasPak™ EZ Anaerobic Container System sachets (table 2.6) to create an anaerobic environment for antimicrobial testing (section 2.2.4). Bioluminescence release from bacterial cultures in a 96 well plate was measured with a VICTOR X Multilabel Plate Reader (sections 2.2.5 – 2.2.7; sections 2.2.9 – 2.2.11). A µQuant™ Microplate Spectrophotometer was used to measure OD$_{600}$ in wells of a 96 well plate during surface testing experiments (section 2.2.5.4; section 2.2.6.3; section
were disinfected using 70% ethanol (table 2.5) and dried in a Herasafe™ KS (NSF) Class II, Type A2 Biological Safety Cabinet.

Table 2.6. Consumable materials used in experiments.

<table>
<thead>
<tr>
<th>Item</th>
<th>Reference number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml tubes</td>
<td>S5016SU</td>
<td>Techno Plas</td>
</tr>
<tr>
<td>50 ml tubes, Falcon™</td>
<td>352070</td>
<td>BD</td>
</tr>
<tr>
<td>dark OptiPlate-96 well microtitre plate</td>
<td>6005270</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>96 well plate, flat bottom, clear, sterile</td>
<td>CLS3370</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cuvettes, semi-micro</td>
<td>613101</td>
<td>Greiner Bio-One</td>
</tr>
<tr>
<td>GasPak™ EZ Anaerobic Container System sachets</td>
<td>260678</td>
<td>BD</td>
</tr>
<tr>
<td>Lid-96, Non-Sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear Lid for 96 well Microplates</td>
<td>6005617</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>Microcentrifuge tube, 1.7 ml</td>
<td>AXYGMC175-L-C</td>
<td>Axygen</td>
</tr>
<tr>
<td>Petri dishes, 90 mm, clean environment manufactured</td>
<td>S9014UV20</td>
<td>Techno Plas</td>
</tr>
<tr>
<td>Serological pipette, 25 ml</td>
<td>760 180</td>
<td>Greiner Bio-One</td>
</tr>
<tr>
<td>Spreadsers, L-shaped</td>
<td>174CS05</td>
<td>Copan</td>
</tr>
<tr>
<td>Syringe filter, 0.2 µm Reliaprep™</td>
<td>760502</td>
<td>Ahlstrom</td>
</tr>
<tr>
<td>Syringe, 10 ml</td>
<td>302149</td>
<td>BD</td>
</tr>
</tbody>
</table>
Table 2.7. Equipment used in experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>µQuant™ Microplate Spectrophotometer</td>
<td>Biotek Instruments, Inc.</td>
</tr>
<tr>
<td>Cubis® Analytical Balance (MSE224S-0CE-DU)</td>
<td>Sartorius</td>
</tr>
<tr>
<td>GasPak™ EZ Small Incubation Container</td>
<td>BD</td>
</tr>
<tr>
<td>Heraeus Biofuge Fresco microcentrifuge</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Herasafe™ KS (NSF) Class II, Type A2 Biological Safety Cabinet</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Mastersizer 2000</td>
<td>Malvern</td>
</tr>
<tr>
<td>QSonica Q700 Sonicator</td>
<td>Alphatech</td>
</tr>
<tr>
<td>Ultrospec 2100 pro UV/Visible spectrophotometer</td>
<td>Amersham</td>
</tr>
<tr>
<td>VICTOR X Multilabel Plate Reader (2030-0050)</td>
<td>PerkinElmer Life and Analytical Sciences</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Growth of bacteria

2.2.1.1 Growth on agar

Bacteria were stored as glycerol stocks at -80°C (224). Single colonies were isolated from these stocks when required using the streak plate method (224). The resulting streak plates were incubated at 37°C until colonies appeared. The E. coli deletion mutants were isolated on LB agar plates containing 30 µg/ml of kanamycin (table 2.3) to select the desired cells (212). The parent strain for the deletion mutants was isolated on LB agar plates (212). E. coli 25922, E. coli 25922 lux and S. aureus 6538 were streaked onto LB agar plates. Mycobacteria were isolated on 7H11 supplemented agar (table 2.3).

Agar plates of E. coli 25922, E. coli 25922 lux and S. aureus 6538 were incubated for 16 h. The E. coli deletion mutant agar plates required 16 h - 24 h of incubation due to the varied growth rates of the strains. Agar plates of M. smegmatis MC²155 and M. smegmatis MC²155 lux were incubated for 2-3 days while all M. tuberculosis strains required 3 weeks of incubation.
2.2.1.2 Growth in liquid
Growth of bacteria to generate inocula was set up as a 10 ml culture in a 50 ml tube while treatments with suspensions were performed as a 1 ml sample in a 5 ml tube. Bacteria grown in liquid growth media were incubated at 37°C with 200 rpm shaking. *E. coli* BW25113 and the *E. coli* deletion mutants were grown in LB broth and in minimal A salts with 0.4% succinate as the carbon source (table 2.4). *E. coli ΔiscS*, *E. coli ΔtonB* and *E. coli ΔsdhB* required supplementation 0.1% casamino acids for growth (table 2.3)(204, 225). *E. coli ΔatpE* was unable to grow in minimal media even with supplementation of casamino acids. LB broth was used to support the growth of *E. coli* 25922, *E. coli* 25922 *lux* and *S. aureus* 6538. Minimal A salts media with 0.7% glycerol and 0.4% succinate was also used to grow *E. coli* 25922 and *E. coli* 25922 *lux*. Mycobacteria were grown in 7H9 supplemented broth with the Tween 80 stock solution added to final concentration of 0.05%(226).

Anaerobic growth was achieved using the GasPak™ EZ Anaerobic Container System, which consists of a GasPak™ EZ Small Incubation Container and GasPak™ EZ Anaerobic Container System sachets(227, 228). The sachets generate anaerobic conditions (≤ 1% oxygen) in the container within 2.5 h at 35°C(227). Lids on the tubes incubated in the GasPak™ EZ Anaerobic Container System were loosened to encourage gas exchange.

2.2.1.3 Preparation of inoculum
Bacterial cultures were set by transferring 4 to 5 colonies from a streak plate to a 50 ml tube containing 10 ml liquid growth media(214). In general, cultures were incubated aerobically at 37°C with 200 rpm shaking until turbid. For fast growing bacteria, such as *E. coli* and *S. aureus*, cultures were incubated for 16 h(229). *M. smegmatis* and the *E. coli* deletion mutants in minimal media were incubated for 24 h - 48 h until an OD$_{600}$ of at least 1 was reached (221, 230). *M. tuberculosis* cultures were incubated for 2-3 weeks until an OD$_{600}$ of at least 0.8 was reached(231, 232). Anaerobic cultures of *E. coli* in rich media were incubated for 24 h while anaerobic cultures of *E. coli* in minimal media were incubated for 48 h, reflecting the slower growth rates in these conditions(233).

All cultures were diluted to approximately $10^6$ colony forming units per millilitre (CFU/ml) to establish the inocula(234). To this end, aerobically grown *E. coli* 25922, *E. coli* 25922 *lux* and *S. aureus* 6538 cultures were diluted 1:1000 by adding 10 µl of turbid culture to 10 ml liquid growth media. *E. coli* deletion mutant turbid cultures were diluted 1:500 by adding 20 µl of
turbid culture to 10 ml liquid growth media. *M. smegmatis* MC$^2$155, *M. smegmatis* MC$^2$155 lux and *M. tuberculosis* cultures were diluted to an OD$_{600}$ of 0.8 (corresponding to $\sim 10^8$ CFU/ml) in 7H9 supplemented broth and then diluted 1:100 by adding 10 µl of the OD$_{600}$ 0.8 culture to 990 µl of 7H9 supplemented broth(235). Anaerobic cultures in rich media and minimal media were diluted 1:100 (100 µl of turbid culture added to 10 ml liquid growth media) or 1:50 (200 µl of culture added to 10 ml liquid growth media), respectively.

### 2.2.2 Enumeration of bacteria

#### 2.2.2.1 Serial dilution

The aim of the serial dilution method is to estimate the number of viable cells in a sample by counting the number of colonies cultured from serial dilutions of the sample and using that number to calculate the cell concentration of the sample (CFU/ml)(236). Serial dilution in this work was performed in a 96 well plate (a microdilution). For enumeration of inocula tested against surfaces, 200 µl of neat culture was added to the first row of a 96 well plate. For enumeration of inocula to be tested against an equal volume of suspension, a 1:1 dilution was performed (to simulate experimental conditions) by adding 100 µl of inoculum to 100 µl of 0.85% saline in the first row of a 96 well plate. The inocula were serially diluted by transferring 20 µl into wells containing 180 µl of 0.85% saline (moving down the columns of the plate) and mixed by pipetting(214). Inocula were diluted as many times as needed to achieve countable colonies when put onto an agar plate. For inocula at $10^6$ CFU/ml, three dilution steps were required.

#### 2.2.2.2 Drop plate and spread plate methods

Serially diluted experimental samples were enumerated using either the drop plate method or the spread plate method(237). Both methods involve aliquoting an amount of each dilution onto an agar plate(237). The drop plate method involves dispensing 20 µl drops of a sample onto the surface of an agar plate(237). One agar plate may contain multiple drops(237). The spread plate method consists of dispensing 50 µl of a sample onto the surface on an agar plate and spreading the liquid over the whole plate using an L-shaped spreader(237). The drop plate method can be performed faster than the spread plate method; however, the former is more susceptible to sampling error as the size of sampling error is inversely related to sample size(236, 237). Therefore, the drop plate method was used for enumeration during time course experiments and the spread plate method was used for enumeration of inocula.
2.2.2.3 Calculation of CFU/ml
Following incubation of enumeration plates, colonies were counted (237). For colonies obtained using the drop plate method, the dilution that gave 3-30 colonies was counted (237). For colonies obtained using the spread plate method, the dilution that gave 30-300 colonies was counted (236). The number of cells in the original sample can be calculated using the formula in Figure 2.1 for both plating methods (214, 238).

\[ N = \frac{C \times 1000}{V \times 10^D} \]

**Figure 2.1. Calculation of the number of cells in a diluted and plated experimental sample.** N is the number of cells per ml (CFU/ml) of the original sample; C is the number of colonies counted; V is the volume of sample deposited onto the agar plate (µl); D is the number of serial dilutions (214, 238).

2.2.3 Cell viability assay to determine sensitivity of bacteria to PANI and P3ABA suspensions

2.2.3.1 Preparation of PANI and P3ABA suspensions
During initial testing, PANI was made in small (~5 g) batches. It was revealed from this work that PANI is subject to batch to batch variation. Following this, three large (~30 g) batches of PANI were made and mixed together to create one batch to reduce batch variation. The large batch was used to generate data presented in this thesis. PANI was finely ground using a mortar and pestle. This insoluble powder requires shaking at 200 rpm to stay in suspension.

The particle size of the large batch of PANI was determined using Malvern Mastersizer 2000 (table 2.7). The Mastersizer 200 measures the size of particles and gives the distribution of different sizes within a sample (239). Particle size is determined by measuring the scattering of a laser beam directed onto the sample (239). The angle at which the beam is scattered is inversely proportional to the soil particle size (239). Most PANI particles were in the range of ~ 5 µm to ~ 80 µm with a peak around 10 µm (Fig. 2.2).
Particle size distribution of PANI. The particle size distribution of PANI was determined using Malvern Mastersizer 2000. Particle diameter (µm) is represented relative to the volume of the sample associated with each particle size.

One batch of P3ABA was used for testing as it is not as susceptible to batch to batch variation. Reflecting the improved solubility of P3ABA, this polymer was suspended in broth using the QSonica Q700 Sonicator at the following settings: amplitude 30, elapsed time 10s, repeat 4×.

2.2.3.2 General experimental protocol
Suspensions of PANI and P3ABA were prepared as 2× suspensions in test broth. To establish 2% (w/v) suspensions (for a final concentration of 1%) 0.06 g of powder was added to 3 ml of broth. To establish 1% (w/v) suspensions (for a final concentration of 0.5%) 0.02 g of powder was added to 2 ml of broth.

A turbid culture of test bacteria was diluted to $10^6$ CFU/ml in broth(238). The inoculum was retrospectively enumerated using the spread plate method. 500 µl of PANI suspension, P3ABA suspension and broth (untreated cells) was aliquoted into 5 ml tubes. These tubes were inoculated with 500 µl of diluted culture. The experimental samples were incubated at 37°C with shaking at 200 rpm. At specified time points, a 100 µl aliquot from each experimental sample was enumerated using the drop plate method(238). Following incubation, colonies were counted and CFU/ml was calculated (section 2.2.2.3)(238). Three biological replicates were obtained for each experiment.
2.2.3.3 Sensitivity of *E. coli* deletion mutants in LB broth to PANI and P3ABA suspensions

*E. coli* deletion mutants grown in LB broth were treated with 1% PANI and 1% P3ABA suspensions (212, 225, 238). Time points for PANI treated cells were 2 h and 4 h while P3ABA treated cells were enumerated at 0.5 h and 1 h time points. Different time points were used for PANI and P3ABA suspensions reflecting the difference in activity levels against target bacteria in LB broth.

2.2.3.4 Sensitivity of *E. coli* deletion mutants in minimal media to PANI and P3ABA suspensions

*E. coli* deletion mutants grown in minimal A salts with 0.4% succinate were treated with 1% PANI and 1% P3ABA suspensions (212, 225). Time points for PANI and P3ABA treated cells were 1 h and 2 h.

2.2.3.5 Sensitivity of *E. coli* and *S. aureus* to PANI and P3ABA suspensions

*E. coli* 25922 *lux* and *S. aureus* 6538 were treated with 0.5% PANI and 0.5% P3ABA suspensions. *S. aureus* 6538 was treated in LB broth while *E. coli* 25922 *lux* was treated in LB broth, minimal A salts with 0.7% glycerol and minimal A salts with 0.4% succinate (204, 225). Time points for all treatments were 0.5 h, 1 h, 2 h and 4 h.

2.2.3.6 Statistical analysis

Linear regression analysis was used to compare the sensitivity of the parent strain to that of each *E. coli* deletion mutant for both PANI and P3ABA (240). Similarly, the sensitivities of *S. aureus* 6538 and *E. coli* 25922 *lux* to PANI and P3ABA in suspension were compared using a linear regression analysis. Linear regression analysis is an approach used to explain the relationship between one dependent variable and one or more independent variables. Statistical analysis by linear regression was performed using GraphPad Prism software version 6 (GraphPad Software, Inc.). Data was graphed in a scatter plot generated with viable cell counts post-treatment (CFU/ml) represented on the y-axis and time (h) represented on the x-axis. Linear regression was used to fit a straight line (regression line) through the data for the categorical factor (bacterial gene mutation) generating the best-fit value of the slope and intercept. An analysis of covariance (ANCOVA) was used to compare the regression lines from the parent strain and a mutant strain to determine if the effect of bacterial gene mutation on
viable cell count post-treatment represented a statistically significant increase or decrease in sensitivity (while controlling for the effect of the covariable, time).

The next step was to compare the slopes of the lines, which represent the decrease in viable cell count overtime and reflect sensitivity to treatment between the two time points. A significant difference (95% level of confidence) between slopes of the lines is denoted by a P value of less than 0.05 while no difference in the slope of the lines was signified by a P value of more than 0.05. Scenario 1 (Fig. 2.3) represents a situation in which the slopes of the regression lines are significantly different, which means that the rate of cell death differs between the two strains. The steeper line of strain 2 is indicative of a higher rate of cell death and greater sensitivity to treatment compared to strain 1 (Fig. 2.3).

Scenario 2 shows a situation in which the slopes of the regression lines are the same; however, the y-intercepts of the lines are significantly different. A significant difference (95% level of confidence) between y-intercepts of the lines is denoted by a P value of less than 0.05 while no difference in the intercepts of the lines is signified by a P value of more than 0.05. The y-intercepts of the regression lines represent the ‘magnitude’ of the viable cell count (inferred from the viable cell counts at the two selected time points) and reflect sensitivity to treatment. Scenario 2 arises when there is early knockdown of one strain, which then dies at the same rate as the other strain during the selected time points. Scenario 3 demonstrates a situation in which both the slopes and the y-intercepts of the two regression lines are not significantly different, denoted by P values for the slopes and intercepts of greater than 0.05. In this scenario both strains are decreasing in viable cell number at the same rate and are present at the same number, which suggests that there is no difference in sensitivity to treatment between the two strains.
Figure 2.3. Schematic showing potential scenarios analysed using a linear regression analysis. Data for two strains is presented on a graph showing viable cell count post-treatment plotted against time. Scenario 1 represents two strains dying at different rates. Scenario 2 represents two strains dying at the same rate but present at different numbers. Scenario 3 represents the two strains dying at the same rate and present at similar cell numbers.

For the *E. coli* deletion mutants, the data was presented in two forms – part A of the relevant figures shows the raw data (in the form that statistical analysis was performed on). Part B shows the relative sensitivity of each deletion mutant plotted as a percent of the parent strain viable cell numbers post-treatment. A line was plotted at 100% to represent no difference in sensitivity relative to the parent strain. Deletion mutant strains that were more sensitive than the parent strain were plotted below this line while less sensitive strains were plotted above the parent strain line.

2.2.4 Assay to determine the activity of PANI and P3ABA suspensions in aerobic and anaerobic conditions against *E. coli* and *S. aureus*

2.2.4.1 General experimental protocol

To prepare the growth media for antimicrobial assays in anaerobic conditions, or comparing activity in aerobic and anaerobic conditions, a 2% cysteine stock solution (table 2.3) was added to liquid growth media to a final concentration of 0.05% (through addition of 2.5 ml cysteine stock solution to 97.5 ml media) to create a reducing environment with less reactive oxygen species present(241).

A range of concentrations of PANI and P3ABA in suspension were established in triplicate (one set for aerobic incubation, one set for anaerobic incubation and one set for blanks). PANI is an insoluble powder that is not suitable for dilution from a stock solution. Each PANI suspension was set up separately by weighing the powder into 5 ml tubes (table 2.8) and adding 500 µl of
broth. A P3ABA stock suspension was established by adding 0.8 g of P3ABA to 5 ml of broth. The P3ABA stock suspension was diluted to 0.125% P3ABA using doubling dilution series to a final volume of 1 ml. The suspensions were established at 2× the final desired concentration (which was achieved following inoculation with an equal volume). 500 µl of each established suspension was aliquoted into a 5 ml tube and 500 µl of LB broth was aliquoted to set up an untreated control.

To monitor the anaerobic environment generated in assays, 0.0002% methylene blue and 0.0002% resazurin solutions were established in duplicate by adding 1 µl of 0.2% stock solutions (table 2.5) to 1 ml deionised water (223). These dyes appear blue when oxidised (indicating oxygen is present) and colourless when reduced (indicating oxygen is absent), which means they can be used as an indicator of the redox state of the solution (223). The methylene blue and resazurin oxygen indicator tubes were set up in duplicate to allow for incubation of each type in both aerobic and anaerobic conditions. The colours of the methylene blue and resazurin oxygen indicator tubes were used to confirm that aerobic and anaerobic conditions were achieved during the experiment. The test bacteria culture, anaerobic suspensions, media used for diluting to 10^6 CFU/ml, methylene blue oxygen indicator tube and resazurin oxygen indicator tube were incubated anaerobically (section 2.2.1.2) at 37°C with 200 rpm shaking. Aerobic suspensions, methylene blue oxygen indicator tube and resazurin oxygen indicator tube were incubated aerobically at 37°C with 200 rpm shaking. Blank tubes were handled as for aerobic suspensions except 500 µl of broth was added to the suspensions in place of inoculum.

Dilution of the turbid anaerobic culture and inoculation of the anaerobic suspensions were performed in ambient conditions as fast as possible to reduce the amount of oxygenation of the experimental samples. To this end, a turbid anaerobic culture of test bacteria was diluted to 10^6 CFU/ml in broth (section 2.2.1.3)(238). The inoculum was retrospectively enumerated using the spread plate method. Each aerobic and anaerobic PANI or P3ABA suspension (and the untreated control) was inoculated with 500 µl of diluted culture. The experimental samples were incubated aerobically and anaerobically (as appropriate, section 2.2.1.2) at 37°C with shaking at 200 rpm (214, 229). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of PANI or P3ABA that was able to inhibit the visible growth of test bacteria following a 24 h or 48 h treatment (214, 228, 229, 242). Tubes that were observed by
eye to have no visible growth were selected for minimum bactericidal concentration (MBC) testing. For this, 20 µl of the experimental sample was spread onto 6 LB agar plates (214, 229). The spread plates were incubated at 37°C for 16 h and growth on these plates was determined. When countable colonies were present, the CFU/ml of the sample was calculated (section 2.2.2.3). Bactericidal activity was defined as 99.9% reduction in cell number relative to the starting inoculum (233, 243). The MBC was defined as the lowest concentration of PANI or P3ABA that prevented the growth of test bacteria following subculture on LB agar plates (229). At least three biological replicates were obtained for each experiment.

Table 2.8. Establishment of PANI suspensions for determining the aerobic and anaerobic MIC and MBC

<table>
<thead>
<tr>
<th>Initial concentration (%)</th>
<th>Final concentration, post-inoculation (%)</th>
<th>Amount of PANI for 1 ml suspension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>8</td>
<td>0.16</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25</td>
<td>0.005</td>
</tr>
<tr>
<td>0.25</td>
<td>0.125</td>
<td>0.0025</td>
</tr>
<tr>
<td>0.125</td>
<td>0.0625</td>
<td>0.00125</td>
</tr>
<tr>
<td>0.0625</td>
<td>0.03125</td>
<td>N/A</td>
</tr>
<tr>
<td>0.03125</td>
<td>0.015625</td>
<td></td>
</tr>
<tr>
<td>0.015625</td>
<td>0.0078125</td>
<td></td>
</tr>
<tr>
<td>0.0078125</td>
<td>0.00390625</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N/A indicates not applicable

Suspensions with an initial concentration of 0.0625% to 0.0078125% PANI were established by doubling dilution of the 0.125% PANI suspension (initial concentration) as the amount of PANI to be weighed for those suspensions exceeded the range of the Cubis® Analytical Balance

2.2.4.2 MIC and MBC of PANI and P3ABA in rich media in aerobic and anaerobic conditions

Test bacteria, including *E. coli* 25922, *E. coli* 25922 lux and *S. aureus* 6538, were grown in LB broth, a rich media, anaerobically for 24 h (244, 245). The anaerobically grown culture was diluted to $10^6$ CFU/ml and was challenged with PANI or P3ABA in aerobic and anaerobic
conditions for 24 h before the MIC was determined. For *E. coli* 25922 *lux*, the MIC and MBC were only determined in aerobic conditions as appropriate for section 5.2.

### 2.2.4.3 MIC and MBC of PANI and P3ABA in minimal media in aerobic and anaerobic conditions

*E. coli* 25922 was grown anaerobically in minimal A salts with 0.7% glycerol or 0.4% succinate (table 2.4) as the carbon source for 48 h(225, 244, 246). To support the growth of cells in anaerobic conditions, potassium nitrate was added at 20 mM to the growth media(225, 244). Nitrate can act as a terminal electron acceptor in *E. coli* facilitating anaerobic respiration (228, 247). For treatment in aerobic conditions no nitrate was present in the growth media(225). To this end, the anaerobic 48 h culture of *E. coli* 25922 was diluted to 10⁶ CFU/ml in minimal A salts with and without nitrate to inoculate the anaerobic and aerobic suspensions, respectively(225). These inoculated PANI or P3ABA suspensions were incubated for 48 h before the MIC was determined. The increased treatment time reflects the slower growth rate of *E. coli* in minimal media(245, 248).

### 2.2.4.4 Statistical analysis

The Mann-Whitney test was used to analyse the difference between the aerobic and anaerobic MIC, and the aerobic and anaerobic MBC of PANI and P3ABA against test bacteria. This test is a non-parametric t-test that compares two unpaired groups. A P value of less than 0.05 is taken to signify that the differences between the two groups is true while a P value of more than 0.05 is taken to indicate that the distribution of data of both groups is the same. The Mann-Whitney test was also used to compare the difference between the influence of the presence of oxygen on the activity of PANI and P3ABA against *E. coli* 25922.

### 2.2.5 Assay to determine the activity of absorbent surfaces containing PANI and P3ABA against *E. coli* and *S. aureus*

#### 2.2.5.1 Determination of the limit of detection for *E. coli* 25922 *lux* and *S. aureus* 6538 growing in a 96 well plate

The limit of detection of *E. coli* 25922 *lux* and *S. aureus* 6538 growing in a 96 well plate was examined by determining the lowest number of cells added to LB broth in a 96 well plate that can grow to detectable levels(249). This was achieved by serially diluting an overnight culture in triplicate in a 96 well plate by transferring 20 µl of culture into wells containing 180 µl of LB broth. A range of inocula were established from ~ 10⁹ CFU/ml to ~ 1 CFU/ml. The overnight
culture was enumerated to confirm the cell numbers tested. The 96 well plate was incubated at 37°C for 16 h in a sealed container with a moist tissue(249). Growth of bacteria was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader for *E. coli* 25922 lux and by measuring OD$_{600}$ using the µQuant™ Microplate Spectrophotometer for *S. aureus* 6538.

### 2.2.5.2 General experimental protocol

Absorbent surfaces containing PANI or P3ABA can be modelled using agar, as drops of liquid containing bacteria will absorb into the agar surface(237). Molten agar was mixed with varying amounts of PANI or P3ABA (table 2.9), which when left to set created absorbent surfaces containing the antimicrobial agents. PANI and P3ABA containing absorbent surfaces were established in triplicate in a 96 well plate by aliquoting 200 µl of each test agar and 200 µl of LB agar (for the untreated control) into individual wells.

A turbid culture of test bacteria was diluted to $10^6$ CFU/ml in broth(238). The inoculum was retrospectively enumerated using the spread plate method. The agar samples were inoculated with 10 µl of diluted culture resulting in $10^4$ CFU in each well, similar to the agar dilution method used to determine MIC values of antibiotics(214, 229). Agar samples for background readings received 10 µl of LB broth. At specified time points the bacterial cells were recovered by washing the agar surface with 100 µl of LB broth, which was transferred to a new 96 well plate containing 100 µl of LB broth(249). The rescued cells in the 96 well plate were incubated at 37°C in a sealed container with moist tissue for 16 h(249). Following this, the viability of treated cells was determined. Three biological replicates were obtained for each experiment.

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Amount of PANI or P3ABA (g)</th>
<th>Volume of molten agar (ml)</th>
<th>Volume of 2× LB broth (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.24</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>4</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>0.06</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>P3ABA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.04</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

P3ABA did not mix well with LB agar so P3ABA was sonicated in 2× LB broth then mixed with equal volume 2× agar

N/A indicates not applicable
2.2.5.3 Activity of PANI and P3ABA in agar against E. coli 25922 lux
E. coli 25922 lux was challenged with PANI in agar for the following treatment times: 15 min, 30 min, 1 h, 2 h, 4 h and 8 h. E. coli 25922 lux was exposed to P3ABA in agar for the following treatment times: 15 min, 30 min, 1 h and 2 h. Each type of absorbent surface was tested for the necessary time to achieve knockdown. Therefore, highly active surfaces, such as 2% P3ABA in agar, were tested only for the shorter treatment times. Rescued cells were incubated in a dark OptiPlate-96 well microtitre plate, following which cell viability was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader.

2.2.5.4 Activity of PANI and P3ABA in agar against S. aureus 6538
S. aureus 6538 was challenged with PANI in agar for the following treatment times: 15 min, 30 min, 1 h, 2 h, 4 h and 8 h. S. aureus 6538 was exposed to P3ABA in agar for the following treatment times: 15 min, 30 min, 1 h, 2 h and 4 h. Each type of absorbent surface was tested for the necessary time to achieve knockdown. Therefore, highly active surfaces, such as 2% P3ABA in agar, were tested only for the shorter treatment times. Rescued cells were incubated in a clear 96 well plate, following which cell viability was assessed by measuring OD$_{600}$ using the µQuant™ Microplate Spectrophotometer.

2.2.5.5 Statistical analysis
The Friedman test was used to analyse the differences between untreated cells and those treated with PANI or P3ABA in agar. The Friedman test is a nonparametric test that compares three or more matched groups; each treatment time for every concentration tested. A P value of more than 0.05 indicates that the differences between groups are due to random sampling. A P value of less than 0.05 indicates that at least one of the groups differs from the rest. When a significant difference was identified (P value less than 0.05), specific groups were compared to each other using Dunn’s multiple comparison test. Dunn’s multiple comparison test was used to compare the treated and untreated cells at each time point with a P value of less than 0.05 indicating a significant difference.

2.2.6 Assay to determine the activity of non-absorbent surfaces containing PANI and P3ABA against E. coli and S. aureus
2.2.6.1 General experimental protocol
Non-absorbent surface samples were prepared using styrene ethylene butylene styrene (SEBS) films containing 5% PANI or 3% PANI or no additive (control film). The films were hole punched
to generate ~ 5 mm diameter circles that fit into the wells of a 96 well plate. The film samples were disinfected by immersion in 70% ethanol for 10 min and dried in the Herasafe™ KS (NSF) Class II, Type A2 Biological Safety Cabinet(65).

A turbid culture of test bacteria was diluted to $10^6$ CFU/ml in broth(238). The inoculum was retrospectively enumerated using the spread plate method. The activity of the PANI and P3ABA containing film samples was determined using a ‘micro-JIS assay’ adapted from Japanese Industry Standard (JIS Z-2801) method(53, 250). A piece of film was placed in an empty well, inoculated with 10 µl of diluted culture (resulting in $10^4$ CFU per well) and a second piece of the same type of film was placed on top of the inoculum(214, 229). This results in the 10 µl drop of inoculum spreading across the surfaces of the film pieces. Film samples for background readings received 10 µl of LB broth. The film treatments were established in triplicate. The 96 well plate was incubated at 37°C in a sealed container with moist tissue. At specified time point(s) bacterial cells were rescued by washing with 190 µl of LB broth and transferred to a new 96 well plate(249). The rescued cells were incubated at 37°C in a sealed container with moist tissue for 16 h(249). Following this, the viability of treated cells was determined. Three biological replicates were obtained for each experiment.

2.2.6.2 Activity of PANI and P3ABA films in 2 h and 24 h against E. coli 25922 lux

E. coli 25922 lux cells were exposed to film treatments for 2 h and 24 h. The viability of cells post-treatment was assessed by using plate counts and measuring bioluminescence. To this end, a 100 µl aliquot of rescued cells was used to enumerate by drop counts and the remaining 100 µl of rescued cells was added to a dark OptiPlate-96 well microtitre plate containing 100 µl of LB broth for measurement of bioluminescence using the VICTOR X Multilabel Plate Reader.

2.2.6.3 Activity of PANI and P3ABA films in 24 h against S. aureus 6538

S. aureus 6538 was exposed to film treatments for 24 h. The viability of cells post-treatment was assessed by incubating 200 µl of rescued cells in a 96 well plate for 16 h and measuring OD$_{600}$ using the µQuant™ Microplate Spectrophotometer.

2.2.6.4 Statistical analysis

The activity of PANI and P3ABA in films against E. coli 25922 lux was analysed using a two-way repeated measures analysis of variation (2-way RM ANOVA)(226). For both the plate counts and the bioluminescence data, the 2-way RM ANOVA determined how E. coli 25922 lux cell
number was affected by two factors, treatment time (2 h and 24 h) and film type (PANI in film, P3ABA in film, no additive). A P value of less than 0.05 indicates that the cell number was significantly affected by at least one of the factors. When a significant difference was identified (P value less than 0.05), treated cells were compared to the untreated control for each time point using Dunnett’s multiple comparison test with a P value of less than 0.05 indicating a significant difference.

The Friedman test was used to analyse the differences between untreated S. aureus 6538 cells and those treated with PANI or P3ABA in film. The Friedman test is a nonparametric test that compares three or more matched groups – cells treated with 5% PANI in film, 3% P3ABA in film and control film. For instances in which all of the differences between groups are due to random sampling the P value will be more than 0.05. A P value of less than 0.05 indicates that at least one of the groups differs from the rest. When a significant difference was identified (P value less than 0.05), specific groups were compared to each other using Dunn’s multiple comparison test. Dunn’s multiple comparison test was used to compare the treated and untreated cells at each time point with a P value of less than 0.05 indicating a significant difference.

2.2.7 Characterisation of the activity of non-absorbent surfaces containing PANI and P3ABA against E. coli and S. aureus

2.2.7.1 Challenge of PANI and P3ABA films with a range of CFU doses of E. coli 25922 lux and S. aureus 6538 in saline

Film punches were prepared and decontaminated as described above (section 2.2.6.1). A 1 ml aliquot of overnight culture of test bacteria was centrifuged at 10,000 rpm for 2 min in the Heraeus Biofuge Fresco microcentrifuge and resuspended in 1 ml 0.85% saline. Washed culture was diluted to 10^6 CFU/ml by adding 10 µl of the culture to 990 µl 0.85% saline. The 10^6 CFU/ml culture was serially diluted to 10^3 CFU/ml by transferring 100 µl into a 5 ml tube containing 900 µl 0.85% saline. The 10^6 CFU/ml culture was also enumerated using the spread plate method. Film treatments were established as described above (section 2.2.6.1). 10 µl of each dilution of culture was inoculated onto the films resulting in challenge with 10^4 CFU, 10^3 CFU, 10^2 CFU and 10 CFU doses. Film treatments were incubated at 37°C in a sealed container with a moist tissue for 2 h. Treated cells were rescued by washing film treatments with 190 µl of LB broth. 200 µl of rescued cells were transferred to a 96 well plate and
incubated at 37°C in a sealed container with a moist tissue for 16 h. Growth of bacteria was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader for *E. coli* 25922 *lux* and by measuring OD$_{600}$ using the µQuant™ Microplate Spectrophotometer for *S. aureus* 6538.

### 2.2.7.2 Assay to evaluate the influence of the presence of organic matter on the activity of PANI and P3ABA films against *E. coli* 25922 *lux*

Film punches were prepared and decontaminated as described above (section 2.2.6.1). Two 1 ml aliquots of overnight culture of *E. coli* 25922 *lux* were centrifuged at 10,000 rpm for 2 min in the Heraeus Biofuge Fresco microcentrifuge and resuspended in 1 ml of LB broth and 1 ml of 0.85% saline(234). The washed cultures were diluted to $10^6$ CFU/ml by adding 10 µl of washed culture to 10 ml of LB broth or to 10 ml 0.85% saline, respectively. The diluted washed cultures were enumerated using the spread plate method. Film treatments were established as described above (section 2.2.6.1) – each type of film was challenged in triplicate with $10^4$ CFU of both the LB broth culture and the saline culture (and media only for background readings). Films treatments were incubated at 37°C in a sealed container with a moist tissue for 2 h. Treated cells were rescued by washing film treatments with 190 µl of LB broth. 200 µl of rescued cells were transferred to a dark OptiPlate-96 well microtitre plate and incubated at 37°C in a sealed container with a moist tissue for 16 h. Growth of bacteria was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader.

### 2.2.7.3 Statistical analysis

The activity of PANI and P3ABA in films against a range of CFU doses of *E. coli* 25922 *lux* and *S. aureus* 6538 was analysed using a 2-way RM ANOVA (see section 2.2.6.4)(226).

### 2.2.8 Activity of PANI and P3ABA suspensions against mycobacteria

#### 2.2.8.1 Activity of PANI and P3ABA in suspension against *M. smegmatis* MC$^2$155

The activity of PANI and P3ABA in suspension against *M. smegmatis* MC$^2$155 was characterised by determining the MIC and MBC(229). A range of concentrations of PANI and P3ABA in suspension were established. PANI is an insoluble powder that is not suitable for dilution from a stock solution. Each PANI suspension was set up separately by weighing the powder into 5 ml tubes (table 2.10) and adding 500 µl of broth. A P3ABA stock suspension was established by adding 0.05 g P3ABA to 5 ml of broth (1% w/v). The P3ABA stock suspension was diluted to 0.125% P3ABA using doubling dilution series to a final volume of 1 ml. The suspensions were
established at 2× the final desired concentration (which was achieved following inoculation with an equal volume). 500 µl of each established suspension was aliquoted into a 5 ml tube and 500 µl of LB broth was aliquoted to set up an untreated control.

A turbid culture of *M. smegmatis* MC^2^155 was diluted to 10^6^ CFU/ml in broth(238). The inoculum was retrospectively enumerated using spread plates. Each PANI or P3ABA suspension (and the untreated control) was inoculated with 500 µl of diluted culture. The experimental samples were incubated at 37°C with shaking at 200 rpm for 24 h(214, 229). The MIC was defined as the lowest concentration of PANI or P3ABA that was able to inhibit the visible growth of *M. smegmatis* following a 24 h treatment(214, 229). Tubes which were observed by eye to have no visible growth were selected for MBC testing. For this, 20 µl of the experimental sample was spread onto 6 LB agar plates(214, 229). The spread plates were incubated at 37°C for 2-3 days and growth on these plates was determined. When countable colonies were present, the CFU/ml of the sample was calculated (section 2.2.2.3). Bactericidal activity was defined as 99.9% reduction in cell number relative to the starting inoculum(233, 243). The MBC was defined as the lowest concentration of PANI or P3ABA that prevented the growth of *M. smegmatis* following subculture on LB agar plates(229).

**Table 2.10. Establishment of PANI suspensions for determining the MIC and MBC for *M. smegmatis*.**

<table>
<thead>
<tr>
<th>Initial concentration (%)</th>
<th>Final concentration, post-inoculation (%)</th>
<th>Amount of PANI for 1 ml suspension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>8</td>
<td>0.16</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
2.2.9 Assay to determine the activity of absorbent surfaces containing PANI and P3ABA against mycobacteria

2.2.9.1 Determination of the limit of detection for \textit{M. smegmatis} MC\textsuperscript{2}155 \textit{lux} growing in a 96 well plate

The limit of detection of \textit{M. smegmatis} MC\textsuperscript{2}155 \textit{lux} growing in a 96 well plate was examined by determining the lowest number of cells added to 7H9 supplemented broth in a 96 well plate that can grow to detectable levels\cite{249,251}. This was achieved by serially diluting a turbid culture in triplicate in a 96 well plate by transferring 20 µl of culture into wells containing 180 µl of 7H9 supplemented broth. A range of inocula were established from \(~10^9\) CFU/ml to \(~1\) CFU/ml. The overnight culture was enumerated to confirm the cell numbers tested. The 96 well plate was incubated at 37°C for 24 h in a sealed container with a moist tissue. Growth of bacteria was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader.

2.2.9.2 General experimental protocol

Absorbent surfaces containing PANI or P3ABA were modelled with agar using the protocol that was optimised with \textit{E. coli} 25992 \textit{lux} and \textit{S. aureus} 6538 (section 2.2.5.2)\cite{237}. Molten agar was mixed with varying amounts of PANI or P3ABA (table 2.11) and 200 µl aliquots of each agar mix were added to wells in triplicate in a 96 well plate. Untreated controls were established in triplicate using 7H11 supplemented agar.

A turbid culture of test bacteria was diluted to \(10^6\) CFU/ml in 7H9 supplemented broth\cite{238}. The inoculum was retrospectively enumerated using the spread plate method. The agar samples were inoculated with 10 µl of diluted culture resulting in \(10^4\) CFU in each well \cite{214,229}. Agar samples for background readings received 10 µl of 7H9 supplemented broth. Following 15 min, 30 min and 120 min treatment times, the bacterial cells were recovered by washing the agar surfaces with 100 µl of 7H9 supplemented broth, which was transferred to a dark OptiPlate-96 well microtitre plate containing 100 µl of 7H9 supplemented broth\cite{249}. The rescued cells in the 96 well plate were incubated at 37°C in a sealed container with moist tissue\cite{249}. Following this, the viability of treated cells was determined by measuring bioluminescence using the VICTOR X Multilabel Plate Reader. Three biological replicates were obtained for each experiment.
Table 2.11. Establishment of PANI and P3ABA in agar for testing against mycobacteria.

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Amount of PANI or P3ABA (g)</th>
<th>Volume of molten agar (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>0.24</td>
<td>3</td>
</tr>
<tr>
<td>P3ABA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>3</td>
</tr>
<tr>
<td>3.5</td>
<td>0.105</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>0.06</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>0.03</td>
<td>3</td>
</tr>
</tbody>
</table>

2.2.9.3 Activity of PANI and P3ABA in agar against *M. smegmatis* MC^2^155 lux

*M. smegmatis* MC^2^155 lux was challenged with agar containing PANI and P3ABA. The rescued cells were incubated in a 96 well plate for 24 h, following which cell viability was determined.

2.2.9.4 Activity of PANI and P3ABA in agar against *M. tuberculosis* H37Rv lux and *M. tuberculosis* Rangipo N lux

*M. tuberculosis* H37Rv lux and *M. tuberculosis* Rangipo N lux were challenged with agar containing PANI and P3ABA. The rescued cells were incubated in a 96 well plate for 21 days, following which cell viability was determined.

2.2.9.5 Statistical analysis

The Friedman test was used to analyse the differences between untreated cells and those treated with PANI or P3ABA in agar. The Friedman test is a nonparametric test that compares three or more matched groups – each treatment time for every concentration tested. For instances in which all of the differences between groups are due to random sampling the P value will be more than 0.05. A P value of less than 0.05 indicates that at least one of the groups differs from the rest. When a significant difference was identified (P value less than 0.05), specific groups were compared to each other using Dunn’s multiple comparison test. Dunn’s multiple comparison test was used to compare the treated and untreated cells at each time point with a P value of less than 0.05 indicating a significant difference.
2.2.10 Assay to determine the activity of non-absorbent surfaces containing PANI and P3ABA against mycobacteria

2.2.10.1 General experimental protocol
Non-absorbent surface samples were prepared from styrene ethylene butylene styrene (SEBS) films containing 5% PANI or 3% PANI or no additive (control film). The films were hole punched to generate ~ 5 mm diameter circles that fit into the wells of a 96 well plate. The film samples were disinfected by immersion in 70% ethanol for 10 min and dried in the Herasafe™ KS (NSF) Class II, Type A2 Biological Safety Cabinet(65).

A turbid culture of test bacteria was diluted to $10^6$ CFU/ml in broth(238). The inoculum was retrospectively enumerated using the spread plate method. The activity of the PANI and P3ABA containing film samples was determined using the ‘micro-JIS assay’ that was optimised using *E. coli* 25922 *lux* and *S. aureus* 6538 (section 2.2.6.1)(53, 250). At specified time point(s) bacterial cells were rescued by washing with 190 µl of 7H9 supplemented broth and transferred to a dark OptiPlate-96 well microtitre plate(249). The rescued cells were incubated at 37°C then bioluminescence was measured. Three biological replicates were obtained for each experiment.

2.2.10.2 Activity of PANI and P3ABA films against *M. smegmatis* MC²155 lux
*M. smegmatis* MC²155 lux cells exposed to film treatments for 24 h. Rescued *M. smegmatis* cells were incubated at 37°C for 24 h, following which cell viability was determined.

2.2.10.3 Activity of PANI and P3ABA films against *M. tuberculosis* H37Rv lux
*M. tuberculosis* H37Rv lux cells were exposed to film treatments for 2 h and 24 h. Rescued *M. tuberculosis* cells were incubated at 37°C for 14 days, following which cell viability was determined.

2.2.10.4 Statistical analysis
The Kruskal-Wallis test was used analyse the activity of films containing PANI, films containing P3ABA and films with no additive against *M. smegmatis* MC²155 lux (see section 2.2.4.4). The activity of PANI and P3ABA in films against *M. tuberculosis* H37Rv lux was analysed using a 2-way RM ANOVA (see section 2.2.6.4)(226).
2.2.11 Characterisation of the activity of non-absorbent surfaces containing PANI and P3ABA against mycobacteria

2.2.11.1 Challenge of PANI and P3ABA films with a range of CFU doses of *M. smegmatis* MC²155 *lux* in saline

Film punches were prepared and decontaminated as described above (section 2.2.6.1). A 1 ml aliquot of turbid culture of test bacteria was centrifuged at 10,000 rpm for 2 min in the Heraeus Biofuge Fresco microcentrifuge and resuspended in 1 ml 0.85% saline(234, 252). Washed culture was diluted to $10^6$ CFU/ml by adding 10 µl of the culture to 990 µl 0.85% saline. The $10^6$ CFU/ml culture was serially diluted to $10^3$ CFU/ml by transferring 100 µl into a 5 ml tube containing 900 µl of 0.85% saline. The $10^6$ CFU/ml culture was also enumerated using the spread plate method. Film treatments were established as described above (section 2.2.6.1). 10 µl of each dilution of culture was inoculated onto the films resulting in challenge with $10^4$ CFU, $10^3$ CFU, $10^2$ CFU and 10 CFU doses. Films treatments were incubated at 37°C in a sealed container with a moist tissue for 2 h. Treated cells were rescued by washing film treatments with 190 µl of 7H9 supplemented broth. 200 µl of rescued cells were transferred to a dark OptiPlate-96 well microtitre plate and incubated at 37°C in a sealed container with a moist tissue for 24 h. Growth of bacteria was assessed by measuring bioluminescence using the Victor Luminescence plater reader.

2.2.11.2 Statistical analysis

The activity of PANI and P3ABA in films against a range of CFU doses of *M. smegmatis* MC²155 *lux* was analysed using a 2-way RM ANOVA (see section 2.2.6.4)(226).
Chapter 3: Sensitivity of E. coli Deletion Mutants to PANI and P3ABA Suspensions

3.1 Introduction

The work described in this chapter sought to further characterise the antimicrobial mechanism of PANI and P3ABA based upon hypotheses formed following a transcriptomic analysis of E. coli MG1655 sublethally treated with a polysulfanilic acid (PSO₃H), a fPANI(163, 185). Genes whose expression is up-regulated in response to PolySO₃H is summarised in table 1a in Appendix 1. Genes whose expression is down-regulated in response to PolySO₃H is summarised in table 1b in Appendix 1. The tables have been annotated further to identify the genes knocked-out in mutants studies found in this chapter. The interpretation of the transcriptomic analysis assumes that for the challenged cells upregulated genes are involved in the responses to any stresses experienced; whereas genes downregulated are possible targets. The PSO₃H challenged E. coli demonstrated upregulation of genes involved in periplasmic and oxidative stress responses as well as iron homeostasis. Periplasmic stress response genes included spy, asr and cpXP(163). Expression of multiple genes in the OxyR regulon, which responds to hydrogen peroxide-based stress were increased, including trxC, grxA, mntH and sufB(163, 186). Some genes in the SoxRS regulon, which responds to superoxide-based stress were also upregulated, such as soxR, soxS and fumC(163, 187). Upregulated genes involved in iron homeostasis included sufC and fes(163, 190). Transcriptomic analysis also revealed downregulation of some genes encoding proteins involved in central metabolism and energy generation including TCA cycle genes, such as sdhB and aceA(163). The transcriptional response of E. coli MG1655 to the fPANI led to the development of several hypotheses pertaining to potential mechanisms of action including induction of oxidative stress, dysregulation of iron homeostasis, targeting of metabolic and respiratory enzymes, and disruption of membrane integrity.

The role of the dysregulation of iron homeostasis in the antimicrobial mechanism of P3ABA has been investigated. EPR spectroscopy was used to detect unpaired electrons in Fe³⁺ permitting quantification of free iron levels in E. coli 25922 sublethally treated with P3ABA(201, 202). Treatment with P3ABA resulted in an increase in intracellular free iron that was comparable to
free iron levels detected following treatment with hydrogen peroxide at 20 mM(200). An increase in intracellular free iron is known to occur following exposure to hydrogen peroxide and superoxide due to oxidation of the exposed iron atom in [4Fe-4S]2+ clusters in metabolic and respiratory enzymes or Fe2+ in mononuclear metabolic iron enzymes, which results in iron dissociation(203–205). The increase in free iron detected in P3ABA treated E. coli cells corroborates hypotheses generated from the transcriptomic analysis of PSO3H treated E. coli cells that relate to induction of oxidative stress, disruption of iron homeostasis and targeting of metabolic and respiratory enzymes. It was also hypothesised that fPANI exposure may result in oxidative damage to DNA, which is a common outcome of oxidative stress(241, 253). P3ABA was also demonstrated to disrupt membrane integrity in E. coli cells as indicated by the increased sensitivity to P3ABA in a hyperosmotic environment (6% NaCl) and the rapid loss of membrane potential, examined using the BacLight Bacterial Membrane Potential Kit and flow cytometry(200, 206, 207). The results generated relating to P3ABA-mediated perturbation of membrane integrity supported the hypothesis formed from the transcriptomic analysis of PSO3H treated cells that fPANI treatment disrupts membrane integrity.

The generated hypotheses, based on results from the transcriptomic analysis of fPANI action on E. coli and previous examination of the action of P3ABA on E. coli, were investigated using E. coli single gene deletion mutants from the Keio collection(212, 213). The deletion mutants were generated from E. coli K12 BW25113 (F- Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514) using Flp-FRT recombination(212, 254). Recombination was achieved through use of a λ Red expression plasmid pKD46 containing a kanamycin resistance cassette flanked by FLP recognition target (FRT) sites and 50-bp homologies to adjacent chromosomal sequences resulting in replacement of specific open-reading frame coding regions with a kanamycin resistance cassette(212). Utilisation of a panel of deletion mutants allowed for a higher throughput testing of multiple hypotheses in one experimental model.

Investigation of antimicrobial mechanism using the Keio collection deletion mutants can be achieved by characterising the susceptibility profiles of selected deletion mutants to PANI and P3ABA. A susceptibility profile describes the sensitivity of a bacterial strain to a range of antimicrobial agents(213, 255). Susceptibility profiles established for an array of bacterial deletion mutants can be used to infer mechanism of the antimicrobial agent(213, 256, 257). This approach is reliant upon the hypothesis that bacteria with mutation of genes involved in
defence against antimicrobial action will have increased sensitivity to treatment (184, 213, 257). Conversely, mutation of genes encoding antimicrobial targets will exhibit a decreased sensitivity to treatment (184, 213, 257). Screening an antimicrobial agent against *E. coli* deletion mutants typically results in the identification of a small subset of these mutants with hypersensitive phenotypes (213). For example, only 3.5% of 4,000 *E. coli* deletion mutants screened against seven antibiotics were identified as being hypersensitive to treatment (213). This approach has been used in work attempting to elucidate the role that reactive oxygen species (ROS) have in the bactericidal mechanism of antibiotics (228, 238, 255).

The media in which bacterial cells are grown can have important effects on the growth and physiological state of the cells and therefore may influence sensitivity to inimical processes. The *E. coli* deletion mutants were first tested in LB broth. The generated results from this led to the selection of a subset of mutants for testing in minimal media with succinate as the carbon source. LB broth is a complex rich media consisting of tryptone, yeast extract and sodium chloride (258, 259). The natural origin of the tryptone and yeast extract means that the exact chemical composition of LB broth is not defined (258). In contrast to this, minimal media is defined and contains only nutrients required for growth (258). These differences in nutrient levels infer that the cells behave differently in rich and minimal media. Bacterial cells growing in LB broth exhibit higher growth rates (a reflection of the greater availability of nutrients) which is associated with increased synthesis of membrane lipid, proteins, and DNA as well as increased respiration (245). Cells growing in minimal media must synthesise all building blocks needed for growth from the single carbon and energy source which involves adjusting the flow of metabolites from central pathways to biosynthetic pathways (245). This is mediated by upregulation of genes involved in biosynthetic pathways for amino acids, vitamins, enzyme cofactors and prosthetic groups (245).

Differences in metabolic and physiological states when *E. coli* is grown in rich or minimal media will be reflected in the associated gene expression profiles. Notably RpoS, a general stress response regulator, is constitutively expressed when *E. coli* is grown in minimal media, which regulates a specific set of genes (a different set to when induced in other environmental conditions) (260, 261). Expression of these genes will change the cell’s physiological state and possibly how it’s affected by PANI or P3ABA treatment (260). For example, *E. coli* grown in minimal media with 0.2% glucose were found to have altered gene expression compared to
growth in LB broth. In minimal media there was upregulation of some periplasmic and acid stress proteins and downregulation of *sodB* (oxidative stress response), *tonB* (iron uptake) and *sdhB* (TCA cycle)\(^{(260)}\). This illustrates how the physiological state of the deletion mutants may be different in both media tested, which may be further influenced by the absence of the protein product from the deleted gene. Investigation of the sensitivity of the deletion mutants in both rich and minimal media will increase the likelihood of identifying a noticeable difference in the relative sensitivity between the parent strain and the mutant strain (see section 4.1).

To investigate the antimicrobial mechanism of PANI and P3ABA, susceptibility profiles for these two antimicrobial agents were determined against a panel of Keio collection deletion mutants in rich and minimal media\(^{(213)}\). This was achieved by challenging the parent strain (*E. coli* K12 BW25113) and selected deletion mutants with 1% PANI or 1% P3ABA suspensions. At particular time points, the treated cells were enumerated using the drop plate method and the sensitivities of the deletion mutants relative to the parent strain were determined. Susceptibility profiles generated from the relative sensitivities of these mutants can be used to infer mechanism and generate hypotheses to be tested more rigorously in *E. coli* and *S. aureus* \(^{(257)}\).

The selected *E. coli* deletion mutants from the Keio collection are outlined below and include deletion mutants lacking genes involved in oxidative stress responses (table 3.1), iron homeostasis (table 3.2), metabolism and respiration (table 3.3), periplasmic stress responses (table 3.4) and DNA repair (table 3.5). Deletion mutants missing oxidative stress response genes included those lacking scavenging enzymes and enzymes that function to restore redox balance. The selected iron homeostasis mutants lack proteins associated with control of intracellular iron levels encompassing iron uptake and storage. Periplasmic stress response mutants were lacking genes involved in the reduction of periplasmic stress through protein chaperone activity. Respiratory and metabolism mutants were deficient in genes involved in metabolic pathways and energy generation.
Table 3.1. Summary of selected *E. coli* oxidative stress response deletion mutants. *E. coli* deletion mutants from the Keio collection were selected for testing against PANI and P3ABA. The functional group, deleted gene name, associated protein product and protein function are outlined.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Protein product</th>
<th>Function of protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>katG</em></td>
<td>Hydroperoxidase I or KatG</td>
<td>Catalase-peroxidase – detoxifies hydrogen peroxide(262, 263)</td>
</tr>
<tr>
<td><em>katE</em></td>
<td>Hydroperoxidase II or KatE</td>
<td>Monofunctional catalase – detoxifies hydrogen peroxide(262, 263)</td>
</tr>
<tr>
<td><em>ahpC</em></td>
<td>Alkyl hydroperoxide reductase subunit C (AhpC)</td>
<td>Subunit of NADH peroxidase – detoxifies hydrogen peroxide and organic hydroperoxides(262)</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>Superoxide dismutase A (SodA) or MnSOD</td>
<td>Cytoplasmic inducible SOD containing manganese that scavenges superoxide(264)</td>
</tr>
<tr>
<td><em>sodB</em></td>
<td>Superoxide dismutase B (SodB) or FeSOD</td>
<td>Cytoplasmic constitutive SOD containing iron that scavenges superoxide(264)</td>
</tr>
<tr>
<td><em>sodC</em></td>
<td>Superoxide dismutase C (SodC) or CuZnSOD</td>
<td>Periplasmic inducible SOD containing copper and zinc that scavenges superoxide(261, 265)</td>
</tr>
<tr>
<td><em>trxC</em></td>
<td>Thioredoxin 2 (Trx2)</td>
<td>Member of thioredoxin system – reduces disulfide bonds(266) Redox balance(267, 268)</td>
</tr>
<tr>
<td><em>grxA</em></td>
<td>Glutaredoxin 1 (Grx1)</td>
<td>Member of glutaredoxin system – reduces disulfides via reduced glutathione(266) Redox balance(266, 267) OxyR autoregulation: GrxA is expressed under control of oxyR and deactivates OxyR by reducing its disulfide bond(269)</td>
</tr>
</tbody>
</table>
### Table 3.2. Summary of selected *E. coli* iron homeostasis deletion mutants.

*E. coli* deletion mutants from the Keio collection were selected for testing against PANI and P3ABA. The functional group, deleted gene name, associated protein product and protein function are outlined.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Protein product</th>
<th>Function of protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tonB</em></td>
<td>TonB</td>
<td>Protein in TonB-ExbB-ExbD transport complex that transduces energy in proton motive force to outer membrane active transporters mediating iron and vitamin B12 import (270, 271)</td>
</tr>
<tr>
<td><em>iscS</em></td>
<td>Cysteine desulfurase (tRNA sulfurtransferase)</td>
<td>FeS protein maturation – provides sulfur during FeS cluster assembly (272) Sulfur trafficking (272) Formation of thiouridine in tRNA (273)</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>Ferric uptake regulator (Fur)</td>
<td>Ferric iron uptake regulon transcriptional repressor that regulates iron uptake and utilisation (274) Functions in iron homeostasis, which is linked to OxyR and SoxRS oxidative stress responses (275) Implicated in DNA synthesis during iron starvation, repression of biofilm formation and redirection of metabolism from oxidative phosphorylation to fermentation (274)</td>
</tr>
</tbody>
</table>

### Table 3.3. Summary of selected *E. coli* metabolism and respiration deletion mutants.

*E. coli* deletion mutants from the Keio collection were selected for testing against PANI and P3ABA. The functional group, deleted gene name, associated protein product and protein function are outlined.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Protein product</th>
<th>Function of protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sdhB</em></td>
<td>Succinate dehydrogenase subunit B</td>
<td>Succinate dehydrogenase oxidises succinate generating fumarate. Electrons from this oxidation are shuttled into the electron transport chain (ETC), coupling the tricarboxylic acid (TCA) cycle to the ETC (195, 196)</td>
</tr>
<tr>
<td><em>atpE</em></td>
<td>F0 sector of membrane-bound ATP synthase, subunit c</td>
<td>ATP synthase uses energy from proton translocation to synthesise ATP (276) This subunit is required for proton translocation and for binding of the F-1 complex</td>
</tr>
</tbody>
</table>
Table 3.4. Summary of selected \textit{E. coli} periplasmic stress response deletion mutants. \textit{E. coli} deletion mutants from the Keio collection were selected for testing against PANI and P3ABA. The functional group, deleted gene name, associated protein product and protein function are outlined.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Protein product</th>
<th>Function of protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{spy}</td>
<td>Spheroplast protein Y (Spy)</td>
<td>Stress-induced periplasmic ATP-independent protein refolding chaperone(277, 278)</td>
</tr>
<tr>
<td>\textit{asr}</td>
<td>Acid shock RNA (Asr)</td>
<td>Protection of cells during acid shock(189) Periplasmic putative protein chaperone(189)</td>
</tr>
</tbody>
</table>

Table 3.5. Summary of selected \textit{E. coli} DNA repair deletion mutants. \textit{E. coli} deletion mutants from the Keio collection were selected for testing against PANI and P3ABA. The functional group, deleted gene name, associated protein product and protein function are outlined.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Protein product</th>
<th>Function of protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{recA}</td>
<td>Recombinase A (RecA)</td>
<td>DNA repair and induction of SOS response(197)</td>
</tr>
<tr>
<td>\textit{nth}</td>
<td>Endonuclease III or Nth</td>
<td>Member of BER pathways that recognises DNA helix disruption(275) DNA glycosylase and apyrimidinic (AP) lyase that functions to remove damaged residues(275)</td>
</tr>
</tbody>
</table>

3.2 Sensitivity of \textit{E. coli} deletion mutants in LB broth to PANI and P3ABA suspensions

Characterisation of the relative sensitivities of \textit{E. coli} deletion mutants to 1\% PANI and 1\% P3ABA suspensions were initially performed in LB broth, a rich media. Time points for enumeration were selected based on the activity of PANI and P3ABA in LB broth so that the treated parent strain may have reduced in number but is above the limit of detection permitting calculation of the relative sensitivity as a percentage of the parent strain. For this reason, PANI treated cells were enumerated at 2 h and 4 h while P3ABA treated cells were enumerated at 0.5 h and 1 h time points. Results presented are from three independent experiments performed on different days.
3.2.1 Hydrogen peroxide scavenger deletion mutants

Transcriptomic analysis of *E. coli* treated with an fPANI suggested that antimicrobial action involves induction of oxidative stress. Hydrogen peroxide is a ROS that can oxidatively damage bacterial cells if it is not successfully scavenged by bacterial enzymes \(^\text{(275)}\). Based on this, the susceptibility of *E. coli* deletion mutants that are lacking genes encoding hydrogen peroxide scavenger enzymes were determined. *E. coli* JW3914-1 Δ*katG* has a deletion of the gene encoding the oxidative stress inducible catalase (HPI) that detoxifies hydrogen peroxide \(^\text{(262, 263)}\). *E. coli* JW1721-1 Δ*katE* has a deletion of the gene encoding the stationary phase induced catalase (HPII) that scavenges hydrogen peroxide \(^\text{(262, 263)}\). *E. coli* JW0598-2 Δ*ahpC* is lacking a gene encoding one subunit of the alkyl hydroperoxide reductase (Ahp), a scavenger of hydrogen peroxide and organic peroxides \(^\text{(262)}\). It was hypothesised that hydrogen peroxide scavenger mutants would have increased sensitivity to treatment by PANI and P3ABA relative to the parent strain as they are deficient in enzymes required for defence against hydrogen peroxide based oxidative stress.

The sensitivity of *E. coli* hydrogen peroxide scavenger mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). *E. coli* Δ*katG* was more sensitive to 1% PANI suspension than the parent strain (Fig. 3.1), which was statistically significant (linear regression analysis, slopes are different, \(P\) value: less than 0.05). The greatest difference in relative sensitivity of the *E. coli* Δ*katG* mutant was observed after treatment for 2 h. This relative difference decreased by 4 h of treatment because the mutant reached the limit of detection (50 CFU/ml), indicated on the graph as a grey data point (Fig. 3.1), which means that the difference between the parent and mutant strain may be underestimated. Therefore, the median line for the *E. coli* Δ*katG* mutant at 4 h time point (Fig. 3.1) may not represent the true relative difference between the parent and mutant strain, and is indicated on the graph as a grey line. This potential underestimation of the relative difference between the parent strain and a deletion mutant due to the experimental limitation of one strain dropping below the limit of detection during treatment is a recurrent theme in this chapter that will influence interpretation of results.

Overall, *E. coli* Δ*katE* had a similar susceptibility to the 1% PANI suspension as the parent strain (Fig. 3.1), which was confirmed by linear regression analysis (slopes and intercepts are similar, \(P\) values: more than 0.05). *E. coli* Δ*ahpC* was less sensitive to treatment with 1% PANI than the
parent strain (Fig. 3.1). This difference was statistically significant (linear regression analysis, slopes are different, P value: less than 0.05). The relative difference between \textit{E. coli} Δ\textit{ahpC} and the parent strain was greater at 4 h than 2 h (Fig. 3.1).

In contrast to the sensitivity to PANI suspension, the \textit{E. coli} Δ\textit{katG} mutant had a comparable response to exposure to P3ABA suspension as the parent strain (Fig. 3.2). Similarly, there was no detectable difference in sensitivity between either \textit{E. coli} Δ\textit{katE} or \textit{E. coli} Δ\textit{ahpC} and the parent strain (Fig. 3.2). The lack of difference was confirmed using linear regression analysis (slopes and intercepts are similar, P values: more than 0.05).

The hypothesis, that mutations in the genes encoding hydrogen peroxide detoxifying enzymes (\textit{katE}, \textit{katG} and \textit{ahpC}) would result in supersensitivity to PANI and P3ABA killing if the mechanism of antimicrobial action involved oxidative stress, is not strongly supported (Fig. 3.1; Fig. 3.2).

For PANI, \textit{E. coli} carrying a deletion of \textit{katG}, encoding an inducible catalase, had increased sensitivity. Mutations in the genes encoding constitutive and stationary phase catalases, \textit{ahpC} and \textit{katE} respectively, had either no effect or were slightly protective(262, 263). It may be that deficiency in the housekeeping removal of H$_2$O$_2$ in \textit{ahpC} and \textit{katE} mutants induces \textit{katG} expression that provides protection against antimicrobial challenges that act through oxidative stress mechanisms. In support of this explanation a number of studies are noteworthy:

i. an \textit{E. coli} \textit{katE} mutant grown in LB broth exhibits increased HPI activity in a spectrophotometric assay, which involves measuring absorbance of crude extracts of \textit{E. coli} cells at 240 nm to detect hydrogen peroxide levels(279, 280).

ii. the sensitivity pattern for \textit{katG} and \textit{katE} mutants challenged with norfloxacin, an antibiotic postulated to exert lethal action by inducing H$_2$O$_2$ oxidative stress, is similar to PANI, i.e. the \textit{katG} mutant is 10- to 100-fold more sensitive to norfloxacin than the wild-type while the \textit{katE} mutant had a similar sensitivity as determined by a cell viability assay involving treatment with 10× MIC of norfloxacin in LB broth(281).

iii. an \textit{E. coli} \textit{ahpCF} mutant contained 7-fold more catalase and scavenged 150 μM H$_2$O$_2$ twice as rapidly as did the wild-type(282). The authors hypothesised that H$_2$O$_2$
accumulated in the *ahpCF* mutant leading to OxyR activation and expression of *katG* (282).

iv. the pre-treatment of *E. coli* cells with small amounts of H$_2$O$_2$ is associated with greater survival following exposure to a second H$_2$O$_2$ challenge (283). This pre-adaptive effect was characterised by a 10-fold induction of the *katG* gene (283).

v. an *E. coli* mutant missing *ahpCF* and *katE* genes has been shown to have H$_2$O$_2$ scavenging abilities while a mutant lacking *ahpCF* and *katG* genes failed to scavenge H$_2$O$_2$ (282). The ability of the *ahpCF katE* mutant to detoxify H$_2$O$_2$ suggests the lack of either of these genes resulted in upregulation of *katG* (282).

a. In conclusion, there is some evidence that H$_2$O$_2$ based oxidative stress is involved in the antimicrobial mechanism of PANI and no evidence to suggest that H$_2$O$_2$ is produced following contact with P3ABA.
Figure 3.1. Sensitivity of *E. coli* 7636 parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants to PANI suspension. Cell viability assays of $\sim 1 \times 10^6$ CFU/ml *E. coli* parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants – *E. coli* ΔkatG, *E. coli* ΔkatE and *E. coli* ΔahpC – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.
Figure 3.2. Sensitivity of *E. coli* 7636 parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants to P3ABA suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants – *E. coli ΔkatG, E. coli ΔkatE* and *E. coli ΔahpC* – challenged with 1% P3ABA suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 0.5 h and 1 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).

### 3.2.2 Superoxide dismutase scavenger deletion mutants

The previous section examining the sensitivity of H\textsubscript{2}O\textsubscript{2} scavenger deletion mutants partially supported hypotheses generated based on upregulation of oxidative stress response genes in *E. coli* sublethally treated with fPANI. The supersensitivity of the *katG* mutant suggested some involvement of H\textsubscript{2}O\textsubscript{2} in the antimicrobial mechanism of PANI. Superoxide (O\textsuperscript{2-}) can also be a mediator of oxidative stress and is formed following transfer of an electron to oxygen\(^{(275)}\). Superoxide can damage biomolecules in bacterial cells in a similar manner to H\textsubscript{2}O\textsubscript{2}\(^{(275)}\). The SoxRS system responds to superoxide associated stress following oxidation of SoxR\(^{(275)}\). This oxidation causes a conformational change in SoxR that facilitates transcription of soxS, which in
turn mediates expression of genes in the SoxRS regulon, including sodA and fur, that function to alleviate the stress and repair damage (264, 275). The sodA gene encodes superoxide dismutase A (MnSOD), a cytoplasmic inducible SOD containing manganese that scavenges superoxide (264). In iron rich conditions Fur negatively regulates sodA expression, although the decrease in transcription is only threefold (253, 264). Fur positively regulates sodB expression in an iron independent manner (253, 284). The sodB gene encodes superoxide dismutase B (FeSOD), a cytoplasmic SOD that contains iron (264). E. coli also has a periplasmic inducible SOD containing copper and zinc, CuZnSOD, encoded by sodC (261, 265). SodC expression is induced by RpoS during stationary phase growth (261). Investigation of the effect of defects in the response to superoxide stress upon the antimicrobial activity of PANI and P3ABA was carried out using E. coli JW3879-1 ΔsodA, E. coli JW1648-1 ΔsodB and E. coli JW1638-1 ΔsodC. It was hypothesised that superoxide scavenger mutants would have increased sensitivity to treatment by PANI and P3ABA relative to the parent strain as they are deficient in enzymes required for defence against oxidative stress.

The sensitivity of E. coli superoxide dismutase scavenger mutants to PANI treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). All three SOD mutants had similar sensitivity to treatment with 1% PANI when compared to the parent strain (Fig. 3.3), which was confirmed to not be statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). For E. coli ΔsodB, during one experimental run the parent strain reached the limit of detection at the 4 h time point, which means that the relative difference detected between the mutant and the parent strain may have been larger than what was detected.

The sensitivity of E. coli superoxide dismutase scavenger mutants to P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). Following treatment with 1% P3ABA suspension, E. coli ΔsodA, E. coli ΔsodB and E. coli ΔsodC had comparable sensitivities to 1% P3ABA treatment as the parent strain (Fig. 3.4), similar to the result observed for 1% PANI suspension treatment. The difference in sensitivity between E. coli ΔsodA, E. coli ΔsodB and E. coli ΔsodC and the parent strain was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05).
The hypothesis that deletion of the genes encoding superoxide detoxifying enzymes (sodA, sodB and sodC) would result in supersensitivity to PANI and P3ABA killing if the mechanism of antimicrobial action involved superoxide-mediated oxidative stress was not supported by these experiments.

The lack of difference in the sensitivities of E. coli ΔsodA, E. coli ΔsodB and E. coli ΔsodC to P3ABA treatment suggests that oxidative stress does not play a major role in the lethal action of this antimicrobial agent, consistent with results obtained for the H$_2$O$_2$ scavenger mutants.

**Figure 3.3. Sensitivity of E. coli 7636 parent strain and E. coli superoxide scavenger deletion mutants to PANI suspension.** Cell viability assays of ~ 10$^6$ CFU/ml E. coli parent strain and E. coli superoxide scavenger deletion mutants – E. coli ΔsodA, E. coli ΔsodB and E. coli ΔsodC – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.
3.2.3 Oxidative stress response deletion mutants

The results obtained in section 3.2.1 and 3.2.2 investigating hydrogen peroxide and superoxide scavenger mutants do not support an increased sensitivity to the antimicrobial effects of PANI and P3ABA in mutant strains of *E. coli* lacking various enzymes responsible for the detoxification of these mediators of oxidative stress as had been hypothesised after the transcriptomics study. The supersensitivity of the Δ*katG* mutant does suggest some role for H$_2$O$_2$ in the antimicrobial actions of PANI. To complete a comprehensive investigation of the effect of defects in the response to oxidative stress upon the antimicrobial activity of PANI and P3ABA, *E. coli* mutants lacking genes required to restore redox balance, *trxC* and *grxA*, were tested.
E. coli JW2566-1 ΔtrxC has a deletion of the gene encoding thioredoxin-2 (Trx2), which forms part of the thioredoxin system (266–268). Trx2 reduces disulfide bonds in oxidatively damaged proteins to restore redox balance in cells challenged with oxidative stress (266–268). Similar to Trx2, the protein product of the deleted gene in E. coli JW0833-1 ΔgrxA, glutaredoxin 1 (Grx1), functions to restore redox balance (266, 267). Grx1 is also involved in autoregulation of OxyR – the effector protein of the hydrogen peroxide stress response (269). GrxA is expressed under control of OxyR and deactivates OxyR by reducing the critical disulfide bond (269). It was hypothesised that loss of proteins involved in restoring redox balance following oxidative stress under the control of OxyR would sensitise the cells to PANI and P3ABA treatment.

The sensitivity of E. coli oxidative stress response mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). E. coli ΔtrxC had a similar sensitivity to 1% PANI suspension to the parent strain (Fig. 3.5), which was confirmed as not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). The difference in sensitivity between E. coli ΔtrxC and the parent strain was greater at the 2 h time point compared to the 4 h time point, which may be in part due to the parent strain reaching the limit of detection (grey data point, Fig. 3.5). E. coli ΔgrxA had a similar sensitivity to 1% PANI suspension as the parent strain (Fig. 3.5), which was confirmed by linear regression analysis (slopes and intercepts are similar, P values: more than 0.05).

E. coli ΔtrxC and E. coli ΔgrxA treated with 1% P3ABA had similar sensitivity to treatment as the parent strain (Fig. 3.6), which was confirmed by linear regression analysis (slopes and intercepts are similar, P values: more than 0.05).

The hypothesis tested – that loss of Trx2 and Grx1 would increase the susceptibility of cells to the bactericidal actions of PANI and P3ABA – was not supported by these results. Cells without Grx1 will have a prolonged OxyR response after hydrogen peroxide treatment (285). This would not necessarily confer an advantage against a H2O2 stress challenge as GrxA acts to inactivate OxyR following a reduction in H2O2 levels and wouldn’t greatly affect the activation state of OxyR in the presence of an oxidant (which would serve to reactivate any OxyR reduced by GrxA). The lack of any notable difference in the sensitivity of E. coli ΔtrxC and E. coli ΔgrxA to
PANI and P3ABA suspensions suggested that antimicrobial action does not involve changes to cellular redox status.

When all the results obtained for the relative sensitivities of *E. coli* oxidative stress response deletion mutants to PANI and P3ABA are taken together, a new hypothesis pertaining to antimicrobial mechanism can be generated. It appears that PANI acts through a H$_2$O$_2$ based oxidative stress mechanism, which was supported by the increased sensitivity of the deletion mutant lacking the OxyR-inducible catalase (*E. coli* ΔkatG). There was no strong evidence suggesting that P3ABA antimicrobial action involves induction of oxidative stress as H$_2$O$_2$ and superoxide scavenger mutants did not have altered susceptibilities to P3ABA treatment.
Figure 3.5. Sensitivity of *E. coli* 7636 parent strain and *E. coli* oxidative stress response deletion mutants to PANI suspension. Cell viability assays of ~ 10^6 CFU/ml *E. coli* parent strain and *E. coli* oxidative stress response deletion mutants – *E. coli* ΔtrxC and *E. coli* ΔgrxA – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.
3.2.4 Iron homeostasis deletion mutants

In the sections examining the response of H$_2$O$_2$ and superoxide scavenger mutants, strong evidence for oxidative stress as the central mechanistic driver of PANI and P3ABA antimicrobial action is not apparent; however, there is transcriptional evidence for oxidative stress responses, namely induction of $\text{trxC}$, $\text{grxA}$, $\text{mntH}$ and $\text{sufC}$ during sublethal fPANI challenge, and the supersensitivity of the $\text{E. coli katG}$ mutant (section 3.2.1). Intracellular oxidative stress is often closely linked with imbalances in iron homeostasis. Unincorporated intracellular iron can be oxidised by H$_2$O$_2$ generating a highly reactive hydroxyl radical, termed Fenton
reaction (261, 275). Therefore, homeostatic control of free intracellular iron levels is important for reducing oxidative stress (261).

Cytoplasmic iron levels are precisely controlled by ferric uptake regulator (Fur), which following binding of Fe$^{2+}$ can act as a transcriptional repressor and activator connecting iron transport with iron utilisation (274). In iron-replete conditions, Fur represses the transcription of iron-uptake genes, such as fhuE, tonB and fiu, and activates iron utilisation enzymes, such as sdhCDAB, uxuAB and hybOA (274, 286, 287). Positive regulation of genes encoding iron utilisation proteins is achieved by Fur-mediated repression of a small (s)RNA named RyhB, which promotes the degradation of mRNAs positively regulated by Fur (286). Therefore, when there is an excess of Fe$^{2+}$ Fur acts to limit further import and promotes iron incorporation reducing free iron levels (194, 286). Conversely, when iron is limited, Fur is inactivated leading to increased iron uptake and decreased iron utilisation (286).

Iron uptake by bacteria is achieved through secretion of siderophores (such as enterobactin and enterochelin) that bind and solubilise the iron (286). The iron-siderophore complex is bound by outer membrane receptors, such as FhuE and Fui, which specifically bind these complexes as well as other iron sources, such as ferric citrate (286). Transport of iron-siderophore complexes across the outer membrane is mediated by the ATP-dependent TonB–ExbB–ExbD complex (286). This complex transduces energy from the proton motive force to the outer membrane receptors resulting in the import of the iron-siderophore complexes into the periplasm (270). TonB is embedded in the cytoplasmic membrane and extends into the periplasmic space (270). Binding of an iron-siderophore complex causes a conformational change in the receptor, which facilitates TonB interaction (270). TonB is responsible for the energy coupling step of iron import (270). Periplasmic iron-siderophore complexes are transported across the inner membrane using different ABC transporter systems (FecBCDE, FepBCDEFG and FhuBCD) (286). Upon reaching the cytoplasm, the iron is released allowing its utilisation by the cell (286).

Iron utilisation in E. coli functions to decrease free iron levels and involves binding by storage proteins and incorporation of iron into metabolic and respiratory enzymes, which can be as part of an iron-sulfur (FeS) cluster or as part of a nonredox mononuclear iron enzyme (194, 204, 205, 274). Iron storage proteins take iron up in the ferrous form, which is converted
to the ferric form for deposition in the central space (194, 288). Stored iron is used by the cell when iron is limited in the environment (194, 288). A common iron storage protein is ferritin, which is positively regulated by Fur (203). Fur induces the expression of genes encoding iron-containing proteins, which suggests that the biosynthesis of some iron-requiring proteins may be geared to iron availability (194, 289). Mononuclear iron enzymes use polypeptide residues to bind a single iron atom, which enables binding of substrate and stabilises the anionic reaction intermediate (203, 204). Mononuclear iron enzymes typically function in metabolic pathways, such as ribulose-5-phosphate 3-epimerase (RPE) in the pentose-phosphate pathway, and catalyse a variety of reaction types including dehydrogenation and deamination (204).

Control of mononuclear iron enzyme expression occurs as part of a greater transcriptional regulatory network that imparts metabolic flexibility in response to a changing environment (290). Oxidation of iron in mononuclear enzymes by either H$_2$O$_2$ or superoxide results in iron dissociation, polypeptide damage and mismetallation of the enzymes leading to loss of function (203–205).

Iron can be incorporated into FeS clusters of metabolic and respiratory enzymes, including TCA cycle enzymes aconitase, succinate dehydrogenase and aerobic fumarase (194, 289). Fur positively regulates the expression of enzymes containing FeS clusters, including acnA (encoding aconitase), fumA (encoding aerobic fumarase) and sdhCDAB (encoding succinate dehydrogenase) (194). Coordinating expression of iron containing proteins with free iron levels would act to reduce free iron levels and avoid the unnecessary synthesis of iron-requiring proteins when there is insufficient iron to combine with them (194, 274, 289). Fur represses the small (s)RNA RyhB, which mediates breakdown of the mRNAs positively regulated by Fur (286, 289). This indirect Fur regulation complements the direct activation by iron-bound Fur (274). Iron in [4Fe-4S]$^{2+}$ clusters is susceptible to oxidation by H$_2$O$_2$, which results in dissociation of the iron and potentially oxidative damage to the enzyme (291). The associated increase in intracellular free iron promotes Fenton reaction and production of the damaging hydroxyl radical (275). Loss of iron from the cluster results in loss of function of the enzyme (291).

*E. coli* has two FeS cluster synthesis systems, ISC (Iron Sulfur Cluster) and SUF (SUlFur assimilation) systems, encoded by the ISC (iscRSUA-hscBA-fdx-iscX) and SUF (sufABCDSE) operons, respectively (191, 292). Fe-S cluster assembly consists of liberating sulfur from
L-cysteine by cysteine desulfurase enzymes IscS or SufSE, which is then donated to a scaffold protein, IscU or SufB, respectively (191, 292). FeS clusters are assembled on the scaffold protein and transferred either directly to apo-protein substrates or to the carrier proteins IscA and SufA, which function to deliver clusters to specific substrates (191, 292). Transfer of clusters is dependent on the ATPase activity of HscBA complex of the ISC pathway or SufC of the SUF pathway (191). The ISC system functions in de novo synthesis of FeS clusters while the SUF system functions to repair oxidatively damaged clusters and synthesise clusters in iron limiting conditions (191, 293). The ISC system is believed to supply more than 150 different proteins with Fe-S clusters in *E. coli* while the SUF pathway only provides clusters to essential Fe-S proteins required for growth (191). The SUF operon is directly positively regulated by Fur and OxyR while the ISC operon is indirectly regulated by Fur via RyhB-mediated mRNA degradation (274, 294, 295). The ISC operon is transcriptionally repressed by IscR, encoded by the first gene in the operon, which is mediated by ligation of a FeS cluster on IscR (191, 289).

The hypothesis that PANI and/or P3ABA induced dysregulation of iron homeostasis is central to the antimicrobial activity, with observed oxidative stress an effect of this, was investigated. Upregulation of genes involved in maintaining iron homeostasis, such as *sufC*, identified by the transcriptomic analysis of *E. coli* sublethally treated with fPANI combined with the increase in intracellular free iron detected in P3ABA treated cells suggests the antimicrobial mechanism of PANI and P3ABA may involve disruption of control of free iron levels and the associated oxidative stress (200). Investigation of this hypothesis was undertaken with mutants lacking genes involved with control of free iron levels (*E. coli JW0669-2 Δfur*), iron uptake (*E. coli JW5195-1 ΔtonB*) and iron utilisation (*E. coli JW2514-4 ΔiscS*).

*E. coli Fur* mutants constitutively express the iron limitation response and have high intracellular free iron concentrations (272). High intracellular free iron is associated with oxidative damage due to hydroxyl radical production following Fenton reaction (253, 275). It was hypothesised that perturbation of iron homeostasis through deletion of the *fur* gene would result in a supersensitivity of the *fur* mutant to PANI and P3ABA treatment. Mutants missing the *tonB* gene cannot take up iron into cells and would have decreased free iron levels (287). It was hypothesised that impairment of exogenous iron uptake in the *E. coli ΔtonB* mutant would render it less sensitive to treatment with PANI and P3ABA suspensions. *E. coli JW2514-4 ΔiscS* mutants cannot form proper FeS clusters resulting in reduced activities of
cluster containing enzymes (273, 293). Therefore, this mutant has fewer potential targets for antimicrobial action involving induction of hydrogen peroxide mediated oxidative stress. It was hypothesised that *E. coli ΔiscS* would be less sensitive to treatment with PANI and P3ABA suspensions than the parent strain.

The sensitivity of *E. coli* iron homeostasis deletion mutants to PANI treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). *E. coli ΔtonB* was less susceptible to the action of 1% PANI suspension compared to the parent strain (Fig. 3.7), which was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05). In contrast to this, *E. coli ΔiscS* was sensitive to 1% PANI suspension treatment than the parent strain (Fig. 3.7). *E. coli ΔiscS* reached the limit of detection by 2 h, which meant that the difference between the mutant and the parent strain may be underestimated at the 4 h time point (Fig. 3.7). A linear regression analysis found that this difference was statistically significant (intercepts are different, P value: less than 0.05). Overall, *E. coli Δfur* had a similar sensitivity to 1% PANI suspension as the parent strain (Fig. 3.7), which was confirmed as no difference (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). The relative sensitivity of *E. coli Δfur* at the 4 h time point was variable with the median relative sensitivity just below the parent strain threshold (Fig. 3.7).

The hypothesis that PANI induced dysregulation of iron homeostasis is central to the antimicrobial activity, with observed oxidative stress an effect of this, was mostly not supported by these experiments. For PANI treatment, only deletion of *tonB*, encoding a protein in the TonB–ExbB–ExbD complex responsible for importing iron into the cell, ratified the hypothesis (286). Deletion of the *tonB* gene had a protective effect following exposure to PANI suggesting induction of oxidative stress, which supports results obtained using a Δ*fur* Δ*recA* mutant of *E. coli* (253). The susceptibility of *E. coli Δfur* to PANI was variable, which may be reflective of the function of Fur as a global transcription factor, implicated in cellular functions including DNA synthesis during iron starvation, repression of biofilm formation and redirection of metabolism from oxidative phosphorylation to fermentation (274). Disruption of the Fur regulatory network in either high or low iron conditions can lead to major changes in transcript levels of many genes (274). Therefore, deletion of the *fur* gene may result in changes to cellular function separate from control of iron homeostasis, which may influence susceptibility to antimicrobial treatment.
The increased susceptibility of *E. coli ΔiscS* to PANI suspension does not support the hypothesis that PANI action involves targeting of FeS clusters in enzymes. Deletion of a target would be expected to decrease sensitivity to antimicrobial action. The ISC operon has functions beyond FeS cluster synthesis involving molybdenum cofactor synthesis and modification, DNA and tRNA modification, and thiamine synthesis; with the IscS cysteine desulfurase having a major role in providing sulfur for these pathways(191, 272). Therefore, deletion of the iscS gene and the consequent inactivation of the ISC operon will have consequences beyond decreasing FeS cluster levels(291). The increased susceptibility of *E. coli ΔiscS* to PANI treatment may reflect loss of one of the other functions mediated by IscS, such as DNA modification. IscS is involved in DNA phosphorothioation, the addition of sulfur to phosphate groups on DNA, which endows DNA with a reducing chemical property believed to confer protection against peroxide damage(296–298). Therefore, IscS mutants would be more susceptible to oxidative damage to DNA(253, 299). The striking sensitivity to PANI may be suggestive of broad targets of action.

The sensitivity of *E. coli* iron homeostasis mutants to P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). All three iron homeostasis deletion mutants were similarly sensitive to P3ABA suspension compared to the parent strain (Fig. 3.8), which was confirmed by linear regression analysis (slopes and intercepts are similar, P values: more than 0.05). *E. coli ΔiscS* reached the limit of detection following treatment for 4 h during one experimental run, which may have underestimated the difference between that strain and the parent strain (Fig. 3.8).

The hypothesis that P3ABA induces perturbation of iron homeostasis alongside inducing intracellular oxidative stress was partially supported by these experiments. The lack of insensitivity of the *E. coli ΔtonB* mutant to P3ABA is not consistent with the release of free iron in P3ABA treated cells as detected by EPR spectroscopy(200). The indifference in relative sensitivity may suggest that the major iron import system does not play a significant role in P3ABA mediated cell death but rather the increase in free iron may be attributed to disruption of internal iron sources. Perturbation of internal iron management would mean that prevention of iron import would not be protective against oxidative stress. A similar situation was found for gyrase inhibitor mediated cell death(238).
The results generated from determining the relative sensitivities of oxidative stress response mutants and iron homeostasis mutants in iron rich media are suggestive of a putative role for the production of hydrogen peroxide during PANI antimicrobial action (indicated by the supersensitivity of the KatG mutant), damage from which is exacerbated by free intracellular iron (indicated by decreased sensitivity of the TonB mutant). This is supported by the increased sensitivity of the IscS mutant (possibly due to a lack of DNA protection by phosphorothioation).
Figure 3.7. Sensitivity of *E. coli* 7636 parent strain and *E. coli* iron homeostasis deletion mutants to PANI suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* iron homeostasis deletion mutants – *E. coli* ΔtonB, *E. coli* ΔiscS and *E. coli* Δfur – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.
Figure 3.8. Sensitivity of *E. coli* 7636 parent strain and *E. coli* iron homeostasis deletion mutants to P3ABA suspension. Cell viability assays of ~ $10^6$ CFU/ml *E. coli* parent strain and *E. coli* iron homeostasis deletion mutants – *E. coli* Δ*tonB*, *E. coli* ΔiscS and *E. coli* Δ*fur* – challenged with 1% P3ABA suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 0.5 h and 1 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.

3.2.5 Metabolism and respiration deletion mutants

Previous work examining fPANI action using transcriptome analysis of sublethally treated *E. coli* cells demonstrated downregulation of some genes encoding proteins involved in central metabolism and energy generation including TCA genes, such as *sdhB* and *aceA* (163). Results of the transcriptome analysis suggested that the mechanism of action may be direct or indirect targeting of metabolic and respiratory enzymes. The study of oxidative stress response mutants (such as *E. coli* Δ*katG*, section 3.2.1) and iron homeostasis mutants (such as *E. coli* Δ*fur*...
ΔtonB, section 3.2.4), and EPR spectroscopic analysis of P3ABA treated E. coli cells lends further support to this hypothesis(200, 291). Targeting of respiratory machinery is supported by previous work that identified disruption of membrane integrity in E. coli cells treated with sublethal levels of P3ABA(200).

Many metabolic and respiratory enzymes can contain FeS clusters or iron cofactors that are susceptible to oxidation by H₂O₂ and superoxide resulting in iron release and enzyme damage(203, 291, 293). Enzymes with FeS clusters susceptible to oxidation include [4Fe-4S]²⁺ containing dehydratase enzymes, such as aconitase, fumarase and SDH from the TCA cycle. Oxidation of the [4Fe-4S]²⁺ cluster results in a [4Fe-4S]³⁺ state, which triggers demetallation to an inactive [3Fe-4S]¹⁺ form(273, 293). The [3Fe-4S]¹⁺ cluster can be converted back to the active form without a sulfur source through the actions of the SUF machinery(273, 293).

Mononuclear iron enzymes, such as those in amino acid synthetic pathways, are known targets of ROS(203). Exposure to H₂O₂ and superoxide results in growth defects that are associated with loss of function of oxidation susceptible metabolic enzymes(203, 291, 293).

To investigate if [4Fe-4S]²⁺ containing TCA dehydratase enzymes are targets of PANI and/or P3ABA challenges, the relative sensitivity of E. coli JW0714-1 ΔsdhB to PANI and P3ABA suspensions was determined. The gene sdhB encodes the b subunit of succinate dehydrogenase (SDH) (195, 196). SDH provides a functional link between the TCA cycle and the ETC as it both oxidises succinate generating fumarate as part of the TCA cycle and transfers electrons to ubiquinone to feed into the ETC(195, 196, 300). SdhB subunit contains a [4Fe-4S]²⁺ cluster, which is susceptible to oxidation by hydrogen peroxide resulting in dissociation of an iron atom and loss of enzyme activity(195, 197). Therefore, deletion of this gene represents loss of both a potential target of PANI and P3ABA action and a source of oxidatively released iron(272). It was hypothesised that E. coli ΔsdhB would be less sensitive to PANI and P3ABA suspensions than the parent strain due to loss of a potential target.

ETC functions to transfer electrons from electron donors through the chain while translocating protons into the periplasmic space(301). Under aerobic conditions, the E. coli ETC consists of two NADH:ubiquinone oxidoreductases – complex I NADH dehydrogenase I (or Nuo) and complex II, NADH dehydrogenase II – different quinone species and three terminal oxidases, ubiquinol oxidoreductase complexes bo, bd-I and bd-II(272, 300, 302). Complex I and the
terminal oxidases couple electron transfer to proton pumping. Quinones, including benzoquinone ubiquinone active under aerobic conditions, provide a functional link between complex I, complex II and the terminal oxidases(302). Under aerobic conditions, the terminal oxidases transfer electrons from quinones to the terminal electron acceptor, oxygen(302). Transfer of electrons through ETC generates a proton gradient, proton motive force (PMF) (272, 303). ATP synthase couples the energy in the PMF to ATP synthesis(272, 276, 303). ATP synthase consists of two parts, F0 and F1, which consist of five subunits (a, b, c, d and e) and three subunits (a, b and c), respectively(276, 303). The F0 subunit spans the inner membrane and contains a proton channel while the F1 subunit contains the three catalytic sites necessary for ATP synthesis(276). The energy derived from the flow of protons through the F0 subunit results in a conformational change in the F1 subunit driving ATP synthesis(276). ATP generation by electron transport is called oxidative phosphorylation(304, 305). ATP can also be generated by substrate level phosphorylation, which occurs during oxidative steps in catabolic pathways, such as glycolysis(305).

The hypothesis that PANI and/or P3ABA antimicrobial activity directly or indirectly targets oxidative phosphorylation was investigated with an E. coli mutant that has a non-functional ATP synthase. E. coli JW3715-1 ΔatpE has deletion of a gene encoding the c subunit of the F0 section of ATP synthase(303). E. coli ΔatpE has impaired oxidative phosphorylation and reduced ATP synthesis(306–308). It was hypothesised that deletion of a potential target of PANI and/or P3ABA action would render E. coli ΔatpE to be insensitive to treatment with PANI and P3ABA. The sensitivity of E. coli metabolism and respiration deletion mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6).

E. coli ΔsdhB was knocked-down to a similar extent as the parent strain after treatment with 1% PANI in suspension (Fig. 3.9), which was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). E. coli ΔatpE had a similar sensitivity to 1% PANI suspension as the parent strain (Fig. 3.9), which was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05).
The hypothesis that PANI antimicrobial action targets metabolic and respiratory machinery was not supported by these results. The SdhB mutant was unchanged in sensitivity relative to the parent strain, which suggests that either SdhB is not targeted by PANI or that loss of this potential target was not sufficient to affect susceptibility to bactericidal activity. Previous work suggests that PANI antimicrobial activity involves H$_2$O$_2$ stress and free iron release. There are many cellular biomolecules that may be targeted and damaged by H$_2$O$_2$ stress including other [4Fe-4S]$^{2+}$ containing enzymes, mononuclear iron enzymes and DNA(197). Loss of one of many putative targets of PANI action may not elicit a protective effect. *E. coli* $\Delta$atpE was as susceptible to PANI action as the parent strain, which infers that ATP synthase is not a direct target.

For 1% P3ABA treatment, *E. coli* $\Delta$sdhB mutant had a similar sensitivity to that of the parent strain (Fig. 3.10). At the 1 h time point *E. coli* $\Delta$sdhB reached the limit of detection on two occasions, which may have led to the underestimation of the difference between the mutant and the parent strain (grey data points, Fig. 3.10). The difference in sensitivity between *E. coli* $\Delta$sdhB and the parent strain was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). *E. coli* $\Delta$atpE was less sensitive to 1% P3ABA suspension than the parent strain (Fig. 3.10). The difference in sensitivity between *E. coli* $\Delta$atpE and the parent strain was statistically significant (linear regression analysis, slopes are different, P value: less than 0.05).

The hypothesised targeting of metabolic and respiratory machinery by P3ABA antimicrobial action was supported by the relative insensitivity the AtpE *E. coli* mutant. Mutation of genes encoding antimicrobial targets is expected to confer a decreased sensitivity to treatment(184, 213, 257). *E. coli* $\Delta$atpE was less sensitive to P3ABA suspension, which infers that respiratory machinery involved in oxidative phosphorylation is a target of P3ABA action.
Figure 3.9. Sensitivity of *E. coli* 7636 parent strain and *E. coli* metabolism and respiration deletion mutants to PANI suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* metabolism and respiration deletion mutants – *E. coli* Δ*sdhB* and *E. coli* Δ*atpE* – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.10. Sensitivity of *E. coli* 7636 parent strain and *E. coli* metabolism and respiration deletion mutants to P3ABA suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* metabolism and respiration deletion mutants – *E. coli* ΔsdhB and *E. coli* ΔatpE – challenged with 1% P3ABA suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 0.5 h and 1 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.

3.2.6 Periplasmic stress response deletion mutants

Previous work examining the responses of *E. coli* mutants lacking genes encoding enzymes involved in metabolic and respiratory functions identified targeting of these enzymes by P3ABA and possible indirect or downstream damage by PANI induced oxidative stress. Damage to pathways that function to produce ATP and maintain cell membrane gradients may result in perturbation of membrane integrity and loss of cell viability(309). Disruption of membrane
Integrity would elicit unfavourable changes in the periplasm resulting in induction of periplasmic stress response(s) that function to minimise defects caused by the stress, such as by inducing expression of protein chaperones (310, 311). Transcriptomic analysis of \textit{E. coli} sublethally treated with an fPANI demonstrated upregulation of the \textit{spy} and \textit{cpxP} genes, which suggests that the protein products, Spy and CpxP, respectively, are involved in bacterial defence against fPANI action as part of periplasmic stress responses (163, 310, 311).

\textit{E. coli} has three signalling pathways that are responsive to periplasmic stress signals, termed extracytoplasmic stress response pathways (310, 311). Two extracytoplasmic stress response pathways are activated through two-component systems, BaeSR and CpxAR, while the third pathway acts via the alternative $\sigma^E$ factor (311). All three extracytoplasmic stress response pathways transmit stress signals from the periplasm to the cytoplasm to modify the expression of a regulon that functions to reduce damage by the periplasmic stress and maintain membrane integrity (310, 311). The $\sigma^E$ and CpxAR envelope stress responses are activated by both common and distinct envelope stresses, with specificity based on distinct classes of misfolded proteins (278, 310). The $\sigma^E$ pathway specifically functions to ensure proper assembly of outer-membrane proteins (278, 310). The CpxAR pathway in the presence of adverse conditions responds to misfolded periplasmic proteins located at the periplasmic face of the inner membrane and acts to ensure proper pilus assembly (278, 310, 312). Activation of both stress responses leads to the upregulation of periplasmic protein folding factors, such as chaperones, and degrading factors, such as the protease DegP, as well as target genes unique to each response (278, 310).

The BaeSR (bacterial adaptive response) pathway consists of the BaeS sensor kinase and the BaeR response regulator (277, 278). The function of the BaeSR system has not been fully elucidated; however, it is known to respond to periplasmic stress signals in a manner that overlaps with the CpxAR pathway (277, 278). The \textit{spy} (Spheroplast protein y) gene is part of both the CpxAR and BaeSR regulons (278, 313). The involvement of the CpxAR and BaeSR pathways in expression of the \textit{spy} gene varies depending on the stress signal (278). Spy is a protein folding chaperone that functions to prevent protein aggregation and mediates protein refolding in an ATP-independent manner (277, 310). Spy forms a flexible cradle-shaped dimer, which allows the chaperone to shield regions of a periplasmic protein that are prone to
aggregation (277). The flexibility in the spy dimer permits it to act on a variety of partially unfolded protein substrates (277).

The periplasm may be subject to acid stress from internal or external sources, which can also cause protein misfolding (314). P3ABA contains 3-aminobenzoic acid groups, which have potential to release protons in solution (163, 315). Transcriptomic analysis of E. coli sublethally treated with an fPANI demonstrated upregulation of the asr (acid shock RNA) gene, which suggests that the protein product Asr is involved in bacterial defence against fPANI action (163, 189). Expression of the asr gene is strongly induced in acidic conditions (pH less than 5) during exponential growth (316). The bacterial two-component regulatory system phoB-phoR, which regulates the E. coli pho regulon inducible by phosphate starvation, positively regulates asr expression in response to low pH (independent of phosphate starvation) (317, 318). Transcription of asr is also positively and negatively regulated by the global transcription regulators RpoS and H–NS, respectively (317, 318). Asr is a small basic periplasmic protein that plays a role in survival under acid conditions (189, 319). At an external pH of 4.5, Asr supports the growth of cells through a putative mechanism of sequestering protons (189, 317). The high number of basic amino acids, such as lysine, in Asr supports this assertion and implies that Asr might act as a proton sink to protect proteins from acid (189). Asr is also involved in the acid tolerance response, which facilitates the survival of cells during extreme acid shock (pH 2.0) after adaptation to moderate acidity (pH 4.5) (189, 317). Based on the upregulation of asr in response to fPANI treatment, it was hypothesised that PANI and P3ABA antimicrobial action may involve acid stress.

The potential role of periplasmic stresses in PANI and P3ABA bactericidal action was characterised using deletion mutants lacking proteins expressed in response to periplasmic stresses. The involvement of misfolded periplasmic proteins in the antimicrobial action of PANI and P3ABA was examined using E. coli JW1732-1 Δspy missing the gene encoding Spy (277, 278). The role of external acid stress in the antimicrobial mechanism of PANI and P3ABA was investigated with E. coli JW5826-1 Δasr, a mutant with a deletion of the gene encoding Asr (189, 212). Both Spy and Asr function to reduce impact of an external stress in the periplasm. It was hypothesised that an absence of a periplasmic stress responsive protein chaperone, such as in E. coli Δspy and E. coli Δasr, would sensitise the cells to the antimicrobial action of PANI and P3ABA. The sensitivity of E. coli periplasmic stress response mutants to
PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). Following treatment with 1% PANI *E. coli* Δspy numbers were reduced to a greater extent than the parent strain (Fig. 3.11). The difference between *E. coli* Δspy and the parent strain was greater at the 2 h time point than the 4 h time point, which reflects the *E. coli* Δspy mutant reaching the limit of detection (Fig. 3.11). A linear regression analysis found that this difference was statistically significant (slopes are different, P value: less than 0.05). *E. coli* Δasr had a similar sensitivity to 1% PANI suspension as the parent strain (Fig. 3.11), which was confirmed as no difference (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05).

The increased sensitivity of *E. coli* Δspy to PANI treatment suggests that Spy is involved in repair of misfolded proteins that are generated during PANI antimicrobial action. Previous work suggests an oxidative stress mechanism for PANI involving production of hydrogen peroxide. Both the σE and CpxAR pathways are implicated in resistance to oxidative stress (188, 312). Oxidative damage of proteins can cause protein misfolding resulting in activation of one or more of the extracytoplasmic stress response pathways (310, 311, 320). Furthermore, the dsbG gene encoding a periplasmic disulfide bond chaperone-isomerase was found to be part of the H2O2 responsive OxyR regulon and was expressed alongside ahpCF encoding a H2O2 scavenger (275, 321). This is suggestive of H2O2 mediated damage to periplasmic proteins causing misfolding. Therefore, the misfolding of periplasmic proteins that is believed to occur during PANI action (based on the supersensitivity of *E. coli* Δspy to PANI suspension) could possibly reflect oxidative damage to proteins.

*E. coli* Δspy was less sensitive to P3ABA treatment than the parent strain (Fig. 3.12). The difference in sensitivity between *E. coli* Δspy and the parent strain was statistically significant (linear regression analysis, intercepts are different, P values: less than 0.05). A 0.5 h treatment with 1% P3ABA suspension demonstrated a variable relative difference between *E. coli* Δasr and the parent strain with each possible outcome found – *E. coli* Δasr as less sensitive, more sensitive and the same sensitivity as the parent strain (Fig. 3.12). Overall, *E. coli* Δasr had a comparable sensitivity to 1% P3ABA suspension as the parent strain (Fig. 3.12), which was confirmed as no difference (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05).
The hypothesis that an absence of the periplasmic stress responsive protein chaperone Spy would sensitisise the cells to the antimicrobial action of P3ABA was not supported by this work. Following treatment, *E. coli Δspy* demonstrated decreased susceptibility to P3ABA suspension. This may reflect the presence of a range of misfolded proteins in P3ABA treated cells that results in activation of more than one periplasmic stress response pathway(310). Loss of the chaperone function of Spy may be compensated by the induction of other chaperones in another extracytoplasmic stress response pathway. Therefore, the overlap that exists between the extracytoplasmic stress response pathways can complicate the analysis of null mutations(310).

It was hypothesised that Asr is involved in bacterial defence against PANI and P3ABA antimicrobial action and that the absence of the *asr* gene would render *E. coli Δasr* more sensitive to treatment compared to the parent strain. The hypothesis was not supported by the results of this experiment. For both PANI and P3ABA treatment, the *asr* mutant had a similar susceptibility to that of the parent strain, which suggests that Asr does not confer resistance to the bactericidal action of PANI and P3ABA and that induction of external acid stress is not part of the antimicrobial mechanism. It is apparent, at this point, that PANI and P3ABA have distinct modes of action as demonstrated by the different responses of oxidative stress response deletion mutants (sections 3.2.1-3.2.3), iron homeostasis deletion mutants (section 3.2.4) and metabolism and respiration deletion mutants (section 3.2.5).
Figure 3.11. Sensitivity of *E. coli* 7636 parent strain and *E. coli* periplasmic stress response deletion mutants to PANI suspension. Cell viability assays of ~10⁶ CFU/ml *E. coli* parent strain and *E. coli* periplasmic stress response deletion mutants – *E. coli* Δspy and *E. coli* Δasr – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.
Figure 3.12. Sensitivity of *E. coli* 7636 parent strain and *E. coli* periplasmic stress response deletion mutants to P3ABA suspension. Cell viability assays of ~ 10^6 CFU/ml *E. coli* parent strain and *E. coli* periplasmic stress response deletion mutants – *E. coli Δspy* and *E. coli Δasr* – challenged with 1% P3ABA suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 0.5 h and 1 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).

### 3.2.7 DNA repair deletion mutants

A number of lines of investigation suggest DNA damage as a lethal event contributing to the killing mechanism of PANI and fPANIs, including:

a) The suggestion that P3ABA targets ATP synthase (Fig. 3.10, section 3.2.5) and the observed loss of membrane potential in sublethally P3ABA treated cells, which would result in decreased ATP production eventuating in ATP depletion (200, 307, 322). A number of DNA repair systems, such as RecA, require ATP to mediate repair (322). Following from this, ATP depletion would reduce the activity of DNA repair systems...
resulting in potentially lethal unrepaired DNA damage (322). P3ABA mediated damage to ATP synthase would cause electron transport to be uncoupled from ATP synthesis leading to the futile cycling state. Futile cycling is associated with increased production of ROS, which can damage DNA in a Fenton reaction mediated manner (197, 226, 322).

b) The hypothesised induction of oxidative stress by PANI, based on the supersensitivity of *E. coli* ΔkatG (Fig. 3.1, section 3.2.1) and the insensitivity of *E. coli* ΔtonB (Fig. 3.7, section 3.2.4) to PANI treatment, could cause lethal DNA damage (197, 201, 323). Oxidative damage to DNA occurs when H$_2$O$_2$ oxidises iron bound to DNA resulting in production of highly reactive hydroxyl radicals. Hydroxyl radicals can oxidise both base and ribose groups of the DNA, generating a variety of DNA lesions (197, 275, 287, 324).

Oxidative DNA lesions include DNA strand breaks (single stranded DNA breaks, ssDNA, and double stranded DNA breaks, dsDNA) and adducts of nucleotide base and sugar groups (such as thymine glycol) (197, 275, 287, 324). Based on this, it was hypothesised that PANI and P3ABA bactericidal action involves damage to DNA. The contribution of DNA damage to the action of PANI and P3ABA was investigated using deletion mutants lacking in an enzyme involved in DNA repair, *E. coli* JW2669-1 ΔrecA and *E. coli* JW1625-1 Δnth (212).

The constitutively expressed base excision repair (BER) pathways scan DNA for absence of duplex integrity (275). Endonuclease III, encoded by nth, is a member of BER pathways that recognises helix disruption, which occurs due to saturation of pyrimidine residues (275). Endonuclease III is a DNA glycosylase and apyrimidinic (AP) lyase that functions to remove damaged residues (275). When DNA lesions accumulate (either due to not being recognised by the BER system or when there are too many lesions for all to be repaired) the SOS response is activated (275).

The SOS response is an inducible global pathway that regulates DNA repair and damage tolerance genes (324, 325). The SOS response functions to excise damaged nucleotides (mediated by uvr genes) and mediate recombinational DNA repair through the action of RecA (275, 324, 326). Additionally, when there is persistent DNA damage the SOS response induces the expression of error-prone repair DNA polymerases PolII (*polB*), PolIV (*dinB*) and PolV (*umuC, umuD*), which facilitate DNA replication across lesions that prevent functioning of the primary replicative DNA polymerase PolIII (275, 324, 326). RecA is a DNA-dependent ATPase.
that uses the energy derived from ATP hydrolysis to mediate dissociation from bound repaired DNA(327). The response is mediated by the recA-lexA genes encoding RecA (an inducer) and LexA (a repressor) resulting in cell cycle arrest, DNA repair and mutagenesis(197, 325). The repressor LexA binds to SOS boxes (20 base pair consensus palindromic DNA sequences upstream from each gene or operon) in the absence of DNA damage resulting in repression of a regulon that contains more than 50 genes, including lexA and recA(324, 325). DNA damage that causes ssDNA lesions results in RecA activation following binding of this protein to these damaged regions(325). RecA mediates autocleavage of LexA resulting in depression of SOS genes and attempted repair of DNA lesions(324). DNA damage that is too severe to repair results in bacterial cell death(325).

**E. coli ΔrecA** and **E. coli Δnth** have a deletion of a gene encoding a protein that functions in DNA repair of ssDNA lesions and damage pyrimidine residues, respectively(197, 275). This repair serves to reduce damage to DNA that may result from application of an external stress, such as oxidative stress, and therefore functions as a means of defence against the stress. It was hypothesised that **E. coli ΔrecA** and **E. coli Δnth** would have greater sensitivity to PANI and P3ABA suspensions than the parent strain reflecting DNA damage by these antimicrobial agents. The sensitivity of **E. coli** DNA repair deletion mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6).

**E. coli ΔrecA** was less susceptible to 1% PANI suspension compared to the parent strain (Fig. 3.13). The difference in sensitivity between these two strains increased over time, reflecting the time required to reduce parent strain numbers and therefore detect the maximum difference (Fig. 3.13). The difference in sensitivity between **E. coli ΔrecA** and the parent strain was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05). **E. coli Δnth** had a similar sensitivity to P3ABA treatment as the parent strain, which was confirmed as no difference (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05).

There was no difference in sensitivity to P3ABA suspension between **E. coli ΔrecA** and the parent strain (Fig. 3.14), which was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). **E. coli Δnth** had a comparable sensitivity
to 1% P3ABA suspension as the parent strain, which was not statistically significant (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05) (Fig. 3.14).

The results do not support the hypothesis that loss of genes encoding DNA repair enzymes, RecA and Endonuclease III, would sensitise the cells to the bactericidal actions of PANI and P3ABA(275, 324, 325). Overall, for P3ABA there was no difference in sensitivity between the mutant strains, *E. coli ΔrecA* and *E. coli Δnth*, and the parent strain, which suggests that generation of DNA lesions is not involved in the action of P3ABA. The decreased sensitivity of *E. coli ΔrecA* to PANI infers that changes in PANI treated cells are associated with DNA repair; however, it is not clear why this resulted in insensitivity to treatment. It is possible that the lack of increased sensitivity of the *E. coli ΔrecA* mutant to PANI bactericidal action is reflective of mode-2 $H_2O_2$ killing. Mode-1 killing occurs at low $H_2O_2$ concentrations (~ 2.5 mM), requires active metabolism and is mediated by DNA damage, particularly single-strand breaks that induce the SOS response(326, 328, 329). Mode-2 killing occurs at higher concentrations (more than 10 mM), does not require active growth and reflects general damage to cellular biomolecules(326, 328, 329). An *E. coli* mutant lacking the *recA* gene was demonstrated to be very sensitive to mode-1 killing and much less sensitive to mode-2 killing(326). The production of $H_2O_2$ that is hypothesised to occur following bacterial cell contact with PANI may occur at a sufficient high rate to facilitate mode-2 killing which is not greatly influenced by the presence of RecA(326).
Figure 3.13. Sensitivity of *E. coli* 7636 parent strain and *E. coli* DNA repair deletion mutants to 1% PANI suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* DNA repair deletion mutants – *E. coli* Δ*recA* and *E. coli* Δ*nth* – challenged with 1% PANI suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 2 h and 4 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.14. Sensitivity of *E. coli* 7636 parent strain and *E. coli* DNA repair deletion mutants to 1% P3ABA suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* DNA repair deletion mutants – *E. coli* Δ*recA* and *E. coli* Δ*nth* – challenged with 1% P3ABA suspension in LB broth. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 0.5 h and 1 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).

### 3.2.8 Summary

*E. coli* deletion mutants treated with 1% PANI and 1% P3ABA suspensions in LB broth demonstrated a range of relative sensitivities. The statistically significant differences are summarised in figure 3.15, which allows for general comparisons between the susceptibility profiles of PANI and P3ABA to be made. From this, it is apparent that PANI action involves induction of oxidative stress and perturbation of iron homeostasis (Fig. 3.15), while P3ABA action involves targeting of ATP synthase. *E. coli* Δ*spy* had contrasting sensitivities to PANI and P3ABA treatment, with increased sensitivity demonstrated with the former and decrease
sensitivity demonstrated with the latter (Fig. 3.15). The differences observed strongly suggest PANI and P3ABA do not act through the same mechanism.

Based on the relative sensitivities of the selected *E. coli* deletion mutants, putative antimicrobial mechanisms for PANI and P3ABA have emerged. PANI action seems to involve production of H$_2$O$_2$ as indicated by the supersensitivity of *E. coli ΔkatG*, which is unable to scavenge exogenous H$_2$O$_2$, and the decreased sensitivity of *E. coli ΔahpC*, which is missing the H$_2$O$_2$ scavenger utilised during normal growth leading to OxyR activation before application of the PANI stress. Free iron can propagate H$_2$O$_2$ stress by participating in Fenton reaction producing hydroxyl radicals. The reduced sensitivity of *E. coli ΔtonB*, which has reduced iron import, infers induction of an oxidative stress state in PANI treated cells. Further evidence towards a H$_2$O$_2$ stress based mechanism of action for PANI is derived from the IscS mutant (which is unable to modify DNA in a protective manner). The supersensitivity of the Spy mutant suggests that at least some of the damage is occurring in the periplasm. Therefore, the PANI antimicrobial mechanism is hypothesised to involve H$_2$O$_2$ generation causing dysregulation of iron homeostasis, which eventuates in damage to cellular biomolecules including periplasmic proteins.

The relative sensitivities of the selected *E. coli* deletion mutants to P3ABA treatment suggested that P3ABA and PANI exert bactericidal activity through different mechanisms. Deletion of genes encoding H$_2$O$_2$ and SOD scavenging enzymes did not change susceptibility to P3ABA treatment. There was no evidence of perturbation of iron homeostasis; however, P3ABA is known to cause an increase in intracellular free iron in *E. coli* cells (200). P3ABA antimicrobial action appears to involve targeting of respiratory machinery, as reflected by the reduced sensitivity of *E. coli ΔatpE* to P3ABA treatment, which suggested that ATP synthase is targeted by P3ABA, akin to the action of bedaquiline (226). Damage to ATP synthase would cause uncoupling of ATP synthesis from electron transport, which can result in futile cycling, increased respiration and an associated increase in ROS production (226, 308, 322). There was evidence to imply damaged proteins may accumulate in the periplasm following P3ABA exposure due to its proximity to the ETC and were inferred to happen based on the insensitivity of the Spy mutant (which was hypothesised to have upregulation of an additional extracytoplasmic stress response resulting in pre-adaption to P3ABA action).
3.3 Sensitivity of *E. coli* deletion mutants in minimal media to PANI and P3ABA suspensions

Following completion of the characterisation of the response of the *E. coli* deletion mutants to PANI and P3ABA in LB broth, a complex rich media, the response of a selection of these deletion mutants was examined in minimal media that contains only essential nutrients required for growth\(^{(258, 259)}\). *E. coli* grown in rich media and minimal media will have different metabolic and physiological states, which will be reflected in the expression patterns of genes\(^{(245)}\). For example, RpoS – an alternative sigma factor critical for the general stress response in *E. coli* – is expressed at much higher levels in minimal media compared to LB broth\(^{(245, 260)}\). RpoS controls the expression of a variety of genes in an environment dependent fashion including upregulation of the SUF operon and downregulation of *tonB* and *sdhB*\(^{(260)}\). This exemplifies how differences in cell state between growth in rich media and growth in minimal media may influence the response of the *E. coli* deletion mutants to PANI and P3ABA treatment\(^{(260)}\). Therefore, *E. coli* deletion mutants were selected for investigation of their responses to PANI and P3ABA in minimal media based on the hypotheses generated from the results obtained in LB broth. Investigation of the sensitivity of the deletion mutants in both rich and minimal media will increase the likelihood of identifying a noticeable difference in the relative sensitivity between the parent strain and the mutant strain.

![Figure 3.15. Summary of the relative sensitivities of *E. coli* deletion mutants to 1% PANI and 1% P3ABA suspensions in LB broth. Deletion mutants that were significantly more or less sensitive to PANI or P3ABA treatment compared to the parent strain are presented. Decreased sensitivity is represented by red and increased sensitivity is represented by blue (see key).](image-url)
To characterise the sensitivity profiles of the *E. coli* deletion mutants to PANI and P3ABA in low nutrient conditions, the selected mutants were challenged in minimal media with 0.4% succinate as the carbon source. Succinate is a TCA cycle substrate that is oxidised by SDH generating fumarate and reduced ubiquinone that feeds electrons into the ETC (see section 4.1)(196). Therefore, energy from succinate can only be derived from oxidative phosphorylation(226). The *E. coli* mutants that were missing genes involved in metabolism and iron homeostasis – *E. coli* ΔtonB, *E. coli* ΔiscS and *E. coli* ΔsdhB – grew poorly on minimal A salts with succinate and required supplementation with 0.1% casamino acids. *E. coli* ΔatpE was unable to grow on minimal A salts with succinate, even with addition of casamino acids, which reflects the dependence of succinate utilisation on a functional ETC and ATP synthase (304, 330). It was hypothesised that the relative sensitivities of PANI and P3ABA that were observed in LB broth would be reflected in results obtained in minimal media.

### 3.3.1 Hydrogen peroxide scavenger deletion mutants

Hydrogen peroxide scavenger deletion mutants in rich media demonstrated variable relative sensitivities to PANI treatment supporting an oxidative stress mode of action (Fig. 3.1). *E. coli* ΔkatG lacking the oxidative stress responsive catalase had increased sensitivity to PANI suspension, which suggested a role for the production of hydrogen peroxide in the mode of action. Mutations in the genes encoding constitutive and stationary phase catalases, *ahpC* and *katE* respectively, had either no effect or were slightly protective, probably due to activation of OxyR before the PANI challenge by endogenously produced H$_2$O$_2$(262, 263). The hydrogen peroxide scavenger mutants in LB broth had similar susceptibilities to P3ABA treatment as the parent strain (Fig. 3.2).

The relative sensitivities of the hydrogen peroxide scavenger mutants in minimal media to PANI suspension were contrasting to those observed in rich media. The sensitivity of *E. coli* hydrogen peroxide scavenger deletion mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). The mutants had similar susceptibilities to PANI treatment as the parent strain (Fig. 3.16) as confirmed by linear regression analysis (slopes and intercepts are similar, P values: more than 0.05). The lack of supersensitivity of *E. coli* ΔkatG to PANI suspension may be reflective of the increased metabolism and respiration of cells growing in rich media, which is associated with increased production of ROS and would sensitise the cells to PANI action(331). The differences in
susceptibilities of the hydrogen peroxide scavenger mutants in minimal media to PANI suspension compared to LB broth may also be due to the action of RpoS(260). RpoS is expressed at much higher levels in minimal media compared to LB broth and is known to positively influence katE expression(260, 261). Perhaps RpoS regulated gene expression in minimal media may reduce sensitivity of cells to oxidative stress. The hydrogen peroxide scavenger mutants in minimal media responded in a similar manner to P3ABA suspension as they did in LB broth (Fig. 3.17). The lack of difference in sensitivity between the parent strain and the hydrogen peroxide scavenger mutants was confirmed by linear regression analysis (slopes and intercepts are similar, P values: more than 0.05).

Figure 3.16. Sensitivity of *E. coli* 7636 parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants to PANI suspension. Cell viability assays of ~ 10^6 CFU/ml *E. coli* parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants – *E. coli* ΔkatG, *E. coli* ΔkatE and *E. coli* ΔahpC – challenged with 1% PANI suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.17. Sensitivity of *E. coli* 7636 parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants to P3ABA suspension. Cell viability assays of ~ 10^6 CFU/ml *E. coli* parent strain and *E. coli* hydrogen peroxide scavenger deletion mutants – *E. coli* ΔkatG, *E. coli* ΔkatE and *E. coli* ΔahpC – challenged with 1% P3ABA suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).

### 3.3.2 Oxidative stress response deletion mutants

*E. coli* ΔtrxC and *E. coli* ΔgrxA are missing genes involved in the thioredoxin and glutaredoxin pathways, respectively, that act to restore redox balance in the cell as well as many other functions including protein folding and deoxyribonucleotide synthesis (266–268, 332).

The sensitivity of *E. coli* oxidative stress response mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). *E. coli* ΔtrxC in minimal media had a similar sensitivity to 1% PANI suspension as the parent strain (Fig. 3.18), which was not statistically significantly different (linear regression analysis, slopes and intercepts are similar, P values: more than 0.05). *E. coli* ΔgrxA had a similar sensitivity to...
1% PANI suspension as the parent strain (Fig. 3.18), which was confirmed using linear regression analysis (slopes and intercepts are different, P values: more than 0.05).

*E. coli* Δ*trxC* in minimal media had a similar sensitivity to 1% P3ABA suspension as the parent strain (Fig. 3.19), which was comparable to that seen in LB broth (Fig. 3.6) and confirmed by linear regression analysis (slopes and intercepts are different, P values: more than 0.05). *E. coli* Δ*grxA* demonstrated increased sensitivity to P3ABA action in minimal media relative to the parent strain (Fig. 3.19), which was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05). The supersensitivity of the Grx1 mutant to P3ABA infers that this protein is involved in defence against P3ABA action. One of the functions of the glutaredoxin system, of which Grx1 is a member, is to scavenge hydroxyl radicals, a highly reactive ROS that causes biomolecule damage (333, 334). Hydroxyl radicals are formed when iron reacts with hydrogen peroxide during Fenton reaction (197). Iron release has been demonstrated to occur in P3ABA treated *E. coli* cells, which implies that hydroxyl radicals may be generated following the increase in free iron (200). Hydroxyl radicals can be scavenged by reduced glutathione (GSH), a member of the glutaredoxin system, in a pathway that involves glutaredoxin and results in formation of superoxide (332, 335). The superoxide can be scavenged by SOD with the overall effect of eliminating the deleterious radical species, hydroxyl radicals (333, 334). Therefore, the increased sensitivity of *E. coli* Δ*grxA* may reflect an inability to efficiently eliminate hydroxyl radicals produced from free intracellular iron in P3ABA exposed cells.
Figure 3.18. Sensitivity of *E. coli* 7636 parent strain and *E. coli* oxidative stress response deletion mutants to PANI suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* oxidative stress response deletion mutants – *E. coli* ΔtrxC and *E. coli* ΔgrxA – challenged with 1% PANI suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.19. Sensitivity of *E. coli* 7636 parent strain and *E. coli* oxidative stress response deletion mutants to P3ABA suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* oxidative stress response deletion mutants – *E. coli* Δ*trxC* and *E. coli* Δ*grxA* – challenged with 1% P3ABA suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 1 h and 2 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.

3.3.3 Iron homeostasis deletion mutants
The hypothesised oxidative stress mode of action of PANI was supported by the reduced sensitivity of *E. coli* Δ*tonB* and increased susceptibility of *E. coli* Δ*iscS* in LB broth to treatment (Fig. 3.7, section 3.2.4). The inability to import iron – a propagator of oxidative stress – in *E. coli* Δ*tonB* provided protection against PANI action. The loss of the sulfur transferase in *E. coli* Δ*iscS* may undermine defence against oxidative stress, such as by DNA phosphorothioation, which is
postulated to confer protection against peroxide damage (296–298). *E. coli Δfur* had a similar sensitivity to 1% PANI suspension in LB broth. The relative sensitivities of the iron homeostasis deletion mutants to P3ABA in LB broth supported a minor role of oxidative stress as demonstrated by the increased sensitivity of *E. coli ΔiscS* and *E. coli Δfur* (Fig. 3.8). Overall, *E. coli ΔtonB* had a similar sensitivity to P3ABA in LB broth as the parent strain, which suggests that the increase in intracellular free iron that occurs in P3ABA treated cells is derived from internal sources.

To determine if impairment of exogenous iron uptake, synthesis of FeS clusters or regulation of iron uptake and utilisation affects the ability of *E. coli* to survive PANI or P3ABA, *E. coli ΔtonB, E. coli ΔiscS* and *E. coli Δfur*, respectively, were challenged in minimal media. The sensitivity of *E. coli* iron homeostasis deletion mutants to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). *E. coli ΔtonB, E. coli ΔiscS* and *E. coli Δfur* had similar sensitivities to PANI suspension as the parent strain (Fig. 3.20), which was confirmed using linear regression analysis (slopes and intercepts are different, P values: more than 0.05). The lack of decreased sensitivity of *E. coli ΔtonB* in minimal media to PANI treatment (compared to rich media) may reflect the reduced availability of iron in minimal media (consistent with RpoS mediated downregulation of *tonB* that occurs during growth in minimal media) (260). The absence of supersensitivity of oxidative stress response mutants in minimal media compared to rich media (such as *E. coli ΔkatG* in Fig. 3.1 and Fig. 3.16) is consistent with the response of *E. coli ΔiscS* to PANI treatment in minimal media (Fig. 3.20).

All three iron homeostasis deletion mutants tested – *E. coli ΔtonB, E. coli ΔiscS* and *E. coli Δfur* – displayed increased sensitivity to P3ABA treatment compared to the parent strain (Fig. 3.21). The difference in sensitivity between *E. coli ΔtonB* and the parent strain was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05). The difference in sensitivity between *E. coli ΔiscS* and the parent strain was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05). The difference in sensitivity between *E. coli Δfur* and the parent strain was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05).
The increased sensitivity of *E. coli Δfur* may be due to the role Fur plays in survival in iron limiting conditions, such as in minimal media. For example, Fur induces expression of the SUF operon in low iron conditions, which can compensate for the loss of iron or sulphur availability for FeS cluster biosynthesis that occurs during iron starvation (294). The effect of the absence of Fur may be exacerbated in minimal media conditions compared to in rich media sensitising the cells to the stress inducing actions of P3ABA. The increased sensitivity of *E. coli ΔiscS* might reflect P3ABA targeting of metabolism and respiration. The deletion mutant is missing a gene from the ISC pathway that functions in *de novo* synthesis of FeS clusters for important metabolic and respiratory enzymes (273, 293). The resulting loss of function of the ISC pathway is only partially compensated by the SUF pathway resulting in synthesis of only essential FeS clusters (191). The associated metabolic defects observed in *E. coli ΔiscS* may sensitise the cells to P3ABA-mediated damage of metabolic and respiratory machinery (273, 293).
Figure 3.20. Sensitivity of *E. coli* 7636 parent strain and *E. coli* iron homeostasis deletion mutants to PANI suspension. Cell viability assays of $\sim 10^6$ CFU/ml *E. coli* parent strain and *E. coli* iron homeostasis deletion mutants – *E. coli* ΔtonB, *E. coli* ΔiscS and *E. coli* Δfur – challenged with 1% PANI suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.21. Sensitivity of *E. coli* 7636 parent strain and *E. coli* iron homeostasis deletion mutants to P3ABA suspension. Cell viability assays of ~10^6 CFU/ml *E. coli* parent strain and *E. coli* iron homeostasis deletion mutants – *E. coli* ΔtonB, *E. coli* ΔiscS and *E. coli* Δfur – challenged with 1% P3ABA suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 1 h and 2 h are presented (A). Statistical significance (P value less than 0.05) is represented by *.* The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.

### 3.3.4 Metabolism and respiration deletion mutant

*E. coli* ΔsdhB, missing a subunit of SDH, did not have a changed susceptibility to PANI treatment in LB broth compared to the parent strain (Fig. 3.9, section 3.2.5), which implied that either SdhB is not targeted by PANI or that loss of this potential target was not sufficient to affect susceptibility to bactericidal activity. The same response was observed for *E. coli* ΔsdhB challenged with PANI in minimal media (Fig. 3.22) (confirmed using linear regression analysis, slopes and intercepts are different, P values: more than 0.05), which corroborates the
conclusions drawn from the results in LB broth. \textit{E. coli} \(\Delta\text{sdhB}\) demonstrated increased sensitivity to P3ABA treatment in minimal media (Fig. 3.23), which is suggestive that SdhB was targeted by P3ABA. SdhB contains an oxidation sensitive \([4\text{Fe}-4\text{S}]^{2+}\) cluster, which would release iron following oxidative damage contributing to the increase in intracellular free iron\((200, 293)\). The difference in sensitivity between \textit{E. coli} \(\Delta\text{sdhB}\) and the parent strain was statistically significant (linear regression analysis, intercepts are different, \(P\) value: less than 0.05). The increased susceptibility of \textit{E. coli} \(\Delta\text{sdhB}\) to P3ABA in minimal media supports the results obtained in rich media and corroborates the hypothesis that P3ABA targets metabolic and respiratory machinery.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_22}
\caption{Sensitivity of \textit{E. coli} 7636 parent strain and \textit{E. coli} metabolism and respiration deletion mutant to PANI suspension. Cell viability assays of \(\sim 10^6\) CFU/ml \textit{E. coli} parent strain and \textit{E. coli} metabolism and respiration deletion mutant – \textit{E. coli} \(\Delta\text{sdhB}\) – challenged with 1% PANI suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).}
\end{figure}
Figure 3.23. Sensitivity of E. coli 7636 parent strain and E. coli metabolism and respiration deletion mutant to P3ABA suspension. Cell viability assays of ~ 10^6 CFU/ml E. coli parent strain and E. coli metabolism and respiration deletion mutant – E. coli ΔsdhB – challenged with 1% P3ABA suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 1 h and 2 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).

3.3.5 Periplasmic stress response deletion mutant

The absence of the asr gene encoding a putative chaperone involved in survival in acidic conditions did not alter the susceptibility to treatment with PANI or P3ABA in LB broth (Fig. 3.11, Fig. 3.12, section 3.2.6) (189, 319). Based on this, it was postulated that induction of external acid stress is not part of the antimicrobial mechanism of PANI or P3ABA. The sensitivity of E. coli Δasr to PANI and P3ABA treatment was compared to that of the parent strain using a linear regression analysis (section 2.2.3.6). In support of this, E. coli Δasr had a similar sensitivity to PANI treatment in minimal media compared to the parent strain (Fig. 3.24), which was confirmed using linear regression analysis (slopes and intercepts are different, P values: more than 0.05). The response of this mutant to P3ABA in minimal media was contrasting (Fig. 3.25), as a supersensitive phenotype was observed, which was statistically significant (linear regression analysis, slopes and intercepts are different, P values: more than 0.05). The supersensitive phenotype suggests that in these conditions Asr confers resistance to the bactericidal action of P3ABA. It is possible that P3ABA targeting of ATP synthase would cause increased respiration resulting in accumulation of more protons in the periplasmic space. The lack of protection provided against acidification by the action of Asr in the mutant would
cause increased sensitivity to P3ABA treatment. The different responses of *E. coli* Δasr in rich and minimal media to P3ABA treatment could be due to differential gene expression in these media. Acid-induced expression of the *asr* gene is reduced (≈15-fold) in LB broth and increased in minimal media, which suggests that other acid tolerance mechanisms operate in rich media (189, 336). Therefore, the loss of Asr action would only be relevant to minimal media conditions and supports mechanism testing in both rich and minimal media.

Figure 3.24. Sensitivity of *E. coli* 7636 parent strain and *E. coli* periplasmic stress response deletion mutant to PANI suspension. Cell viability assays of ≈10^6 CFU/ml *E. coli* parent strain and *E. coli* periplasmic stress response deletion mutant – *E. coli* Δasr – challenged with 1% PANI suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% PANI for 1 h and 2 h are presented (A). The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B).
Figure 3.25. Sensitivity of *E. coli* 7636 parent strain and *E. coli* periplasmic stress response deletion mutant to P3ABA suspension. Cell viability assays of ~ $10^6$ CFU/ml *E. coli* parent strain and *E. coli* periplasmic stress response deletion mutant — *E. coli Δasr* — challenged with 1% P3ABA suspension in minimal A salts with 0.4% succinate. The viable cell counts (CFU/ml) of the parent and each mutant strain challenged with 1% P3ABA for 1 h and 2 h are presented (A). Statistical significance (P value less than 0.05) is represented by *. The relative cell counts for each mutant strain at each treatment time are expressed as the percent of the associated parent strain cell counts with a line indicating the median (B). The grey dot indicates a time point at which the difference between the parent and mutant strain may be underestimated due to one of the strains decreasing in number below the limit of detection (50 CFU/ml). Therefore, the median represented by the grey line may not represent the true relative difference between the parent and mutant strain.

### 3.3.6 Summary

*E. coli* deletion mutants treated with 1% PANI and 1% P3ABA suspensions in minimal media demonstrated a range of relative sensitivities. The statistically significant differences are summarised in figure 3.26 to allow for comparisons between the susceptibility profiles of PANI and P3ABA to be made.

The *E. coli* deletion mutants were more greatly affected by P3ABA treatment in minimal media compared to PANI treatment (Fig. 3.26) or to treatment in rich media (Fig. 3.15). Increases in sensitivity were attributed to *E. coli ΔgrxA*, *E. coli ΔtonB*, *E. coli ΔiscS*, *E. coli Δfur*, *E. coli ΔsdhB* and *E. coli Δasr* treated with P3ABA suspension (Fig. 3.26) suggesting perturbation of metabolism and iron homeostasis as well as acid stress is occurring in P3ABA treated cells. The increased susceptibilities of mutants missing genes involved in metabolism and iron homeostasis to P3ABA in minimal media supports targeting of metabolic and respiratory machinery, which would be expected to elicit greater responses for cells that have the increased metabolic demand of growth on minimal media. The different responses seen in
**E. coli** deletion mutants in rich and minimal media highlight the advantages for antimicrobial testing in more than one type of media. Overall, the antimicrobial susceptibility profiles for the **E. coli** deletion mutants in rich and minimal media led to the refinement of hypotheses pertaining to the antimicrobial mechanism of PANI and P3ABA.

![Figure 3.26. Summary of the relative sensitivities of **E. coli** deletion mutants to 1% PANI and 1% P3ABA suspensions in minimal A salts with 0.4% succinate.](image)

Deletion mutants that were significantly more or less sensitive to PANI or P3ABA treatment compared to the parent strain are presented. Decreased sensitivity is represented by red and increased sensitivity is represented by blue (see key).

### 3.4 Discussion

The susceptibility profiles of PANI in rich media and minimal media can collectively be examined and used to form hypotheses on antimicrobial mechanism. The most striking responses from **E. coli** deletion mutants to PANI were observed in LB broth (Fig. 3.15). The behaviour of these mutants in defined minimal media deviated less from the parental behaviour and was less informative (Fig. 3.26). Thus, interpretation of the susceptibility profiles of PANI focussed on that which was obtained in rich media.

The attenuated relative differences in sensitivity of the deletion mutants in minimal media to PANI may be indicative of an oxidative stress mechanism. Growth in rich media is characterised by high metabolic and respiratory flux associated with the utilisation of high levels of nutrients, which results in increased generation of ROS\(^{(331, 337)}\). This implies that bacterial cells would be more susceptible to an oxidative stress antimicrobial mechanism in rich media and supports the hypothesis relating to the PANI mode of action. Additionally, the higher level of iron in LB broth compared to minimal media is reflected in the internal iron concentration of bacterial
cells grown in this media(328). Increased intracellular iron concentration of cells grown in LB broth has been associated with increased sensitivity to hydrogen peroxide(328). Incubation of *E. coli* in minimal media before challenge with hydrogen peroxide has been demonstrated to be protective against bactericidal action(338). Therefore, the susceptibility profiles of PANI in LB broth and minimal media are suggestive of induction of oxidative stress.

In this study, we sought to identify mechanisms that directly or indirectly contribute to the antimicrobial action of P3ABA using the susceptibility profiles obtained using rich and minimal media. Overall, the results in both media were suggestive of targeting of metabolic and respiratory enzymes, with the strongest evidence coming from the reduced sensitivity of *E. coli ΔatpE* to P3ABA in LB broth. There was limited evidence to suggest induction of oxidative stress and perturbation of iron homeostasis in P3ABA treated cells in LB broth. Acid stress was identified as a potential mechanism of P3ABA by the supersensitivity of *E. coli Δasr* in minimal media (Fig. 3.25). ATP synthase and ETC are important for regulation of intracellular pH, thus perturbation of this machinery following P3ABA exposure may cause acid stress in the cells(303). ATP synthase mutants are known to have lower internal pH values compared to the parent strain, which supports this hypothesis(303). Overall, the metabolism, respiration and iron homeostasis deletion mutants had increased sensitivity to P3ABA treatment in minimal media (Fig. 3.26) and decreased sensitivity in rich media (Fig. 3.15). The increased metabolic demands that are associated with growth on minimal media may sensitise the cells to perturbations of metabolism and respiration, such as following P3ABA mediated damage to target enzymes. Therefore, the susceptibility profiles of P3ABA in LB broth and minimal media are suggestive of targeting of metabolism and respiration resulting in downstream effects of oxidative stress and acid stress.

The approach of using single gene deletion mutants to investigate the mode of action of an antimicrobial agent is based on the idea that disruption of a gene encoding a protein product that is either a target or is involved in defence against antimicrobial action will render the mutant less or more sensitive to treatment than the parent strain, respectively. The physiological state of a bacterial cell is highly regulated by complex intracellular networks, which involves control of gene transcription, translation, post-transcriptional regulation and post-translational modification of proteins(289, 339). The complexity of cellular physiology entails that knockout of a single gene may not elicit an expected or explainable phenotype due
to gene pleiotropy, redundancy of enzymes and overlapping of complex regulatory systems(289, 339). Understanding of these complex systems and the effects of gene deletion can be hampered by a paucity of relevant information in the current literature(339, 340). To further complicate matters, bacterial cells respond to stresses by regulating the physiology of the cell at all different levels of the system(341). The immediate response of a bacterial cell exposed to a stress is mediated by metabolic changes, which promote survival of the cell for the duration of time needed to change gene expression patterns and synthesise the associated proteins (estimated ~ 30 min)(341, 342). Translational regulation allows cells to respond to stress within minutes(342). For example, exposure to ROS results in nonspecific and irreversible tRNA degradation in *E. coli* results in slowed translation elongation and decreased protein synthesis(342). Impedance of translation reduces the chance of protein misfolding and amino acid mis-incorporation occurring thereby protecting against the deleterious accumulation of misfolded proteins(342). Thus, experiments based on responses at a genomic level may oversimplify the situation, which might explain the lack of an expected mutant phenotype.

Care needs to be taken during interpretation of the phenotypic responses of single gene deletion mutants to environmental stresses. Loss of a gene and the associated protein product can have a profound effect on the cell’s physiological state(289, 339). For example, during construction of the Keio collection of *E. coli* single gene deletion mutants, 303 genes were unable to be disrupted and were deemed to be essential genes(212). Essential genes tend to be metabolic genes and metabolic regulators that are expected to be indispensable under any environmental conditions(212, 340). Deletion mutants, especially those missing genes involved in metabolism, respiration and biomolecule synthesis, will grow at slower rates compared to the parent strain(293, 308). For example, doubling times of an *E. coli* parent strain (MG1655) and an isogenic *iscS* mutant grown in minimal media with 40 mM succinate were 84 min and 200 min, respectively(293). During antimicrobial challenge, a deletion mutant that grows at a noticeably slower rate than the parent strain will be expressing growth rate dependent genes, which may influence the sensitivity of the deletion mutant (separate to the loss of the gene)(245). Thus, the comparisons made between the sensitivity of the parent strain and the deletion mutant strain may not be representative of the true difference. The differences in cellular physiology in deletion mutants compared to the parent strain may reflect indirect or
downstream effects that act in a supplementary manner to loss of the main function of the absent protein (328). DNA damage following hydrogen peroxide exposure was investigated using DNA repair-deficient strains (328). These strains were demonstrated to have increased sensitivity to the bactericidal action of hydrogen peroxide; however, this may not mean that DNA damage is occurring (328). It is possible that lethal DNA damage is only an outcome in the DNA repair-deficient strains and that bactericidal action in wild-type strains involves DNA damage combined with another microbicidal mechanism (328).

The mechanisms that are involved in the action of antimicrobial agents have been the focus of much investigation over the years (343–345). Single targets of antibiotics have been identified and are deemed to reflect essential bacterial cell functions, including synthesis of DNA, RNA, the cell wall or proteins (183, 184). However, the mode of action of antimicrobial agents is more complicated than it has been deemed (184). Functional genomic studies have demonstrated that different compounds acting against the same target can have different mechanisms of action (184). Experiments examining gene expression profiles of antibiotic treated cells have raised the possibility that antibiotics may have several targets (213, 346). Indeed it appears more likely that antibiotic mediated cell death is a complex process involving multiple primary and secondary molecular targets (183, 184, 347). Reduced sensitivity of a knockout mutant may reflect loss of an initial target and/or process or it may be due to involvement of secondary targets and downstream effects (213). While the utilisation of deletion mutants is suited for screening of potential mechanisms, the complexity of antimicrobial action necessitates more specific testing to determine if targets identified represent the initiation of action or a downstream effect (184, 213). The sensitivity profile for an antimicrobial agent should be determined at early and later time points, in rich and minimal media, and at a range of concentrations, including a sub-inhibitory concentration (184, 348).

The media that antimicrobial testing is performed in can affect the physiological state of the bacterial cells and/or affect the antimicrobial agent and thus impact results. The complex interactions between growth conditions and antimicrobial exposure resulting in changes in gene expression necessitate that the influence of the media used for testing is considered when designing tests and interpreting results (349). For example, E. coli Δasr was demonstrated to have the same sensitivity as the parent strain to P3ABA treatment in LB broth (Fig. 3.12) and showed supersensitivity in minimal media (Fig. 3.25). The difference in sensitivities observed
between the two media is a reflection of the expression pattern of the *asr* gene – reduced in LB broth and increased in minimal media (189, 336). Distinct gene expression patterns following ciprofloxacin exposure has been observed for *E. coli* growing in LB broth and minimal media (349). Expression of *marA* and *soxS* genes encoding transcriptional activators of a drug efflux pump in minimal medium was reduced relative to in rich media (349). The decreased expression of these genes was counterintuitive as they confer ciprofloxacin resistance (349). It was concluded that reduced expression of *marA* and *soxS* was an outcome of a global stress response to nutrient limitation in cells growing in minimal media (349). Therefore, the outcome of exposure to an antimicrobial agent is dependent on the media used.

The effect of media conditions on the activity of antimicrobial agents has been previously demonstrated (214, 350, 351). A prime example being the influence of Ca$^{2+}$ and Mg$^{2+}$ ions on the activity of aminoglycoside antibiotics and cationic antimicrobial peptides (214, 350, 351). The MIC of tetracycline, kanamycin and gentamycin against *E. coli* increased 3-4 fold in the presence of 30 mM Ca$^{2+}$ ions relative to no calcium (352). Cations are known to stabilise the lipopolysaccharide and outer member of *P. aeruginosa* possibly reducing antimicrobial agent uptake (353, 354). Ca$^{2+}$ and Mg$^{2+}$ ions can also complex with tetracycline, which reduces uptake into cells (355). Thus, the concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions in antimicrobial testing media can affect the results obtained through acting on the bacteria and the antimicrobial agent. The activity of cationic AMPs is greatly or completely reduced in the presence of physiological concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions as the cations can interfere with the binding of AMPs to the negatively charged bacterial surface (214, 350, 351, 356). The MIC of α-helical peptides against *P. aeruginosa* increased 8-fold following addition of 3 mM MgCl$_2$ (357). For physiological relevance, the cations should be present at the concentrations found in the human body, such as 1 mM Mg$^{2+}$ and 2 mM Ca$^{2+}$ present in serum (214, 357). Thus, performing antimicrobial testing in both rich and minimal media alongside elucidation of the regulation of genes of interest is important for characterising the response of the bacteria to antimicrobial treatment.

A final point to discuss is the variability of the results observed for the parent and mutant strains challenged with PANI in this chapter. It is clear that there is variability between independent experiments, but also that the results obtained are valid, despite the variability, as the aim of these experiments was to compare the sensitivity of at least two strains to the
same PANI suspension. Therefore, although each suspension made may exhibit variable activity when compared to other suspensions, the relative sensitivity of a mutant strain compared to the parental strain is similar for each suspension, despite the raw data seeming variable. The testing of mutants was done with one large batch of PANI that was made from the combination of three smaller batches (section 2.2.3.1) to reduce batch variation. Testing multiple mutants at once helped to reduce the impact of PANI variability. Potential reasons for heterogeneity of results may include:

i. A failure to thoroughly mix PANI batches leading to variations in the activity of PANI suspensions made at different times, although this is unlikely as the individual batches were thoroughly mixed;
ii. A settling of particles in the mix of PANI batches leading to variations in the activity of PANI suspensions made at different times;
iii. A gradual change in activity of PANI in the mixed batches over time;
iv. Differences in the physiological state of the bacteria challenged, despite care being taken to always take bacteria from cultures grown for the same length of time;
v. Differences in the susceptibility of the bacterial strains challenged to small changes in PANI suspensions or small differences in the phase of growth due to background mutations present in these strains.

3.5 Conclusion
Based on the results presented in this chapter, it is hypothesised that the mode of action of PANI involves formation of hydrogen peroxide resulting in oxidative stress and dysregulation of iron homeostasis. The supersensitivity of E. coli Δspy to PANI treatment in LB broth is suggestive of perturbation of proteins in the periplasm. Thus, it is possible that following contact with PANI, hydrogen peroxide is produced, which can participate in Fenton reaction with free iron to form highly reactive hydroxyl radicals(328). Hydroxyl radicals and other related ROS can oxidatively damage biomolecules, such as iron containing proteins(328). Oxidation of iron atoms in certain proteins promotes release of iron leading to further Fenton reaction and oxidative damage. The proximity of electron flow in the ETC, flavoproteins and oxygen to the periplasm infers that ROS may accumulate in the periplasm causing protein misfolding and toxicity.
It is hypothesised that P3ABA targets ATP synthase perturbing oxidative phosphorylation and possibly intracellular pH levels causing acid stress. Disruption of oxidative phosphorylation, such as by targeting ATP synthase, would cause uncoupling of ATP synthesis from electron transport, which can result in futile cycling, increased respiration and an associated increase in ROS production (226, 308, 322). The generated ROS can target iron containing metabolic enzymes resulting in enzyme inactivation and iron release, promoting further ROS production by Fenton reaction (328).
Chapter 4: The Role of Oxygen in the Antimicrobial Mechanism of PANI and P3ABA

4.1 Introduction
The relative sensitivities of \textit{E. coli} deletion mutants to PANI, and specifically the – increased sensitivities of \textit{E. coli} \textit{ΔkatG} and \textit{E. coli} \textit{Δspy}, and the reduced sensitivity of \textit{E. coli} \textit{ΔtonB} in LB broth, – support the hypothesis that the antimicrobial mechanism of PANI involves induction of hydrogen peroxide based oxidative stress and the perturbation of iron homeostasis (chapter 3). The greatly reduced sensitivity of the \textit{ΔatpE} deletion mutant considered with the more modest reduction in sensitivity of the \textit{Δspy} and \textit{ΔsdhB} deletion mutants to P3ABA led to the hypothesis that P3ABA is targeting metabolic and respiratory machinery (section 3.5), which is thought to result in disruption of internal iron homeostasis, based on the slightly increased sensitivity of an \textit{E. coli} \textit{Δfur} mutant (Fig. 3.8, section 3.2.4; Fig. 3.21, section 3.3.4) and the increase in intracellular free iron detected in P3ABA treated cells(200). Perturbation of respiratory machinery function may result in product of ROS as a downstream effect, as occurs with bedaquiline that targets ATP synthase(226). This is supported by the localisation of electrons and oxygen to the ETC and the identification of the sources of ROS in \textit{E. coli} (359–361). Therefore, the activity of P3ABA may include production of ROS but at a lower extent than for PANI. The potential involvement of oxygen and its associated reactive species in the antimicrobial action of PANI and P3ABA warranted investigation.

The role of ROS in the antimicrobial mechanism of bactericidal antibiotics is contentious (228, 238, 362, 363). Many papers have been published that examine the contribution ROS make to the lethal action of antibiotics, with some arguing that all antibiotics kill through inducing oxidative damage, some arguing a partial and common contribution, and other groups contesting any role for oxidative damage(228, 238, 362, 363). A range of approaches have been used to this end including examining the sensitivities of gene knockout mutants, the activity of antibiotics in aerobic and anaerobic conditions, transcriptomics, measurement of
oxygen consumption, protection by antioxidants and iron chelators, and free intracellular iron levels(228, 238, 362, 363).

To investigate the contribution of ROS to the antimicrobial action of PANI and P3ABA, the sensitivity of *E. coli* and *S. aureus* to these compounds in aerobic and anaerobic conditions was determined(228, 362). *E.coli* 25922 and *S. aureus* 6538 were selected as they are standard antibiotic sensitivity testing strains, routinely used as control organisms to verify that antibiotic susceptibility results are accurate(214–217). One important consideration in these investigations is that if endogenously produced ROS are important part of the killing mechanism, then cells should experience some protection from the effects of the antimicrobial in anaerobic conditions. The measures of sensitivity to treatment utilised were the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For an antimicrobial agent, the MIC is the lowest concentration (defined by test conditions) that inhibits visible growth of bacteria tested while the MBC is the lowest concentration that prevents growth of an organism after rescue from treatment (inferring that the organism has been killed)(214, 364). The MIC is a ‘gold standard’ for determining the susceptibility of bacteria to an antimicrobial agent; however, the MBC has greater relevance to examining antimicrobial mechanism(214, 364).

The MIC and MBC of PANI and P3ABA in aerobic and anaerobic conditions were determined for *E. coli* in LB broth and minimal A with glycerol or succinate as the carbon source. Potassium nitrate was added to the minimal A at 20 mM to act as a terminal electron acceptor facilitating anaerobic respiration(244). For *S. aureus* 6538 the experiment was only carried out in LB broth. The central role for ROS in the killing mechanism was tested in challenges to determine whether anaerobiosis was protective. The details of contributory antimicrobial mechanisms were further investigated in different media conditions that favour different routes for ATP generation. Comparison of the MIC and MBC of PANI or P3ABA in the various conditions allowed for inferences to be made about the mechanism of action. LB broth is a rich media that contains amino acids, which are used as the carbon source as well as other nutrients used for growth(245, 258). *E. coli* cells growing in LB broth can generate ATP through substrate level phosphorylation, oxidative phosphorylation or fermentation(365–367). The increased growth rate and respiration of cells growing in LB broth means that they should be able to quickly respond to an external stress by synthesising defence proteins as the building blocks required
to do this are found in the media (245). Minimal A is a defined medium that contains only the essential nutrients for growth (258). Glycerol and succinate can function as the sole carbon and energy source for *E. coli* cells growing in minimal media (245). All building blocks needed for *E. coli* to grow, such as amino acids, must be synthesised from the carbon source (245). Energy can only be derived from these non-fermentable carbon sources mostly through oxidative phosphorylation; however, substrate level phosphorylation is relevant as well for glycerol (304, 368, 369).

Based on the metabolic and physiological states of *E. coli* growing in rich LB media or minimal media, hypotheses can be formed in reference to the antimicrobial action of PANI and P3ABA. If PANI induces oxidative stress in cells then it would be expected that cells in aerobic minimal media would be more sensitive than cells in aerobic LB broth, reflecting the ability of the cells growing in rich media to be able to quickly synthesise proteins to combat the oxidative stress. *E. coli* cells growing in minimal media would first have to synthesise amino acids and cofactors before assembling proteins to defend against oxidative stress. The extra steps and time taken to achieve this would likely render the cells more sensitive to an exogenous oxidative stress challenge. It would be expected that this difference in sensitivity to PANI based on the availability of nutrients does not exist in anaerobic conditions as oxidative stress would not be occurring. The nature of the carbon source for minimal media should not affect the susceptibility of cells to PANI as the lack of amino acids and other nutrients is hypothesised to mediate the increased sensitivity.

P3ABA is hypothesised to target respiratory machinery, such as the ATP synthase, based on the greatly reduced sensitivity of *E. coli* ΔatpE and the loss of membrane potential measured in P3ABA treated *E. coli* cells (200). Bedaquiline is a novel antibiotic, primarily used against *M. tuberculosis*, that targets ATP synthase resulting in uncoupling of electron transport from ATP synthesis (226). The uncoupling that follows bedaquiline exposure causes loss of the proton gradient and ATP depletion (226, 370). It is hypothesised that loss of oxidative phosphorylation results in ATP depletion, which causes cell death (370, 371). Considering the effects of P3ABA challenge on *E. coli* that correspond to bedaquiline effects upon mycobacteria, it is possible that ATP synthase is a target for P3ABA. Based on this, it was hypothesised that *E. coli* cells would exhibit greater sensitivity to P3ABA in energy poor conditions (compared to aerobic, high nutrient, energy rich conditions). Energy rich conditions
describes the ability of a cell to synthesise ATP through means besides oxidative phosphorylation while energy poor conditions are associated with a more limited ability to synthesise ATP (330). Therefore, P3ABA challenged with *E. coli* in LB broth was expected to have higher MIC and MBC values compared to *E. coli* in minimal media. Similarly, it was postulated that the decreased capacity to synthesise ATP in anaerobic conditions, especially in minimal media, compared to aerobic conditions, would sensitise the *E. coli* cells to the antimicrobial action of P3ABA (330). Following from this, cells grown on the non-fermentable, non-glycolytic carbon source, succinate, would be expected to demonstrate greater sensitivity compared to the glycolytic glycerol as energy from succinate can only be derived from oxidative phosphorylation (226). More energy is derived from aerobic metabolism of glycerol (more than 11 μmol ATP/g dry cells) compared to succinate (8 μmol ATP/g dry cells) (372). Aerobic breakdown of glycerol and succinate is outlined in Fig. 4.1.
Figure 4.1. Aerobic breakdown of glycerol and succinate to generate ATP. Glycerol is converted to dihydroxyacetone phosphate (DHAP), which can be fed into the glycolytic pathway generating ATP by substrate level phosphorylation\(^{373}\). Glycolysis generates pyruvate, which is converted to acetyl-CoA to enter the TCA cycle\(^{365}\). The TCA cycle functions to produce metabolites, reduced electron carriers and ATP by substrate level phosphorylation\(^{365}\). The reduced electron carriers feed electrons (e\(^{-}\)) into the ETC, which couples electron transport to proton pumping\(^{365}\). Oxygen is the terminal electron acceptor and is converted to water following reduction. ATP synthase uses the energy stored in the proton gradient to synthesise ATP, termed oxidative phosphorylation. Succinate is oxidised by succinate dehydrogenase (SDH) generating fumarate and reduced ubiquinone that feeds electrons into the ETC.

4.2 Activity of PANI and P3ABA against \textit{E. coli} and \textit{S. aureus} in aerobic and anaerobic conditions

To investigate the potential role of oxygen in the antimicrobial mechanism of PANI and P3ABA, the MIC and MBC against \textit{E. coli} and \textit{S. aureus} cells was determined in aerobic and anaerobic conditions (section 2.2.4). MIC values were obtained following a 24 h treatment of bacterial cells with PANI or P3ABA suspension. MBC values were obtained by spreading 20 µl aliquots from experimental samples that did not have visible cell growth onto LB agar plates. For \textit{E. coli} 25922 the activity of PANI and P3ABA was elucidated in rich media (LB broth), minimal media with glycerol and minimal media with succinate. The differences in MIC and MBC against \textit{E. coli}
25922 in the various types of media in aerobic and anaerobic conditions may inform the hypotheses relating to antimicrobial mechanism. Results presented were from independent experiments performed on different days.

**4.2.1 Activity of PANI and P3ABA against E. coli 25922 in aerobic and anaerobic conditions**

To characterise the activity of PANI and P3ABA in aerobic and anaerobic conditions, the MIC and MBC against *E. coli* 25922 was determined. Oxygen can undergo univalent reduction to form superoxide followed by further reductions to form hydrogen peroxide, hydroxyl radical and finally water (Fig. 4.2)(262, 374). Hydrogen peroxide is formed from different sources; however, this has yet to be fully elucidated(225). Autoxidation of redox enzymes occurs when molecular oxygen collides with and adventitiously oxidises a reduced, solvent-exposed flavin cofactor(225, 244). Only 1% of electron flow through redox enzymes is intercepted by oxygen; however, the high fluxes of the associated pathways can result in substantial hydrogen peroxide production(244). Higher oxygen concentrations and higher titres of flavin containing enzymes are associated with higher rates of autoxidation(197, 262). Autoxidation of redox enzymes results in the formation of hydrogen peroxide (and superoxide)(225, 244). Complex II of the ETC has been demonstrated to produce hydrogen peroxide when exposed to oxygen; however, it is believed to be only a minor contributor (less than 15% of total) to hydrogen peroxide generation(225).

\[
\text{O}_2 \rightarrow \text{O}_2^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} \rightarrow \text{H}_2\text{O}
\]

*Figure 4.2. Univalent reduction of oxygen gives rise to reactive species.* Oxygen (O$_2$) can undergo four successive reductions involving extraction of one electron to form reactive intermediates. Univalent reduction of oxygen generates superoxide (O$_2^-$). Addition of another electron with two protons gives rise to hydrogen peroxide (H$_2$O$_2$). Donation of a third electron to hydrogen peroxide generates the hydroxyl radical (•OH) accompanied by release of a hydroxide ion (OH$^-$). Addition of a fourth electron to this radical produces a non-reactive water molecule (H$_2$O).

Adapted from Davies, (1995)(374)

Hydrogen peroxide is formed as a reaction product of oxidase enzymes, although they are not considered to play an important role in endogenous hydrogen peroxide production. The lack of
significant hydrogen peroxide formation is due to the low flux through the oxidase associated pathways and saturation at low oxygen concentrations (meaning that the rate of hydrogen peroxide production would not increase in response to an increase in oxygen concentration) (225, 244). One exception is aspartate oxidase, an abundant oxidase enzyme (~ 2 mM) that experiences high flux through the nicotinamide formation pathway(244). Aspartate oxidase contributes about 25% of the endogenously produced hydrogen peroxide in E. coli(244, 361). Hydrogen peroxide is also formed as a by-product of the catalytic activity of a periplasmic copper-dependent monoamine oxidase (CuMAO); however, less than 10 µM/s reaches the cytoplasm as permeability of the outer membrane to hydrogen peroxide is more than 30-fold higher than that of the inner membrane(244). The rate of endogenous hydrogen peroxide formation is dependent on the concentration of dissolved oxygen(225, 244). The requirement for the presence of oxygen in the formation of hydrogen peroxide (and superoxide) denotes that in anaerobic conditions ROS cannot form(197).

The relative activity of an antimicrobial agent in aerobic and anaerobic conditions can signify the involvement of ROS in the mode of action. Greater activity of an antimicrobial agent (shown by lower MIC and MBC values) in aerobic conditions compared to anaerobic conditions would imply induction of oxidative stress is part of the mode of action(228). Similar activity of an antimicrobial agent in aerobic and anaerobic conditions would suggest that oxygen and its associated reactive species are not involved in the antimicrobial mechanism. Reduced activity of an antimicrobial agent (shown by higher MIC and MBC values) in aerobic conditions compared to anaerobic conditions would infer that the antimicrobial mechanism does not involve generation of ROS.

4.2.1.1 In aerobic conditions PANI is more active against E. coli 25922 in rich media and minimal media compared to anaerobic conditions
To investigate the hypothesis that the mode of action of PANI involves production of hydrogen peroxide in treated bacterial cells, E. coli 25922 was challenged in rich and minimal media with PANI suspensions in the presence and absence of oxygen. In rich media, PANI demonstrated greater activity in aerobic conditions compared to anaerobic conditions as indicated by the lower aerobic MIC and MBC values (Fig. 4.3). The difference between the MIC and MBC of PANI in aerobic and anaerobic conditions against E. coli 25922 in LB broth was statistically significant (Mann-Whitney test, P value: less than 0.05). A similar trend was seen for the activity against
E. coli 25922 in minimal A salts media with 0.7% glycerol and minimal A salts media with 0.4% succinate as a carbon source (Fig. 4.4). The difference between the aerobic and anaerobic MIC and MBC of PANI against E. coli 25922 in minimal media with glycerol was statistically significant (Mann-Whitney test, P value: less than 0.05). The difference between the MIC and MBC of PANI against E. coli 25922 in minimal media with succinate in aerobic and anaerobic conditions was statistically significant (Mann-Whitney test, P value: less than 0.05). The reduced sensitivity of E. coli 25922 to PANI treatment in anaerobic conditions infers that oxygen and its associated reactive species are involved in the mode of action of PANI.

Aerobically, PANI had increased antimicrobial efficacy in minimal media compared to rich media as demonstrated by the lower MIC and MBC values in the former (Fig. 4.3, Fig. 4.4). The increased sensitivity of E. coli 25922 in minimal media to the bactericidal activity of PANI was abolished in anaerobic conditions (Fig. 4.3, Fig. 4.4), which indicates that the E. coli 25922 cells were more susceptible to the antimicrobial action of PANI in both aerobic and low nutrient conditions. The increased susceptibility to PANI action in minimal media compared to LB broth may reflect the E. coli cells ability to respond efficiently to PANI induced stress in rich media as cells in minimal media must synthesise all building blocks, the enzymes for which are susceptible to oxidative damage.

The aerobic MBC of PANI against E. coli 25922 in minimal media with succinate as the carbon source (0.0625% PANI) was higher than that in minimal media with glycerol as the carbon source (0.015625% PANI). The difference in susceptibility to PANI in these conditions may be reflective of the metabolic state of E. coli growing on the particular carbon source. The succinate concentration used to support cell growth in this experiment was ~ 15 mM. High concentrations of succinate (more than 8.8 mM) have been shown to reduce hydrogen peroxide formation by fumarate reductase, a flavin containing enzyme, by preventing autoxidation(375). The increased aerobic MBC of PANI against E. coli grown on succinate (compared to growth on glycerol) may be due to succinate somewhat reducing hydrogen peroxide production and following from this, susceptibility to PANI action. The lack of a difference in the bactericidal activity of PANI in the presence of succinate or glycerol in anaerobic conditions is supportive of this notion (Fig. 4.4).
Figure 4.3. The effect of oxygen on the activity of PANI against *E. coli* 25922 in rich media. The MIC (circles) and MBC (squares) of PANI in aerobic (O₂) and anaerobic (AnO₂) conditions against *E. coli* 25922 in LB broth. Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).
Figure 4.4. The effect of oxygen on the activity of PANI against *E. coli* 25922 in minimal media. The MIC (circles) and MBC (squares) of PANI in aerobic (O₂) and anaerobic (AnO₂) conditions against *E. coli* 25922 in minimal A salts with 0.7% glycerol (A) or 0.4% succinate (B) as the carbon source. Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).

4.2.1.2 In aerobic conditions P3ABA is more active against *E. coli* 25922 in rich media and less active against *E. coli* 25922 in minimal media compared to anaerobic conditions

The hypothesis that *E. coli* cells would exhibit greater sensitivity to P3ABA in energy poor conditions (i.e. low nutrient and no oxygen conditions) compared to energy rich conditions was mostly not supported, with the exception of the low anaerobic MIC in minimal media with
succinate. It has been recently argued that ATP depletion following antimicrobial treatment does not cause bacterial cell death but rather delays the onset of killing \cite{226, 376, 377}. It has been demonstrated that mycobacterial cells depleted of ATP do not loose viability \cite{378}. The impact of ATP depletion on P3ABA action was indirectly examined by challenging \emph{E. coli} cells grown on a non-glycolytic substrate, succinate, and \emph{E. coli} cells grown on a glycolytic substrate, glycerol. Cells growing on succinate can only generate ATP through oxidative phosphorylation, which requires a functioning ATPase while energy can be derived from glycerol through oxidative phosphorylation as well as substrate level phosphorylation through the glycolytic pathway \cite{226, 330, 379}. The similar MIC and MBC values obtained for P3ABA with challenge on succinate and on glycerol carbon sources \cite{Fig. 4.6} suggest that the ability to synthesise ATP by alternate methods does not prevent P3ABA bactericidal action. Consistent with this, bedaquiline treatment of \emph{M. smegmatis} has been shown to result in downregulation of genes involved in glycolysis \cite{226}. Therefore, the antimicrobial mechanisms of P3ABA separate to the putative ATP depletion may be involved in the bactericidal mode of action.

One important consideration for examining antimicrobial mechanism that involves targeting of metabolism and respiration is that the metabolic state of the cell may influence how it responds to bactericidal treatment \cite{376}. The bactericidal action of antimicrobial agents is associated with increased respiration while bacteriostatic action is characterised by suppressed cellular respiration \cite{376}. The bacteriostatic effect reduces ATP demand and is often the dominant effect blocking bactericidal action \cite{376}. Following from this, if cellular energy output is readily inhibited, such as in cells in energy poor conditions, antimicrobial action may result in inhibition of growth rather than bactericidal killing \cite{376}. Bacterial cells that are highly active may therefore be more susceptible to antimicrobial exposure because of accelerated respiration.

It has been postulated that bedaquiline exerts lethal activity by uncoupling of respiration-driven ATP synthesis causing increased respiration, loss of the proton gradient and futile cycling \cite{226}. The greater activity of P3ABA in rich media relative to minimal media – as shown by the lower MIC and MBC values obtained in LB broth \cite{Fig. 4.5} compared those obtained in minimal media \cite{Fig. 4.6} – may be due to the lower energy state of the \emph{E. coli} cells in minimal media. It is possible that the increased respiration and metabolic activity associated with \emph{E. coli} cells in LB broth would predispose the cells to the bactericidal action of P3ABA (resulting in
accelerated respiration and futile cycling) while the cells in minimal media may experience more inhibitory action(245). Indeed, the MIC of bedaquiline against *M. smegmatis* MC²155 in LB broth (0.01 µg/ml) was lower compared to that in minimal media (0.025µg/ml)(226).

Succinate is a non-fermentable carbon source that necessitates ATP production to be carried out by the ETC (rather than by fermentation) compared to components in LB broth. The notable difference in the anaerobic MIC and MBC of P3ABA in minimal media with succinate (4 doubling dilutions) compared to that in other media (~ 2 doubling dilutions) may be reflective of the effective inhibition of cells by P3ABA in a very energy-poor environment contributing to less effective bactericidal action (Fig. 4.5, Fig. 4.6).

In this thesis, the hypothesis that the mode of action of P3ABA may eventuate in ROS production has been proposed (section 3.2.8). To investigate this hypothesis, *E. coli* 25922 was challenged with P3ABA suspensions in rich and minimal media in the presence and absence of oxygen. In rich media, the MIC and MBC of P3ABA against *E. coli* 25922 were higher in anaerobic conditions compared to aerobic conditions (Fig. 4.5). The greater MIC and MBC of P3ABA against *E. coli* 25922 in anaerobic conditions relative to aerobic conditions were statistically significant (Mann-Whitney test, P value: less than 0.05). The decreased activity of P3ABA in anaerobic conditions suggests that in rich media the antimicrobial activity of P3ABA involves a small amount of ROS production. Uncoupling activity, such as what is postulated to occur during P3ABA action, causes futile cycling, which is associated with increased oxygen consumption and production of ROS(226, 322). Therefore, the minor role of induction of oxidative stress in P3ABA antimicrobial action may be due a downstream consequence of uncoupling electron transport from ATP synthesis. P3ABA activity was less affected by oxygenation compared to PANI activity, which implies that P3ABA acts through other more important antimicrobial mechanisms.

The trend observed in rich media was distinct from that observed in minimal media. Reduced activity of P3ABA was demonstrated when oxygen was present (as shown by higher MIC and MBC values) (Fig. 4.6). The difference between the MIC in minimal media with succinate in aerobic and anaerobic conditions was statistically significant (Mann-Whitney test, P value: less than 0.05) while all other differences observed for *E. coli* 25922 in minimal media were not statistically significant (Mann-Whitney test, P value: more than 0.05). These results suggest
that induction of ROS-mediated stress is not occurring in *E. coli* cells treated with P3ABA in minimal media.

**Figure 4.5.** The effect of oxygen on the activity of P3ABA against *E. coli* 25922 in rich media. The MIC (circles) and MBC (squares) of P3ABA in aerobic (O$_2$) and anaerobic (AnO$_2$) conditions against *E. coli* 25922 in LB broth. Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).
Figure 4.6. The effect of oxygen on the activity of P3ABA against *E. coli* 25922 in minimal media. The MIC (circles) and MBC (squares) of P3ABA in aerobic (O\(_2\)) and anaerobic (AnO\(_2\)) conditions against *E. coli* 25922 in minimal A salts with 0.7% glycerol (A) or 0.4% succinate (B) as the carbon source. Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).

### 4.2.2 Activity of PANI and P3ABA against *S. aureus* 6538 in aerobic and anaerobic conditions

Following testing with *E. coli*, the activity of PANI and P3ABA in the presence and absence of oxygen was determined against *S. aureus* 6538 in LB broth. It was hypothesised that the activity of PANI and P3ABA in aerobic and anaerobic conditions against *S. aureus* 6538 is similar to that against *E. coli* 25922 (section 4.2.1).
4.2.2.1 In aerobic conditions PANI is more active against *S. aureus* 6538 compared to anaerobic conditions

The trend observed for activity of PANI against *S. aureus* 6538 in aerobic and anaerobic conditions was similar to that observed against *E. coli* 25922 (Fig. 4.3). The MIC and MBC of PANI against *S. aureus* 6538 was lower in aerobic compared to anaerobic conditions, which indicate that the presence of oxygen positively influences the activity of PANI (Fig. 4.7). The difference between the aerobic and anaerobic MIC and MBC of PANI against *S. aureus* 6538 was statistically significant (Mann-Whitney test, P value: less than 0.05). In aerobic conditions, PANI showed greater activity against *S. aureus* 6538 (MBC 0.0625% PANI; Fig. 4.7) compared to *E. coli* 25922 (MBC 0.25% PANI; Fig. 4.3) while in anaerobic conditions this trend is not apparent. Therefore, the activity of PANI against *S. aureus* 6538 was greatly affected by the presence of oxygen, which supports the hypothesis of induction of oxidative stress as a mode of action of PANI.

![Figure 4.7](image-url)  
*Figure 4.7. The effect of oxygen on the activity of PANI against *S. aureus* 6538 in rich media.* The MIC (circles) and MBC (squares) of PANI in aerobic (O\(_2\)) and anaerobic (AnO\(_2\)) conditions against *S. aureus* 6538 in LB broth. Statistical significance is represented by * (Kruskal-Wallis test, P value less than 0.05; Dunn’s multiple comparison post-hoc test). Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).

4.2.2.2 In aerobic conditions P3ABA has similar activity against *S. aureus* 6538 compared to anaerobic conditions

The inhibitory activity of P3ABA against *S. aureus* 6538 in LB broth (Fig. 4.8) was comparable to that of *E. coli* 25922 (Fig. 4.5) as shown by the similar MIC values in the range of...
0.0625%-0.125% P3ABA. P3ABA had reduced bactericidal activity against *S. aureus* 6538, demonstrated by the higher aerobic and anaerobic MBC values (2%-4%; Fig. 4.8), compared to *E. coli* 25922 (MBC 0.125%-0.5%; Fig. 4.5). The bactericidal activity of P3ABA against *S. aureus* 6538 in anaerobic conditions was similar to that in aerobic conditions, which implies that oxygen and its associated reactive species are not involved in the mode of action of P3ABA and suggests involvement of other mechanisms.

![Figure 4.8. The effect of oxygen on the activity of P3ABA against *S. aureus* 6538 in rich media.](image)

The MIC (circles) and MBC (squares) of P3ABA in aerobic (O$_2$) and anaerobic (AnO$_2$) conditions against *S. aureus* 6538 in LB broth. Data obtained from aerobic conditions is represented by filled data points while data obtained from anaerobic conditions is represented by unfilled data points.

### 4.2.3 Activity of PANI and P3ABA in aerobic and anaerobic conditions

The overall aim of the determination of the MIC and MBC of PANI and P3ABA against *E. coli* and *S. aureus* in aerobic and anaerobic conditions was to examine the influence oxygen has on the activity of these antimicrobial agents. A higher MBC in anaerobic conditions compared to aerobic conditions is indicative of a reduction in bactericidal activity when no oxygen is present, which would suggest that, taken with other evidence, the antimicrobial mechanism involves induction of oxidative stress. The number of doubling dilutions difference between the aerobic MBC and anaerobic MBC reflects the extent oxygenation influences lethal activity. The difference in activity between aerobic and anaerobic conditions is presented as a graph in Figures 4.9 and 4.10 with the number of doubling dilutions between MBC O$_2$ and MBC AnO$_2$ on the y-axis and treatment on the x-axis. Greater activity in aerobic conditions (MBC O$_2$ less than MBC AnO$_2$) is represented by a positive number while greater activity in anaerobic conditions
(MBC O₂ more than MBC AnO₂) is represented by a negative number. No difference in activity between aerobic and anaerobic conditions (MBC O₂ = MBC AnO₂) is represented by 0.

4.2.3.1 PANI is less active in anaerobic conditions while P3ABA is less affected by the absence of oxygen

The activity of PANI against *E. coli* 25922 and *S. aureus* 6538 in all media tested was decreased in anaerobic conditions as indicated by a positive number of doubling dilutions difference (Fig. 4.9, Fig. 4.10). In contrast to PANI, the activity of P3ABA against these strains in LB broth was not affected as much by the presence and absence of oxygen (Fig. 4.9, Fig. 4.10). The difference between the influence of the presence of oxygen on the activity of PANI compared to P3ABA against *E. coli* 25922 and *S. aureus* 6538 was statistically significant (Mann-Whitney test, P value: less than 0.05). These results demonstrate that the antimicrobial activity of PANI is more greatly influenced by the presence of oxygen compared to P3ABA, which supports the hypothesis that PANI mediates induction of oxidative stress.
Figure 4.9. The difference between the MBC of PANI and P3ABA against *E. coli* 25922 in aerobic and anaerobic conditions. The number of doubling dilutions between the MBC of PANI and P3ABA against *E. coli* 25922 in aerobic and anaerobic conditions. *E. coli* 25922 cells were challenged in LB broth (circles), minimal A salts with 0.7% glycerol (squares) and minimal A salts with 0.4% succinate (triangles). The dotted line represents no difference in the aerobic MBC and anaerobic MBC of PANI and P3ABA. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).

Figure 4.10. The difference between the MBC of PANI and P3ABA against *S. aureus* 6538 in aerobic and anaerobic conditions. The number of doubling dilutions between the MBC of PANI and P3ABA against *S. aureus* 6538 in LB broth in aerobic and anaerobic conditions. The dotted line represents no difference in the aerobic MBC and anaerobic MBC of PANI and P3ABA. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).
4.3 Discussion

The contribution of oxidative stress to lethal action of antibiotics – mostly focused on norfloxacin, ampicillin and kanamycin – has been thoroughly investigated. It was hypothesised that ROS facilitate cell death through oxidising iron atoms in enzymes, releasing iron that may participate in Fenton reaction with hydrogen peroxide to produce hydroxyl radicals (238, 344, 363). Hydroxyl radicals can cause significant damage to cellular biomolecules including protein, lipid and DNA (238, 344). Damage to DNA can occur through the direct action of hydroxyl radicals or by oxidising the deoxynucleotide pool before incorporation into DNA (228, 363). Deoxynucleotide triphosphates bind iron facilitating localised production of hydroxyl radicals (228, 298). Guanine is particularly susceptible to oxidation, which usually generates 8-oxo-guanine (298, 347, 380). Closely spaced 8-oxo-guanine nucleotides are often unable to be repaired leading to lethal double strand DNA breaks (298, 380, 381). Only one unrepaired single strand break is sufficient to kill a bacterial cell (347). Therefore, the lethality of oxidative stress is dependent on free iron generating hydroxyl radicals that damage DNA. Investigation of the involvement of oxidative stress in the bactericidal action of antibiotics focussed on these factors.

Intracellular hydroxyl radicals were detected in antibiotic treated cells using fluorescent dyes, which are oxidised by the radicals causing an increase in fluorescence (as measured using flow cytometry) (238, 344, 382). The most commonly used dye, hydroxyphenyl fluorescein (HPF), is thought to be oxidised selectively by hydroxyl radicals and peroxynitrite with high specificity (344, 382). Several groups have demonstrated increases in HPF fluorescence in *E. coli* treated with bactericidal antibiotics including norfloxacin, ampicillin and kanamycin (238, 344, 383). HPF fluorescence in antibiotic treated cells has been correlated with increases in mutation rate, which is indicative of oxidative stress (384). These results have been contested, however, as dyes may be oxidised by species other than hydroxyl radicals including high-valence metal centres of enzymes, disruption of cell membrane integrity can facilitate dye penetration and fluorescence from dyes is influenced by cell dimensions (363, 381). It is possible that during antibiotic treatment, metal centres of enzymes are responsible for dye oxidation as their levels can increase as metabolism fails (363, 381). One group used a range of dyes to increase sensitivity of the assay and reduce the potential impact of confounding chemical events that can generate false positive results (228). Overall, treatment with
ampicillin, gentamycin and norfloxacin was demonstrated to cause increased fluorescence compared to controls(228). Increases in cell size (in antibiotic treated cells) are associated with increased HPF signals and thereby may not reflect the hydroxyl radical concentration(385). However, increased HPF fluorescence has been detected in gentamicin treated cells, which were also demonstrated to have unchanged cell dimensions(383). The use of HPF fluorescence as an indicator of oxidative stress is further compounded by the potential antioxidant activity of the dyes, which would protect against ROS mediated lethality(228). Therefore, while there is evidence to suggest that hydroxyl radicals are produced in antibiotic treated cells, the potential ambiguity of using fluorescent dyes, such as HPF, necessitates use of other supporting techniques, such as examining the influence of iron chelators and ROS scavengers on antibiotic activity.

The role of iron in propagating oxidative stress and producing hydroxyl radicals led to the investigation of the influence of iron chelators – which would inhibit Fenton reaction – on antibiotic activity(382). In a similar vein, the effect of ROS scavengers, such as thiourea, was examined(382, 383). Iron chelators, o-phenanthroline, 2,20-dipyridyl and bipyridyl, have been shown to prevent formation of hydroxyl radicals in antibiotic treated cells (detected using HPF) and increase bacterial survival(238, 281, 344). Similarly, thiourea, a hydroxyl radical scavenger, reduced hydroxyl radical formation and rates of cell death in antibiotic exposed cells(281, 344). Pre-treatment with the antioxidants, glutathione and ascorbic acid, both provided protection against the lethal action of antibiotics(228). Thiourea has also been shown to reduce mutation rates of antibiotic treated bacterial cells to that of untreated cells(384). Reduction in bactericidal action in the presence of iron chelators and ROS scavengers was used to support the hypothesis that antibiotics cause oxidative stress in bacterial cells, eventuating in cell death. There has, however, been work published that argues against this hypothesis, including the observation of protection from thiourea under anaerobic conditions (in which ROS would not be produced)(362, 363). It has been found that thiourea mildly inhibits cell growth, which can mediate tolerance to lethal action and may explain thiourea protection against killing in anaerobic conditions(362, 363). Iron chelators may also protect cells in this manner as they may cause decreased growth rates(363, 382).

The reliance of ROS formation on the presence of oxygen allows for investigation of the involvement of oxidative stress in antibiotic action by challenging bacterial cells in aerobic and
anaerobic conditions (228, 383, 384, 386). Challenge of bacterial cells in anaerobic conditions will provide protection (relative to aerobic conditions) against bactericidal action if that action involves ROS-mediated damage. It has been found that anaerobic conditions protected against antibiotic lethal action and reduced mutation rates in treated cells close to that of untreated cells (228, 383, 384, 386). *E. coli* cells were challenged with nalidixic acid, norfloxacin and ciprofloxacin in aerobic and anaerobic conditions for 2 h and enumerated using plate counts (386). Nalidixic acid completely lost activity, while the other antibiotics had reductions in activity, in anaerobic conditions supporting the proposed production of ROS in treated bacterial cells (in aerobic conditions) (386). Similarly, *E. coli* cells treated with gentamicin, norfloxacin and ampicillin in aerobic conditions were reduced in cell viability (as determined using plate counts) to a greater extent compared to treatment in anaerobic conditions (228).

Other groups, however, have demonstrated similar, and increased, bactericidal activity of antibiotics in anaerobic conditions relative to aerobic conditions (362, 363). For example, the MIC of norfloxacin in aerobic conditions (0.125 µg/ml) was found to be similar to that in anaerobic conditions (0.25 µg/ml) (362). Challenge of a range of concentrations of norfloxacin in aerobic and anaerobic conditions, however, yielded conflicting results (362). Two norfloxacin concentrations (1.25 µg/ml and 2.5 µg/ml) were less effective at reducing *E. coli* cell viability in anaerobic conditions while the other concentrations (0.5 µg/ml and 1.5 µg/ml) had similar activity in both conditions (362). The discrepancies in the impact of oxygen on the bactericidal action of antibiotics in published literature may suggest that oxidative stress is involved in the action of antibiotics under certain conditions.

The investigation into the role of oxidative stress in the lethal action of antibiotics has yielded inconsistent results. The complexity of metabolic perturbations resulting from antibiotic exposure has been underestimated and likely underpins the inconsistencies (298, 382). In combination with this, specifics of experimental methods used to assay oxidative stress may explain conflicting results, such as differences in flask size, which influence aeration of incubated cells and test media (228, 382). To further this point, incubation in lower-volume cultures is associated with higher oxyR expression, thus the precise experimental conditions can expose bacterial cells to various stresses and affect their response to antibiotic exposure, influencing results (387). It is also probable that antibiotic exposure in different bacteria results in distinct molecular events that eventuate in ROS production, complicating elucidation of
these events(228, 298). Additionally, the degree to which a ROS-dependent mechanism contributes to overall bactericidal activity can vary hugely between antibiotics(228, 298). It is also likely that additional bactericidal mechanisms operating will influence the role of oxidative stress. Therefore, no overall conclusion on whether oxidative stress is a common mechanism in the lethal action of antibiotics can be made at this time. It is apparent that oxidative stress is involved, at least for some antibiotics in certain conditions; however, the extent and essentiality of this involvement remains to be elucidated(382).

The results presented in this chapter support the hypothesis that PANI antimicrobial action involves induction of oxidative stress based on activity in aerobic and anaerobic conditions. Based on the debate surrounding the role of oxidative stress in the action of antibiotics, it is likely that oxidative stress is only one of several mechanisms, which may act concurrently or sequentially. A reduction in, but not a complete loss of, bactericidal action of PANI in anaerobic conditions infers that other modes of action are also involved(228, 383). Further work is required to elucidate the molecular events involved in PANI mode of action.

4.4 Conclusion
The results presented in this chapter lend further support towards the hypothesis that the antimicrobial action of PANI involves induction of hydrogen peroxide stress. The reduction in activity of PANI against *E. coli* and *S. aureus* observed in anaerobic conditions relative to aerobic conditions demonstrates the impact of the presence of oxygen. Oxygen may undergo adventitious univalent reduction to form superoxide followed by hydrogen peroxide at catalytic sites in redox enzymes. Thus, in aerobic conditions, oxygen is available to create ROS, which may be produced at higher rates following exposure to PANI. The antimicrobial activity of PANI is more affected by the presence of oxygen compared to P3ABA, which supports the hypothesis that PANI mediates induction of oxidative stress.

P3ABA was more active against *E. coli* in rich media compared to minimal media, which may reflect the higher energy state of the cells in rich media sensitising them to the putative uncoupling action of P3ABA. Bacterial cells with increased respiration and metabolic activity would be more likely to develop accelerated respiration and lethal futile cycling following exposure to an uncoupler compared to bacterial cells with low metabolic and respiratory
activity, as is found with growth on minimal media. There is limited evidence for a small amount of ROS production in P3ABA treated *E. coli* cells grown in rich media, which is a known downstream effect of respiratory uncoupling. There was no evidence to support involvement of ROS in the P3ABA mode of action in minimal media, which implies that P3ABA has other more important antimicrobial mechanisms.
Chapter 5: Characterisation of the Antibacterial Activity of PANI and P3ABA Surfaces

5.1 Introduction
Antimicrobial surfaces are a useful tool for controlling the spread of bacteria due to surface contamination and preventing the formation of biofilms.(388) Thus, antimicrobial surfaces have the potential to disrupt transmission pathways and eliminate pathogen surface reservoirs. Antimicrobial surfaces may release the antimicrobial agent to elicit an effect or the agent may be immobilised on the surface so that killing only occurs on contact(388, 53). Surfaces that release the active agent are limited to short term applications only due to the eventual loss of the agent and are not suitable for food processing applications due to safety concerns (outlined in section 1.2.5)(388, 68). Antimicrobial agents immobilised onto surfaces must be able to exert bactericidal activity without penetrating the bacterial cell(53). Surfaces with immobilised antimicrobial agents are better suited to applications involving long term and repeated challenges(52). Antimicrobial polymers are good candidates for immobilised biocides and can be described as either polymeric biocides or biocidal polymers(388, 53). The first class is based on the concept that biocidal groups attached to a polymer act in the same way as analogous low molecular weight compounds, i.e., the repeating unit is a biocide(388). For biocidal polymers the active principle is embodied by the whole macromolecule, not necessarily requiring antimicrobial repeating units(388). In this chapter, we investigate the antimicrobial activity of PANI and P3ABA as surface-immobilised biocidal polymers.

Surfaces in the hospital environment are now recognised as a potential reservoir of pathogenic bacteria(389–391). Included in these types of surfaces are fabrics, such as the apparel worn by healthcare workers and patient privacy curtains(54, 389, 390). One study found that up to 60% of hospital staff uniforms were contaminated with drug resistant bacteria, including MRSA(392). Privacy curtains are high-touch areas that are contacted by the hands of the healthcare worker before, during and after patient care and are infrequently changed(54, 393).
It has been demonstrated that more than 90% of privacy curtains can become contaminated within a week of use (393). Natural fabrics can be particularly susceptible to contamination as they bind water and nutrients, which promote bacterial persistence (394). Contaminated absorbent surfaces in hospitals may be involved in pathogen transmission (389, 390). Surfaces near infectious patients can become contaminated with pathogens, which may be spread to other surfaces or patients through hand contact events resulting in disease transmission (29–31, 37). Absorbent surfaces are harder to clean or disinfect than non-absorbent surfaces while the latter facilitates greater transfer of bacteria (5, 90, 393). Therefore, the development of both absorbent and non-absorbent antimicrobial surfaces would help curtail the spread of infection in hospitals; and complement other initiatives aimed at reducing the public health burden of antibiotic resistant pathogens (1). For example, a ‘complex element compound’ metal alloy made by PurThread Technologies was incorporated into polyester fibres, which were used to make privacy curtains (54). These curtains took 7-times longer to be colonised by pathogenic bacteria than standard curtains demonstrating the potential for antimicrobial absorbent surfaces to reduce contamination (54).

To create an antimicrobial surface, we can take one of two basic approaches. First, a coating may be applied to a material or a modification of the surface chemistry of the material made to provide an antimicrobial surface (84). Alternatively, the material may be fabricated by incorporating an antimicrobial into the material; this can be challenging as many factors exist that can negatively impact on bactericidal activity (53, 68, 395). If the selected antimicrobial agent is not compatible with manufacturing procedures, such as extrusion or lamination, antimicrobial activity would be lost (53, 68, 395, 396). Extrusion is an example of a thermal polymer processing method for incorporation of a compound into surfaces (63, 68). Extrusion involves high temperatures, shear forces and pressures; all of which may cause degradation of the antimicrobial agent (63, 68). Another manufacturing approach is dissolving or dispersing the polymer and the antimicrobial agent into an appropriate solvent mixture (63). The resulting homogeneous solution of antimicrobial agent and polymer can be used to generate uniform antimicrobial films (by evaporation of solvent, which is commonly used for food packaging) or to produce antimicrobial nanofibers (by using an electrospinning method) (63, 167, 397). Electrospinning is an economic, fast and simple process to generate a highly porous bactericidal material, such as for food and biomedical applications (167, 397). Antimicrobial
agents may be covalently attached to a surface; however, that may cause side reactions that result in conformational changes in the agent, ultimately causing loss of activity(396). PANI and its associated fPANI are good candidates for incorporation into surfaces because they have thermal stability up to 300°C, environmental stability in the conducting form and inexpensive synthetic procedures(163, 173). PANI and fPANI have been successfully incorporated into films(166, 168, 173).

The activity of an antimicrobial surface is also influenced by the nature of the surface. Surfaces can be absorbent allowing water droplets to move into the surface or they can be non-absorbent, in which water droplets sit on top of the surface. These surface properties may affect the antimicrobial activity as a bacterium in a water droplet would have more contact with the antimicrobial agent if it has absorbed into the surface. Non-absorbent surfaces in hospitals are frequently contaminated with pathogens and include walls, door handles and bed frames. Much of the focus of development of antimicrobial surfaces in the published literature is on model non-porous surfaces, such as metal coupons and plastic films(389, 398, 399).

Characterising the activity of antimicrobial surfaces can be achieved through several approaches, including determining the zone of inhibition, direct inoculation of the surface with a liquid sample, direct inoculation of the surface with a thin film and direct inoculation of the surface with an inoculum that rapidly dries(53, 126). Zone of inhibition is only relevant for antimicrobial agents that leach from the surface(53). Direct inoculation of a surface is suitable for both immobilised and non-immobilised antimicrobial agents(53). One common test that involves direct inoculation with a liquid sample is the Japanese Industry Standard (JIS Z-2801) method(53, 250). This method involves application of a drop of inoculum onto a surface, which is then covered by a coverslip or film(53, 250). The JIS method was developed for testing non-immobilised agents; however, it can be applied to antimicrobial agents that are fixed onto a surface(53, 250). Application of a thin film of bacteria can be useful as it allows very close contact between the bacteria and the antimicrobial surface(53). This type of test is suited for emulating ambient contamination of surfaces and the associated activity of the antimicrobial surface(53). Following exposure of an inoculum to an antimicrobial surface for a specified period of time, the bacterial cells are recovered and enumerated to determine the extent of knockdown(53). Fingertip contamination of surfaces is characterised by transfer of bacteria and biomolecules on the skin (including proteins, salt and sugar) in a low volume of liquid that
dries in seconds(125, 126). One study developed a model for examining surface activity of dry contamination by spreading $10^7$ CFU/µl of concentrated inoculum over the surface of a copper coupon resulting in rapid drying of inoculum(126). Desiccation may contribute to the reduction in bacterial cell number and influence the results in dry fomite tests(12, 141).

In this chapter, the antimicrobial activities of PANI and P3ABA containing surfaces were investigated by challenge with *E. coli* 25922 Lux and *S. aureus* 6538. Both *E. coli* and *S. aureus* have been isolated from surfaces in hospitals(30, 400). Furthermore, both species are able to persist on surfaces, making them prime candidates for the testing of PANI and P3ABA surfaces(7). Characterisation of antimicrobial surfaces involves two approaches, first to determine the activity of those antimicrobial agents as part of a surface against relevant bacteria, and second to determine the antimicrobial mechanism to facilitate optimisation of the antimicrobial agents incorporated into a surface(53). The first step is to validate the action of an antimicrobial agent in ideal testing conditions, which in most cases involves determining the activity of an antimicrobial agent in suspension(401). If an antimicrobial agent requires contact to elicit action then it will have the greatest efficacy in suspension and less when part of a surface, as the amount of antimicrobial agent present on a surface is limited(53). Following validation of activity in suspension, the activity of antimicrobial agent incorporated into surfaces is examined against target bacteria. It is important to determine how bactericidal activity may change due to incorporation into a material or onto a surface(53, 396).

The activity of PANI and P3ABA in both suspension and at surfaces against *E. coli* 25922 Lux and *S. aureus* 6538 was determined allowing for a comparison of the reduction in viability of these two strains. It also provided an opportunity to produce data that could be used to support or challenge hypotheses regarding the antimicrobial mechanism of PANI and P3ABA. To this end, the sensitivity of *E. coli* 25922 Lux to PANI and P3ABA suspensions was evaluated in LB broth, minimal A salts with 0.7% glycerol and minimal A salts with 0.4% succinate allowing comparison between these conditions. This was consistent with the media used to investigate the activity of PANI and P3ABA in aerobic and anaerobic conditions against *E. coli* 25922 (section 4.2.1). LB broth is a rich media (on which cells grow at high rates) while minimal A salts only contains nutrients that are essential for growth(245, 258, 259). Glycerol and succinate are glycolytic and non-glycolytic carbon sources, respectively, which entails that more energy is derived from aerobic metabolism of glycerol (outlined in section 4.1). Differences in the
composition of media affects cell physiology and may affect susceptibility to treatment (331). Additionally, slow growth of bacteria in a minimal media environment is similar to what may occur in nature(260).

Short treatment times (up to 4 h) were used to determine the activity of PANI and P3ABA suspensions in this work. Disrupting transmission pathways through surface decontamination would be best achieved with an antimicrobial agent that kills over a short period of time(401). A single contact event is sufficient to transfer pathogens, including *E. coli*, between a surface and a person’s hand(3, 8). The longer a bacterium persists on a surface, the greater the opportunity to be spread(7). Therefore, rapid decontamination times will decrease the chance that bacteria may be transferred to a new surface before sterilisation is achieved. Rapid activity would also decrease the likelihood of resistance developing(126).

Protocols were developed to assess the activity of absorbent and non-absorbent surfaces containing PANI and P3ABA using techniques derived from the JIS method and from standard antimicrobial testing methods(214, 229, 250). A laboratory model of an absorbent surface is solidified agar, as drops of liquid containing bacteria will absorb into the agar surface(237). Molten agar was mixed with varying amounts of PANI or P3ABA, which when left to set created absorbent surfaces containing the antimicrobial agents. PANI and P3ABA in agar were set up in wells of a 96 well plate allowing for the testing of many concentrations and treatment times against one inoculum. Following optimisation of this protocol with absorbent surfaces, the activity of non-absorbent surfaces containing PANI and P3ABA, in the form of Styrene Ethylene Butylene Styrene (SEBS) films, against test bacteria was determined using the micro-JIS assay (section 2.2.6)(53, 63, 250). The PANI and P3ABA films were cut into circles ~ 5 mm in diameter to allow for incubation of many test samples in a 96 well plate. Recovered cells were enumerated through bioluminescent or optical density means, as opposed to plate counts, to allow for high-through testing and to evaluate the method for future testing against slow growing bacteria, such as *M. tuberculosis*.

Utilisation of the protocol for testing PANI and P3ABA films verified their activity in standard antimicrobial testing conditions, namely bacteria at a high inoculum (10^6 CFU/ml) grown in a rich broth. This can be quite different to the conditions experienced in real world settings. Generally surfaces come into contact with a range of bacterial concentrations, usually less than
10⁶ CFU/ml, and there are few nutrients available if the surfaces are not soiled with organic matter(260). Surfaces may be soiled with organic matter, which may affect the activity of the incorporated antimicrobial agents, usually in a negative manner(65). To characterise the activity of PANI and P3ABA films in more realistic settings these films were challenged with a range of CFU doses of bacteria in saline solution. PANI and P3ABA films were also challenged with bacteria in rich media and bacteria in saline solution to determine how the presence of general organic matter influences the activity of the films. Taken together these experiments demonstrated the activity of surfaces containing PANI and P3ABA against target bacteria in rich media and application relevant settings.

5.2 Activity of PANI and P3ABA suspensions against E. coli and S. aureus

Standard antimicrobial susceptibility testing is performed on suspensions(214, 229). To determine the potential of PANI and P3ABA as surface antimicrobial agents active against the target organisms, E. coli and S. aureus, activity was first confirmed in suspension. The action of PANI and P3ABA suspensions was determined against two antimicrobial susceptibility testing bacterial strains, E. coli 25922 and S. aureus 6538(214–216). For testing of surfaces containing PANI and P3ABA, a lux-tagged version of E. coli 25922 was used as the released bioluminescence can be detected and serves as a marker of cell viability(53, 218, 219). The protocol for determining the activity of PANI and P3ABA in surfaces was optimised using the bioluminescent E. coli 25922 strain as detection of bioluminescence is a practical alternative to enumeration by plate counts for future testing of these surfaces against slow growing bacteria, such as M. tuberculosis.

To elucidate the activity of PANI and P3ABA in suspensions, cell viability assays were performed on S. aureus 6538 and E. coli 25922 lux treated with 0.5% PANI or 0.5% P3ABA suspensions (section 2.2.3). A 0.5% concentration was selected (as opposed to the 1% suspensions tested against the E. coli deletion mutants) because there needs to be detectable cells present when experimental samples were enumerated to generate data to analyse. S. aureus 6538 and E. coli 25922 lux were both challenged in LB broth to allow comparison of their susceptibilities to the bactericidal actions of PANI and P3ABA in suspension. E. coli 25922 lux was also challenged in minimal A salts with either 0.7% glycerol or 0.4% succinate as the
carbon source. Determination of the activity of PANI and P3ABA in suspension against *E. coli* 25922 *lux* in rich media and minimal media with different carbon sources permitted comparisons to be made between these conditions. At 0.5 h, 1 h, 2 h and 4 h time points the treated cells were enumerated using the drop count method. Results presented are from three independent experiments performed on different days. The sensitivity of each tested strain in each media condition was compared using a linear regression analysis (section 2.2.3.6).

5.2.1 PANI has similar activity against *E. coli* 25922 and *E. coli* 25922 *lux* while P3ABA is less active against the latter

*E. coli* 25922 *lux* has a chromosomal insertion of the bacterial luciferase (*lux*) operon (*luxCDABE*) into the 16S locus(219). This operon encodes bacterial luciferase (*luxAB*), which catalyses the oxidation of substrate (aldehyde) resulting emission of blue-green light (bioluminescence)(219, 221). A cell expressing the *lux* operon (*luxCDABE*) will be in an altered state compared to the non-tagged version as cellular energy is diverted in order to generate the luminescence(53). Synthesis of the proteins in the *lux* operon will require energy and metabolites. The bioluminescence reaction consumes reduced flavin mononucleotide (FMNH$_2$) and a long chain fatty aldehyde(402). The products of the *luxC*, *luxD* and *luxE* genes divert tetradeanoic acid away from the fatty acid biosynthesis pathway to regenerate the aldehyde substrate(402). Thus, it is possible that the *lux*-tagged version of *E. coli* 25922 may have different sensitivities to PANI and P3ABA compared to the non-bioluminescent version. Therefore, the activity of PANI and P3ABA in suspensions was determined against *E. coli* 25922 and *lux*-tagged *E. coli* 25922.

The MICs and MBCs of PANI and P3ABA against *E. coli* 25922 and *E. coli* 25922 *lux* in LB broth were determined (section 2.2.4.2). The sensitivity of *E. coli* 25922 and the *lux*-tagged version to PANI in suspension were demonstrated to be very similar (Fig. 5.1). P3ABA had a similar MIC against *E. coli* 25922 and *E. coli* 25922 *lux* (Fig. 5.2); however, the MBC of P3ABA against *E. coli* 25922 (0.125%) was lower than that for *E. coli* 25922 *lux* (1%-4%), which was statistically significant (Mann-Whitney test, P value: less than 0.05). The difference in activity of P3ABA observed against *E. coli* 25922 and *E. coli* 25922 *lux* may be reflective of the metabolic burden of light production. The *lux*-tagged *E. coli* 25922 may be less susceptible to the bactericidal action of P3ABA over a 24 h treatment time. Overall, these results support the use of *lux*-tagged *E. coli* for testing of PANI and P3ABA.
Figure 5.1. Activity of PANI against *E. coli* 25922 and *E. coli* 25922 lux. The MIC (circles) and MBC (squares) of PANI against *E. coli* 25922 (EC) and *E. coli* 25922 lux (EC lux) in LB broth. Data obtained from *E. coli* 25922 is represented by filled data points while data obtained from *E. coli* 25922 lux is represented by unfilled data points.

Figure 5.2. Activity of P3ABA against *E. coli* 25922 and *E. coli* 25922 lux. The MIC (circles) and MBC (squares) of P3ABA against *E. coli* 25922 (EC) and *E. coli* 25922 lux (EC lux) in LB broth. Data obtained from *E. coli* 25922 is represented by filled data points while data obtained from *E. coli* 25922 lux is represented by unfilled data points. Statistical significance is represented by * (Mann-Whitney test, P value: less than 0.05).
5.2.2 PANI and P3ABA suspensions are active against *S. aureus* 6538 and *E. coli* 25922 lux in rich media

The sensitivity of *E. coli* 25922 lux and *S. aureus* 6538 growing in LB broth to 0.5% PANI and 0.5% P3ABA suspensions was examined. *S. aureus* 6538 treated with PANI suspension decreased slightly less than 0.5 log over the 4 h treatment time (Fig. 5.3). The levels of *S. aureus* and *E. coli* treated with PANI were comparable at the earlier time points, 0.5 h and 1 h (Fig. 5.3). PANI suspension mediated a moderate knockdown (~ 2 log) of *E. coli* at longer treatment times, 2 h and 4 h (Fig. 5.3). The overall difference in sensitivity between *E. coli* and *S. aureus* to 0.5% PANI suspension was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05).

*S. aureus* was more sensitive to P3ABA suspension than PANI suspension as reflected in the greater knockdown over 4 h (~ 2 log) observed with P3ABA treatment (Fig. 5.4). Similarly, *E. coli* was more sensitive to P3ABA suspension compared to PANI suspension, with the former reducing the inoculum to below the limit of detection within 2 h (Fig. 5.4). P3ABA suspension was much more active against *E. coli* than *S. aureus* at all time points measured (Fig. 5.4). The difference in susceptibility between *E. coli* and *S. aureus* to 0.5% P3ABA suspension was statistically significant (linear regression analysis, intercepts are different, P value: less than 0.05).
Figure 5.3. Sensitivity of *S. aureus* 6538 and *E. coli* 25922 *lux* to PANI suspension. Cell viability assay of ~10^6 CFU/ml *S. aureus* 6538 and *E. coli* 25922 *lux* treated with 0.5% PANI suspension in LB broth. Viable cell counts (CFU/ml) were obtained for each strain at 0.5 h, 1 h, 2 h and 4 h time points. The limit of detection is 50 CFU/ml.

Figure 5.4. Sensitivity of *S. aureus* 6538 and *E. coli* 25922 *lux* to P3ABA suspension. Cell viability assay of ~10^6 CFU/ml *S. aureus* 6538 and *E. coli* 25922 *lux* treated with 0.5% P3ABA suspension in LB broth. Viable cell counts (CFU/ml) were obtained for each strain at 0.5 h, 1 h, 2 h and 4 h time points. The data presented are from three independent experiments. The limit of detection is 50 CFU/ml.
5.2.3 PANI and P3ABA suspensions are active against *E. coli* 25922 *lux* in rich media and minimal media

The activity of 0.5% PANI and 0.5% P3ABA suspensions against *E. coli* 25922 *lux* in rich media and minimal media was determined. *E. coli* in LB broth treated with PANI suspension decreased in cell number after 2 h with an overall 1-2 log reduction by 4 h (Fig. 5.5). In contrast to the activity in LB broth, *E. coli* in both types of minimal media was reduced below the limit of detection by the 2 h time point (Fig. 5.5). PANI mediated a greater knockdown of *E. coli* in minimal media with glycerol (~3 log) compared to minimal media with succinate (~1 log) after treatment for 0.5 h (Fig. 5.5). The overall more efficacious activity of PANI suspensions against *E. coli* in minimal media compared to LB broth was statistically significant (linear regression analyses; minimal media with glycerol: intercepts are different, P value: less than 0.05; minimal media with succinate: intercepts are different, P value: less than 0.05).

P3ABA suspension showed greater activity against *E. coli* in LB broth than *E. coli* in minimal media with the former being reduced beyond detection by the 2 h time point (Fig. 5.6). The level of viable *E. coli* in minimal media with glycerol was stable at the 0.5 h and 1 h time points and decreased to just above the limit of detection by 4 h (Fig. 5.6). P3ABA suspension facilitated a ~2 log reduction in *E. coli* in minimal media with succinate by the 4 h time point (Fig. 5.6). The difference in activity of P3ABA against *E. coli* in LB broth and *E. coli* in minimal media with succinate was statistically significant (linear regression analysis, slopes are different, P value: less than 0.05). The sensitivity of *E. coli* in both minimal media was similar at the earlier time points and differed by more than 1 log at the 4 h time point (Fig. 5.6).
Figure 5.5. Sensitivity of *E. coli* 25922 *lux* to PANI suspension in rich and minimal media. Cell viability assay of $\sim 10^6$ CFU/ml *E. coli* 25922 *lux* treated with 0.5% PANI suspension in LB broth, minimal A salts with 0.7% glycerol and minimal A salts with 0.4% succinate. Viable cell counts (CFU/ml) were obtained for experimental sample at 0.5 h, 1 h, 2 h and 4 h time points. The limit of detection is 50 CFU/ml.

Figure 5.6. Sensitivity of *E. coli* 25922 *lux* to P3ABA suspension in rich and minimal media. Cell viability assay of $\sim 10^6$ CFU/ml *E. coli* 25922 *lux* treated with 0.5% P3ABA suspension in LB broth, minimal A salts with 0.7% glycerol and minimal A salts with 0.4% succinate. Viable cell counts (CFU/ml) were obtained for experimental sample at 0.5 h, 1 h, 2 h and 4 h time points. The limit of detection is 50 CFU/ml.

5.3 Activity of absorbent surfaces containing PANI and P3ABA against *E. coli* and *S. aureus*

Following confirmation of the activity of PANI and P3ABA suspensions against *E. coli* 25922 *lux* and *S. aureus* 6538 in LB broth, absorbent surfaces containing several concentrations of PANI and P3ABA were tested for their bactericidal activity. LB agar containing PANI or P3ABA was
used as a model of an absorbent surface. Many antimicrobials are incorporated into materials at 0.1%-5% (w/w)(63). Agar surfaces containing 1% and 2% concentrations of PANI and P3ABA in agar were established. A high concentration of PANI (8% PANI) was also tested because PANI in suspension was less active against *E. coli* 25922 *lux* and *S. aureus* 6538 than P3ABA in suspension (Fig. 5.3; Fig. 5.4). One aim of this work was to determine the concentration of an absorbent surface containing PANI or P3ABA that can mediate knockdown of test bacteria in a short treatment time. Therefore, the 8% PANI concentration was included to allow for a PANI containing absorbent surface to reduce bacterial load in a short exposure time.

To test the activity of agar containing PANI and P3ABA, *E. coli* 25922 *lux* cells were inoculated onto the experimental agar, rescued at a range of time points and recovered in fresh media (section 2.2.5). Each type of absorbent surface was tested for the necessary time to achieve knockdown. Therefore, highly active surfaces, such as 2% P3ABA in agar, were tested only for the shorter treatment times. Survival of the PANI or P3ABA in agar challenge was based on growth of rescued cells following recovery. Growth of *E. coli* 25922 *lux* was assessed by measuring bioluminescence using the VICTOR X Multilabel Plate Reader. Growth of *S. aureus* 6538 was determined measuring OD$_{600}$ using the µQuant™ Microplate Spectrophotometer. It should be noted that a *lux*-tagged *S. aureus* 6538 strain was not available for testing, which necessitated use of OD$_{600}$ as a measure of growth in a 96 well plate. Results presented are from three independent experiments performed on different days.

### 5.3.1 The limit of detection for *E. coli* 25922 *lux* and *S. aureus* 6538 recovered in a 96 well plate is 100 CFU/ml

Determination of the activity of PANI and P3ABA in surfaces was performed in a 96 well plate to facilitate high-throughput testing of many concentrations and treatment times against one inoculum(249). Cells recovered from test surfaces were grown in a 96 well plate, which necessitated elucidation of the limit of detection of *E. coli* 25922 *lux* and *S. aureus* 6538 growing in this manner. The limit of detection a 96 well plate was used to inform further work examining the activity of PANI and P3ABA in surfaces. The limit of detection was examined by determining the lowest number of cells added to 180 µl of LB broth in a 96 well plate that can grow to detectable levels(249). The limit of detection is presented as CFU/ml to relate back to the initial inoculum concentration. The absolute number of cells present in the 180 µl volume in the wells would be roughly 5-fold lower than the CFU/ml value.
After a 16 h incubation of *E. coli* 25922 *lux* in a 96 well plate the wells inoculated with between $\sim 10^9$ down to $\sim 10^3$ CFU/ml released bioluminescence 2 log or more above background light levels indicating bacterial growth (Fig. 5.7). The median bioluminescence level from a starting inoculum of $\sim 10^2$ CFU/ml was 2 log above background levels; however, two points were only slightly above background levels (Fig. 5.7). Therefore, the limit of detection of *E. coli* 25922 *lux* in a 96 well plate was 100 CFU/ml, which would correspond to $\sim 20$ CFU in the well.

After a 16 h incubation of *S. aureus* 6538, the wells inoculated with between $\sim 10^9$ down to $\sim 10^2$ CFU/ml grew well above the threshold set for growth (OD$_{600}$ above 0.05) indicating bacterial growth (Fig. 5.8). The median OD$_{600}$ value from starting inoculum of $\sim 10$ CFU/ml was above the threshold for growth; however, two points were at background levels (Fig. 5.8). Therefore, the limit of detection of *S. aureus* 6538 in 96 well plate was 100 CFU/ml, which would correspond to $\sim 20$ CFU in the well.

Figure 5.7. The limit of detection of *E. coli* 25922 *lux* when grown in a 96 well plate. *E. coli* 25922 *lux* was serially diluted in LB broth from $\sim 10^9$ CFU/ml to $\sim 1$ CFU/ml in a 96 well plate and incubated at 37°C for 16 h. Light release (RLU s$^{-1}$) was measured from each dilution and detectable light levels above 10 RLU s$^{-1}$ indicates growth. The data presented are from four independent experiments.
Figure 5.8. The limit of detection of *S. aureus* 6538 when grown in a 96 well plate. *S. aureus* 6538 was serially diluted in LB broth from ~ 10⁹ CFU/ml to ~ 1 CFU/ml in a 96 well plate and incubated at 37°C for 16 h. OD₆₀₀ was measured from each dilution and OD₆₀₀ readings above 0.05 are considered as growth. The data presented are from four independent experiments.

5.3.2 PANI and P3ABA in agar are active against *E. coli* 25922 lux
Agar containing 8% PANI reduced bioluminescence levels of *E. coli* 25922 lux to background levels within 1 h of treatment whereas 2% PANI required a longer exposure time (2 h) to reduce surface bacterial load (Fig. 5.9). The limit of detection for this assay is ~ 20 CFU (Fig. 5.7). 1% PANI in agar did not mediate a reduction of *E. coli* with bioluminescence levels from these cells being similar to that of untreated cells (Fig. 5.9). Surface incorporated 2% P3ABA reduced *E. coli* surface load to background levels by the first time point, 15 min (Fig. 5.10). 1% P3ABA in agar also mediated knockdown of *E. coli*; however, this required exposure for 1 h (Fig. 5.10). These results demonstrate the extent of the activity against *E. coli* 25922 lux of PANI and P3ABA incorporated into an absorbent surface.
Figure 5.9. Sensitivity of *E. coli* 25922 lux to PANI in agar. ~ $10^4$ CFU of *E. coli* 25922 lux was exposed to 8% PANI, 2% PANI and 1% PANI incorporated into LB agar. Following treatment for 15 min, 30 min, 1 h, 2 h, 4 h and 8 h the cells were rescued by washing the agar surface with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and light release was measured. The vertical axis shows the bioluminescence measurements (relative light units per second, RLU s$^{-1}$) from the recovered cells with each data point representing an independent experiment and the line representing the median. Background luminescence readings are ~ 10 RLU s$^{-1}$.

Figure 5.10. Sensitivity of *E. coli* 25922 lux to P3ABA in agar. ~ $10^4$ CFU of *E. coli* 25922 lux was exposed to 2% P3ABA and 1% P3ABA incorporated into LB agar. Following treatment for 15 min, 30 min, 1 h and 2 h the cells were rescued by washing the agar surface with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s$^{-1}$) from the recovered cells with each data point representing an independent experiment and the line representing the median. Background luminescence readings are 10 RLU s$^{-1}$. 
5.3.3 PANI and P3ABA in agar are active against *S. aureus* 6538

Agar containing PANI was less active against *S. aureus* 6538 than *E. coli* 25922 lux reflecting the trend observed with PANI suspensions (Fig. 5.3, Fig. 5.11). 8% PANI and 2% PANI in agar effectively decontaminated *S. aureus* on a surface following a 4 h exposure and 8 h exposure, respectively (Fig. 5.11). The limit of detection for this assay is ~ 20 CFU (Fig. 5.7). Similar to activity demonstrated against *E. coli*, agar containing 1% PANI did not reduce bacterial load within the time constraints (8 h) of the experiment (Fig. 5.11). The viability of *S. aureus* cells following treatment with 8% PANI in agar for 2 h, 4 h and 8 h, and following treatment with 2% PANI in agar for 8 h was significantly different from that of untreated cells (Friedman test, P value: less than 0.05, Dunn’s multiple comparison test).

Similar to results obtained with *E. coli*, surface incorporated 2% P3ABA mediated knockdown *S. aureus* cells following a 15 min treatment (Fig. 5.12). Agar containing 1% P3ABA was less effective than that containing 2% P3ABA with the former requiring a 4 h exposure to reduce bacterial load (Fig. 5.12). The activity of surface incorporated 2% P3ABA in 15 min and 1% P3ABA in 4 h was statistically significant (Friedman test, P value: less than 0.05, Dunn’s multiple comparison test). These results show the efficacy of PANI and P3ABA incorporated into an absorbent surface in mediating knockdown of *S. aureus* 6538.
Figure 5.11. Sensitivity of *S. aureus* 6538 to PANI in agar. ~10⁴ CFU of *S. aureus* 6538 was exposed to 8% PANI, 2% PANI and 1% PANI incorporated into LB agar. Following treatment for 15 min, 30 min, 1 h, 2 h, 4 h and 8 h the cells were rescued by washing the agar surface with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and optical density at 600nm (OD<sub>600</sub>) was measured. The vertical axis shows the OD<sub>600</sub> measurements from the recovered cells with each data point representing an independent experiment and the line representing the median. OD<sub>600</sub> readings above 0.05 are considered as growth.

Figure 5.12. Sensitivity of *S. aureus* 6538 to P3ABA in agar. ~10⁴ CFU of *S. aureus* 6538 was exposed to 2% P3ABA and 1% P3ABA incorporated into LB agar. Following treatment for 15 min, 30 min, 1 h, 2 h and 4 h the cells were rescued by washing the agar surface with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and OD<sub>600</sub> was measured. The vertical axis shows the OD<sub>600</sub> measurements from the recovered cells with each data point representing an independent experiment and the line representing the median. OD<sub>600</sub> readings above 0.05 are considered as growth.
5.4 Activity of non-absorbent surfaces containing PANI and P3ABA against *E. coli* and *S. aureus*

The activity of PANI and P3ABA incorporated into an absorbent surface against *E. coli* and *S. aureus* was demonstrated. Following from this, the surface antibacterial activity of PANI and P3ABA incorporated into a non-absorbent material was determined using SEBS films containing 5% PANI and 3% P3ABA (63). The concentrations of the additive in these films are within the range typically used for incorporation into surfaces (0.1%-5%) and reflect the greater activity of P3ABA against test organisms compared to PANI as demonstrated in suspension (Fig. 5.3, Fig. 5.4) and in agar (Fig. 5.9-Fig. 5.12) (63). Results presented are from three independent experiments performed on different days.

5.4.1 Activity of PANI and P3ABA films against *E. coli* 25922 lux and *S. aureus* 6538

PANI and P3ABA films were tested in a micro-JIS assay in which 10 µl of inoculum in LB broth is placed between two pieces of film and recovered at particular time points in fresh LB broth in a 96 well plate (section 2.2.6) (53, 250). Cell viability was determined by measuring bioluminescence for *E. coli* 25922 lux and OD_{600} for *S. aureus* 6538.

5.4.1.1 P3ABA films are active while PANI films are not active against *E. coli* 25922 lux in rich media

The protocol for determining the activity of 5% PANI and 3% P3ABA films against bacteria in rich media (LB broth) was optimised using *E. coli* 25922 lux. Following contact with PANI and P3ABA films for 2 h and 24 h, the *E. coli* cells were rescued by washing with LB broth. A 100 µl aliquot of the recovery broth was transferred to a 96 well plate for 16 h incubation after which bioluminescence was measured. The bacteria present in the remaining recovery broth were enumerated using plate counts to verify the ability to bioluminescence levels to infer cell number.

The exposure of *E. coli* 25922 lux with PANI and P3ABA films for 2 h did not reduce cell numbers as indicated by plate counts and bioluminescence levels (Fig. 5.13). PANI films were also not active during a 24 h exposure as the viable cell counts and bioluminescence levels were comparable to the untreated cells (Fig. 5.13). P3ABA films demonstrated activity against *E. coli* following treatment for 24 h. These treated cells were reduced by 2 logs and released...
~ 2 log less light than untreated cells (Fig. 5.13). The activity of 3% P3ABA films against *E. coli* after 24 h treatment was statistically significant (2-way RM ANOVA; CFU/ml P value: less than 0.05; RLU s⁻¹ P value: less than 0.05). The similarity in trends seen between the plate counts and bioluminescence measurements from *E. coli* 25922 *lux* treated with PANI and P3ABA films confirmed that the bioluminescence-based experimental approach to determining activity of a non-absorbent surface was appropriate to use for further testing.
Figure 5.13. Sensitivity of \textit{E. coli} 25922 \textit{lux} to PANI and P3ABA films. \(\sim 10^4\) CFU of \textit{E. coli} 25922 \textit{lux} in 10 \(\mu\)l LB broth was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h and 24 h. The cells were rescued by washing the film samples with LB broth. The rescued cells were enumerated with plate counts and were also transferred to a 96 well plate. The cells in the 96 well plate were incubated at 37°C for 16 h and light release was measured. The vertical axis shows the viable cell counts (A) and bioluminescence measurements (RLU s\(^{-1}\)) (B) from the recovered cells with each data point representing an independent experiment and the line representing the median. The limit of detection for the plate counts is 50 CFU/ml. Background luminescence readings are 10 RLU s\(^{-1}\).

5.4.1.2 PANI and P3ABA films are not active against \textit{S. aureus} 6538 in rich media

Results from treating \textit{E. coli} 25922 \textit{lux} with PANI and P3ABA films using the optimised protocol demonstrated that growing rescued cells in a 96 well plate and detecting overall growth is a valid means of establishing activity of the films. Following from this, the activity of 5\% PANI in films and 3\% P3ABA in films against \textit{S. aureus} 6538 was determined with an increase in OD\(_{600}\).
above 0.05 indicating the presence of viable cells. *S. aureus* 6538 was treated for only 24 h as PANI and P3ABA films were not active against *E. coli* 25922 lux following 2 h treatments (Fig. 5.13). Both 5% PANI in films and 3% P3ABA in films displayed no activity against *S. aureus* 6538 in LB broth as indicated by the similarity of the OD$_{600}$ values from the treated and untreated cells (Fig. 5.14).

**Figure 5.14. Sensitivity of *S. aureus* 6538 to PANI and P3ABA films.** ~ 10$^4$ CFU of *S. aureus* 6538 in 10 µl LB broth was sandwiched between two pieces of PANI film, P3ABA film or control film for 24 h. The cells were rescued by washing the film samples with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and OD$_{600}$ was measured. The vertical axis shows the OD$_{600}$ measurements from the recovered cells with each data point representing an independent experiment and the line representing the median. OD$_{600}$ readings above 0.05 are considered as growth.

### 5.4.2 Characterisation of the action of PANI and P3ABA films against *E. coli* and *S. aureus*

Following examination of the activity of 5% PANI and 3% P3ABA films against *E. coli* and *S. aureus*, in standard testing conditions, the activity of these films was characterised in conditions more similar to those experienced in potential applications, such as in hospital patient rooms (391). PANI and P3ABA films were challenged with a range of concentrations of *E. coli* 25922 lux and *S. aureus* 6538 in saline, modelling the varied challenges a surface might be exposed to. The bacterial cells were washed with 0.85% saline so that testing was done in a low nutrient environment. *E. coli* and *S. aureus* were exposed to PANI and P3ABA films for 2 h, which is a more useful contact time than 24 h. The shorter contact time of 2 h is more pertinent to applications in hospitals and food processing plants involving disruption of
transmission pathways. Challenging the films with a range of CFU doses also allowed for elucidation of the maximum dose of *E. coli* and *S. aureus* that the films were able to clear. The influence of the presence of organic matter was examined by challenging PANI and P3ABA films with *E. coli* 25922 lux washed in LB broth or in 0.85% saline for 2 h. Results presented are from three independent experiments performed on different days.

### 5.4.2.1 PANI and P3ABA films are active against a range of CFU doses of *E. coli* 25922 lux in saline

Bioluminescence levels from all CFU doses (~ $10^4$, ~ $10^3$, ~ $10^2$, ~ $10$) of *E. coli* 25922 lux that were treated with 5% PANI in films were below background levels whereas bioluminescence from untreated cells was above $10^3$ RLU s$^{-1}$ (Fig. 5.15). 3% P3ABA in films had the same efficacy as 5% PANI in films with the exception of ~ $10^4$ CFU and ~ $10^3$ CFU during one experimental run (Fig. 5.15). All CFU doses tested were significantly different from the control film for both PANI and P3ABA in films (2-way RM ANOVA, interaction of film type and CFU dose P value: less than 0.05).

![Figure 5.15. Activity of PANI and P3ABA films against a range of CFU doses of *E. coli* 25922 lux.](image)

~ 10 CFU - ~ $10^4$ CFU of *E. coli* 25922 lux in 10 µl 0.85% saline was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h. The cells were rescued by washing the film samples with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s$^{-1}$) from the recovered cells with each data point representing an independent experiment and the line representing the median. Background luminescence readings are 10 RLU s$^{-1}$. 

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5.4.2.2 PANI and P3ABA films are active against low CFU doses of *S. aureus* 6538 in saline

OD$_{600}$ values from $\sim 10^4$ CFU and $\sim 10^3$ CFU of *S. aureus* 6538 treated with 5% PANI and 3% P3ABA films were above the threshold for growth, OD$_{600}$ of 0.05, indicating that the films were not active against these higher CFU doses (Fig. 5.16). The $\sim 10^2$ CFU and $\sim 10$ CFU doses of *S. aureus* were below the threshold for growth (Fig. 5.16). 3% P3ABA films only mediated knockdown of $\sim 10$ CFU of *S. aureus* (Fig. 5.16). Activity of PANI films against $\sim 10^2$ and $\sim 10$ CFU, and P3ABA films against $\sim 10$ CFU was statistically significant (2-way RM ANOVA, film type P value: less than 0.05, CFU dose P value: less than 0.05). It can be concluded that PANI and P3ABA films are active against low inocula of *S. aureus* in saline.

![Graph showing OD$_{600}$ values for PANI, P3ABA, and control films for different CFU doses.](image)

**Figure 5.16. Activity of PANI and P3ABA films against a range of CFU doses of *S. aureus* 6538.** ~ 10 CFU - ~ $10^4$ CFU of *S. aureus* 6538 in 10 µl 0.85% saline was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h. The cells were rescued by washing the film samples with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and optical density at 600nm (OD$_{600}$) was measured. The vertical axis shows the OD$_{600}$ measurements from the recovered cells with each data point representing an independent experiment and the line representing the median. OD$_{600}$ readings above 0.05 are considered as growth.

5.4.2.3 The activity of PANI and P3ABA films against *E. coli* 25922 lux is influenced by the presence of organic matter

To test the effect of organic matter on the efficacy of an antimicrobial surface containing PANI or P3ABA, PANI in film and P3ABA in film were challenged with *E. coli* 25922 lux in LB broth and 0.85% saline. Bioluminescence levels from *E. coli* 25922 lux treated with 5% PANI films in 0.85% saline were below background levels whereas *E. coli* 25922 lux in LB broth released the same amount of light as the untreated cells (Fig. 5.17). A similar result was found for P3ABA films,
with the *E. coli* in LB broth releasing light at the same level as untreated cells while bioluminescence from the cells in saline was below background levels (Fig. 5.17). This indicates that *E. coli* in saline was much more sensitive to PANI and P3ABA films than *E. coli* in LB broth. The constituents of LB broth interfere with the contact killing of *E. coli* on films containing PANI and P3ABA.

![Figure 5.17. Activity of PANI and P3ABA films against *E. coli* 25922 lux in the presence and absence of organic matter.](image)

~10^4 CFU of *E. coli* 25922 lux in 10 µl LB broth or 10 µl 0.85% saline was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h. The cells were rescued by washing the film samples with LB broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 16 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s^{-1}) from the recovered cells with each data point representing an independent experiment and the line representing the median. Background luminescence readings are ~10 RLU s^{-1}.

### 5.5 Discussion

The antimicrobial activity of PANI and P3ABA against *E. coli* and *S. aureus* was investigated in suspension, in agar and in films. Overall, *E. coli* had greater susceptibility to both PANI and P3ABA compared to *S. aureus*. Following a 4 h treatment with 0.5% PANI in suspension *E. coli* was knocked-down to a greater extent – 2 log lower – than *S. aureus* (Fig. 5.3). Similarly, 0.5% P3ABA in suspension reduced *E. coli* 4 log more than *S. aureus* after a 2 h exposure (Fig. 5.4). From this it is also apparent that there is a bigger difference in susceptibility between *E. coli* and *S. aureus* for P3ABA compared to PANI. 8% PANI in agar required a 1 h exposure to knock *E. coli* down to background levels (Fig. 5.9) while a 4 h treatment was needed to achieve
the same for *S. aureus* (Fig. 5.11). The time required to reduce *S. aureus* on 1% P3ABA in agar (4 h, Fig. 5.12) was more than what was need to kill *E. coli* (1 h, Fig. 5.10). 3% P3ABA in films after a 24 h treatment reduced *E. coli* (Fig. 5.13) by ~2 log while *S. aureus* was unaffected (Fig. 5.14). These results demonstrate that PANI and P3ABA are active against the model Gram-negative and Gram-positive bacteria, *E. coli* and *S. aureus*, respectively, in suspension and in different types of surfaces. The differing levels of activity observed against *E. coli* and *S. aureus* highlight how a broad spectrum antimicrobial agent may be more or less effective against a range of bacteria.

The sensitivity of *E. coli* to PANI and P3ABA in suspensions in rich and minimal media were determined. *E. coli* was more susceptible to the antimicrobial action of 0.5% PANI in suspension when incubated in minimal media compared to LB broth, with a 4 log reduction observed in the former and a 2 log decrease observed in the latter (Fig. 5.5). The greater sensitivity to PANI of *E. coli* in minimal media is consistent with the lower MIC and MBC values of PANI (section 4.2.1) in minimal media against *E. coli* (0.015625%-0.0625% PANI) compared to those obtained in LB broth (0.25% PANI). It has been postulated that the metabolic burden of *E. coli* growing in minimal media would undermine the cell’s ability to mount an effective oxidative stress response against PANI action. The more efficacious activity of PANI in a low nutrient environment supports the incorporation of this antimicrobial agent in surfaces for applications that are associated with only minor contamination with organic matter.

The antimicrobial activity of P3ABA demonstrates a different trend on rich and minimal media compared to PANI with greater P3ABA action observed in rich media. 0.5% P3ABA in LB broth was able to reduce *E. coli* by 4 log following a 2 h treatment while *E. coli* in minimal media was reduced only by 2 log (Fig. 5.6). The greater sensitivity of *E. coli* to P3ABA in rich media is consistent with the lower MIC and MBC values demonstrated for P3ABA (section 4.2.1) against *E. coli* in LB broth (0.0625-0.125% P3ABA) compared to those obtained in minimal media (0.25%-2% P3ABA). It was hypothesised that *E. coli* cells treated in minimal media would be more likely to be inhibited by P3ABA (leading to suppression of respiration and protection against killing) while cells in rich media would be sufficiently active to be susceptible to P3ABA bactericidal action. P3ABA antimicrobial action is postulated to involve uncoupling of respiration-driven ATP synthesis causing increased respiration, loss of the proton gradient and futile cycling eventuating in cell death. The greater activity of P3ABA in the
presence of nutrients that facilitate bacterial cell growth supports the use of P3ABA in surfaces in settings that are associated with contamination of organic matter, such as surfaces in the vicinity of patients with gastrointestinal infections, which are commonly contaminated with faecal matter containing the bacteria.

Overall, both PANI and P3ABA are most active in suspension followed by in agar and in films. 0.5% PANI in suspension (Fig. 5.5) mediated a 2 log reduction of E. coli in 4 h while 1% PANI in agar (Fig. 5.9) did not decrease the E. coli burden after an 8 h exposure. Thus, PANI in suspension demonstrated greater activity despite the higher concentration of PANI in agar. For surface incorporated PANI to achieve comparable activity to PANI in suspension a higher concentration is required. For example, 2% PANI in agar (Fig. 5.9) achieved total knockdown of E. coli after a 4 h treatment. The reduction in activity of surface incorporated PANI and P3ABA is reflective of how immobilisation in a surface can affect bactericidal activity and how different surface matrixes may influence this in different ways(53). It is believed that PANI and P3ABA exert antimicrobial action following contact with a bacterial cell. Thus, the reduced contact that occurs between a bacterial cell and surface incorporated PANI and P3ABA (relative to in suspension) would mediate the decrease in antimicrobial efficacy. The least amount of contact between the antimicrobial agent and a bacterial cell would occur for non-absorbent surfaces, which mirrors the decreased activity observed for PANI and P3ABA in films(390). 2% PANI and 2% P3ABA in agar (Fig. 5.11; Fig. 5.12) were able to mediate knockdown of S. aureus in 8 h and 15 min, respectively, while 5% PANI and 3% P3ABA in films were unable to reduce bacterial cell numbers after a 24 h treatment (Fig. 5.14). In this example, higher concentration and treatment time did not ameliorate the reduction of activity for polymers incorporated into a non-absorbent surface. The results of this work demonstrate why it is important to test antimicrobial agents first in suspension (associated with quick and reproducible testing) before testing as part of a surface, which should reflect the final application(401).

The effect of the size of the inoculum on the activity of films containing PANI and P3ABA was investigated. In real world settings antimicrobial surfaces may be challenged with a range of inocula. It is apparent in susceptibility testing of antimicrobial agents that the size of the inoculum used can influence the level of antimicrobial activity(214). High inocula can lead to an increase in the MIC while low inocula can cause reduced MIC values(214). In general, higher
inocula need a higher concentration of antimicrobial agent and/or a longer treatment time to achieve knockdown while the opposite is true for lower inocula (403). The decreased surface activity in the presence of high numbers of bacteria may be mediated by the piling of bacterial cells on top of each other, thereby reducing direct contact with the antimicrobial agent for a portion of the population (133). In line with this trend, the surface activity of films containing PANI and P3ABA were affected by the size of the bacterial challenge. 5% PANI in films was able to knockdown inocula containing 10 CFU and $10^2$ CFU of *S. aureus* but not the higher inocula containing $10^3$ CFU and $10^4$ CFU (Fig. 5.16). Similarly, 3% P3ABA in films were only able to clear 10 CFU of *S. aureus* and not the higher inocula tested (Fig. 5.16). These results demonstrate the necessity to perform antimicrobial surface testing with appropriate inocula to simulate the potential challenges that would occur in the real world application. Surfaces in hospitals are considered to be contaminated when aerobic colony counts exceed 2.5 CFU/cm$^2$; however, sampling of objects in patient hospital rooms has demonstrated contamination with a range of bacterial loads (up to $10^4$ CFU/m$^2$, equivalent to $10^2$ CFU/cm$^2$) including $10^3$ CFU/m$^2$ (equivalent to 10 CFU/cm$^2$) of MRSA on door handles (70, 143, 404, 405). Therefore, antimicrobial surfaces in hospitals would need to be active against up to $10^4$ CFU/m$^2$ of contaminants to prevent bacterial spread.

The presence of organic matter can affect the activity of antimicrobial surfaces and thus was investigated for films containing PANI and P3ABA (65). 5% PANI in film and 3% P3ABA in film eliminated surface contamination of *E. coli* in saline following a 2 h treatment while *E. coli* in the presence of organic matter was not reduced in number (Fig. 5.17). Organic matter can interfere with contact between the bacterial cell and the antimicrobial agent – particularly for charged proteins and polysaccharides that can disrupt charge based interactions – thus providing protection from antimicrobial action (42, 53, 89). Additionally, contaminating organic matter may inactivate antimicrobial agents (89). Typical organic contaminants on hospital surfaces include blood and faecal matter (65, 401). It is important that antimicrobial surfaces are tested in conditions, including contamination with organic matter, relevant to the application to verify that the surfaces will be sufficiently active in these settings (65).
5.6 Conclusion

PANI and P3ABA both demonstrated bactericidal activity against *E. coli* and *S. aureus* in suspension and as part of an absorbent surface, with greater activity observed with the former. PANI in films was not active against *E. coli* or *S. aureus* while P3ABA in films reduced the viability of *E. coli*. The results presented in this chapter support the use of PANI and P3ABA to create contamination resistant surfaces.
Chapter 6: Antimycobacterial Action of Surface Incorporated PANI and P3ABA

6.1 Introduction

PANI and P3ABA incorporated into both absorbent and non-absorbent surfaces are active to varying degrees against model Gram-negative and Gram-positive bacteria, *E. coli* and *S. aureus* respectively (section 5.3; section 5.4). This activity has been characterised in terms of treatment times, CFU doses that can be cleared and effect of the presence of organic matter. To determine how well this characterisation of PANI and P3ABA surfaces translates into a potential real-world application, the activity of these surfaces against mycobacteria, particularly the highly important pathogen *M. tuberculosis*, was determined. The aim was to test whether PANI and P3ABA surfaces may reduce the survival of mycobacteria on surfaces, which could be utilised to improve infection control.

*M. tuberculosis* was selected for investigation of its sensitivity to PANI and P3ABA surfaces as a means of interrupting transmission because despite concerted efforts from the World Health Organisation (WHO) over the last 20 years, tuberculosis (TB) remains a major worldwide threat to public health(406). Tuberculosis ranks alongside human immunodeficiency virus (HIV) as a leading cause of death worldwide(406). The WHO estimated that in 2014 9.6 million people contracted and 1.5 million people died from TB(406). The burden of TB has been decreasing at an average rate of 1.5% per year since 2000; however, this improvement is being undermined by antimicrobial resistance(406). It is estimated that 3.3% of new TB cases and 20% of previously treated cases are due to multidrug resistant TB (MDR-TB), which accounted for 480,000 new cases and 190,000 deaths in 2014(406). MDR-TB is resistant to both front-line drugs isoniazid and rifampin and is associated with decreased treatment success rates (from more than 85% for susceptible TB to ~ 50% for resistant TB)(406, 407). Extensively drug-resistant TB (XDR-TB) is MDR-TB that is resistant to at least one fluoroquinolone and a second-line injectable drug(406). By the end of 2015, 105 countries had reported cases of
XDR-TB(406). Emergence of MDR-TB and XDR-TB in both patients who have been previously treated and new untreated TB patients suggests that this is occurring due to both antibiotic exposure in the patient and from transmission of drug resistant TB.

Improved TB infection control is essential to curtail the development and spread of MDR-TB and XDR-TB(407). One means of improving infection control is the utilisation of anti-tubercular surfaces. More than 95% of TB cases and deaths occur in low- and middle-income countries, notably in South-East Asia and African regions(406). In these countries, healthcare facilities lack the resources to adequately manage infectious patients or reliably supply personal protective equipment, such as facemasks, to staff to reduce nosocomial transmission of the disease.

Availability issues with facemasks results in healthcare workers undertaking non-standardised practises, such as reuse of facemasks, despite their potential role in TB transmission(408–411). Consequently, healthcare workers (HCWs) in resource-limited settings are at an increased risk of becoming infected with TB. A South African study found that HCWs had 6-fold and 7-fold greater chances of contracting MDR-TB and XDR-TB, respectively, compared to the general population(412). Facemasks impregnated with an antimicrobial agent would add an extra level of protection against the spread of tuberculosis and are a cost effective strategy as the facemasks would not need to be replaced as frequently.

Characterisation of the action of PANI and P3ABA incorporated surfaces against *M. tuberculosis* may also result in evidence towards elucidating antimicrobial mechanisms. Determination of the response of *E. coli* deletion mutants to P3ABA led to the hypothesis that P3ABA may mediate its antimicrobial activity through acting on respiratory machinery, possibly as an uncoupler. Bedaquiline is a promising new antibiotic to which both susceptible and MDR-TB are sensitive(413). Bedaquiline has a novel mechanism of action – it uncouples respiration-driven ATP synthesis leading to futile proton cycling(226). Uncoupling activity is initiated by binding to the a-c subunit interface of the ATP synthesis machinery(226). The c subunit of ATP synthase is encoded by *atpE*, homologous to that in *E. coli*(226). Mutations in the *atpE* gene are associated with resistance to bedaquiline(413). The reduced sensitivity of the *E. coli ΔatpE* deletion mutant to P3ABA suggests that P3ABA may act in a similar manner (Fig. 3.10, section 3.2.5). Based on this, it was hypothesised that *M. tuberculosis* would be susceptible to the antimicrobial action of P3ABA.
The manner in which *M. tuberculosis* coordinates its response to oxidative stress differs from that of *E. coli* and even *Mycobacterium smegmatis*(414, 415). In *E. coli*, OxyR detects the presence of hydrogen peroxide and mediates the expression of genes in the OxyR regulon(414). Genes in this regulon (such as *katG*, *ahpCF* and *fur*) encode proteins functioning to mitigate this stress (such as hydrogen peroxide scavengers and proteins involved in iron homeostasis). *M. smegmatis* has been shown to have an inducible protective oxidative stress response, which is regulated by an as yet undetermined regulator(414, 415). The *ahpC* gene in *M. smegmatis* is under the control of a separate promoter and has been detected during normal growth and in response to peroxide treatment(415).

In contrast to this, *M. tuberculosis* does not have an inducible oxidative stress response and only has a single catalase enzyme (KatG) encoded by the *katG* gene(416). The *M. tuberculosis* equivalent of OxyR (central regulator of the response to oxidative and nitrosative stress) was found to be a pseudogene that has multiple aberrations in its coding region rendering it non-functional(414). The *ahpC* gene encoding a subunit of alkyl hydroperoxide reductase (a peroxide scavenger) is expressed under the control of OxyR and is undetectable in *M. tuberculosis* cells(417). It is hypothesised that *ahpC* is also actively silenced via repression mechanisms separate to OxyR control(417). This is supported by increased *ahpC* expression in *M. tuberculosis* H37Rv grown *in vitro* in conditions of static growth(417).

The *katG* gene of *M. tuberculosis* is under the control of two promoters resulting in distinct transcripts expressed in response to different conditions(416). The first transcript is controlled by FurA, one of two ferric uptake regulator (Fur) orthologues, and consists of the *katG* gene and the *furA* gene(232, 416). FurA is thought to be an oxidative stress sensor that autoregulates its own expression(418). Upon binding Fe^{2+} FurA is activated and binds DNA, repressing the expression of *katG* and *furA*(418). Oxidation of FurA leads to a conformational change disrupting DNA binding and resulting in derepression of the downstream genes(418). During long-term oxidative stress it can be inferred that *katG* would be expressed at high levels. The second transcript consists only of the *katG* gene and has also been shown to be induced in response to peroxide treatment(416). Therefore, the only protein induced in response to peroxide exposure is KatG. *In vivo* the first transcript is expressed during initial stages of infection while the second transcript is activated following extended growth in
macrophages, a highly oxidative environment(416). AhpC is not involved in establishing infections (as determined in a murine model of TB)(417).

Inactivation of the oxyR gene in *M. tuberculosis* may seem paradoxical given how important protection against ROS – and reactive nitrogen intermediates – is for this bacterium(417). In the host *M. tuberculosis* infects macrophages and persists in granulomatous lesions, which can result in continuous exposure to ROS(232, 414). However, the lack of a strong inducible response (such as that found in *E. coli*) suggests that *M. tuberculosis* doesn’t require new protein products to protect itself against oxidative stress. It has been suggested that defence against oxidative stress in *M. tuberculosis* appears to be constitutive, as KatG is expressed from two promoters during different stages of growth – the effect of which is cumulative – and AhpC is expressed during stasis(414, 416, 419). Defence against damage from peroxides is so crucial to the survival of *M. tuberculosis* that isoniazid-resistant mutants that have non-functional or deleted katG genes accumulate mutations in the promoter of ahpC leading to overexpression of AhpC(420, 421). AhpC is not involved in isoniazid resistance and functions purely to enhance survival in oxidative conditions(420). Indeed, KatG is an important virulence factor – loss of the katG gene attenuates virulence in murine models of tuberculosis(422). It is postulated that PANI exerts antimicrobial activity through induction of oxidative stress based on the reduction in activity observed in anaerobic conditions compared to aerobic conditions (section 4.2). The effective means by which the obligate aerobe *M. tuberculosis* alleviates hydrogen peroxide stress led to the hypothesis that *M. tuberculosis* would not be susceptible to killing by PANI.

The methods used to characterise the activity of absorbent and non-absorbent PANI and P3ABA surfaces were optimised using *E. coli* and *S. aureus*. These methods were applied to *M. smegmatis*, which served as a model of *M. tuberculosis*, and further optimised for testing against mycobacteria. Optimisation of experiments with *M. smegmatis* was performed as *M. tuberculosis* is highly pathogenic and therefore requires more time and safety procedures than the less pathogenic *M. smegmatis*. Following this, the activity of absorbent and non-absorbent PANI and P3ABA surfaces against *M. tuberculosis* was characterised.
6.2 Activity of PANI and P3ABA suspensions against mycobacteria

Initial investigation of the antimycobacterial activity of PANI and P3ABA was performed using *M. smegmatis* MC²155 challenged with PANI and P3ABA suspensions. The antimicrobial efficacy of PANI and P3ABA in suspension and as part of surfaces against *E. coli* and *S. aureus* was examined in chapter 5. A greater susceptibility was observed for treatment with suspensions possibly due to increased contact between the polymer and the bacterial cells. Verification of the activity of PANI and P3ABA in suspension was therefore a logical step preceding elucidation of surface activity.

The MIC and MBC of PANI and P3ABA suspensions against *M. smegmatis* MC²155 were determined. The MIC and MBC values were obtained following a 24 h treatment of *M. smegmatis* cell with a range of concentrations of PANI or P3ABA (section 2.2.8). The MIC was defined as the lowest concentration that can inhibit visible growth while the MBC was determined as the lowest concentration that killed cells (229). Results presented are from three independent experiments performed on different days.

**6.2.1 PANI and P3ABA suspensions are active against *M. smegmatis* MC²155**

For *M. smegmatis* cells challenged with PANI, the MIC was 2% PANI and the MBC was 4% PANI (Fig. 6.1). For *M. smegmatis* cells challenged with P3ABA, the MIC was 0.125% P3ABA and the MBC was 0.25% P3ABA (Fig. 6.1). These results demonstrate that both PANI and P3ABA when incorporated into a suspension are active against *M. smegmatis*. The lower MIC and MBC of P3ABA indicates that *M. smegmatis* is more susceptible to P3ABA than to PANI. This difference in susceptibility may be a reflection of the particular mode by which P3ABA kills bacterial cells. Confirmation of the activity of PANI and P3ABA suspensions against *M. smegmatis* led into investigations of the activity of surfaces containing PANI and P3ABA against mycobacteria.
Figure 6.1. Sensitivity of *M. smegmatis* to PANI and P3ABA suspensions. The MIC and MBC for PANI and P3ABA in suspensions against $10^6$ CFU/ml of *M. smegmatis* MC\textsuperscript{2}155. This strain was challenged with PANI (circle) and P3ABA (square) suspensions in a range of concentrations for 24 h to determine the activity of these suspensions as reflected by the MIC (filled points) and MBC (unfilled points) values. The data presented are from three independent experiments with a line at the median value.

6.3 Activity of absorbent surfaces containing PANI and P3ABA against mycobacteria

PANI and P3ABA in suspension were able to mediate knockdown of *M. smegmatis*. Following this, the susceptibilities of *M. smegmatis* and *M. tuberculosis* to absorbent surfaces containing PANI and P3ABA were determined. 7H11 supplemented agar containing PANI or P3ABA was used as a model of an absorbent surface that would support mycobacterial viability\citep{237,423}. The agar surface decontamination assay established for testing against *E. coli* 25922 \textit{lux} and *S. aureus* 6538 was optimised for testing against mycobacteria using *M. smegmatis* MC\textsuperscript{2}155 \textit{lux}. The assay involved inoculating $\sim 10^4$ CFU of *M. smegmatis* or *M. tuberculosis* onto agar containing PANI or P3ABA in a 96 well plate and rescue of these cells at a range of time points (section 2.2.9)\citep{251}. Rescued cells were incubated in 7H9 supplemented broth in a 96 well plate at 37°C. *M. smegmatis* MC\textsuperscript{2}155 \textit{lux} rescued cells were recovered for 24 h while *M. tuberculosis* cells were recovered for 21 days, reflecting the slower growth rate of pathogenic *M. tuberculosis*\citep{424,425}. Survival of the PANI or P3ABA in agar challenge was based on bioluminescence levels measured using the VICTOR X Multilabel Plate Reader. Results presented are from three independent experiments performed on different days.
6.3.1 The limit of detection for *M. smegmatis* MC\(^2\)155 lux recovered in a 96 well plate is 100 CFU/ml

Determination of the activity of PANI and P3ABA in surfaces was performed in a 96 well plate to facilitate high-throughput testing of many concentrations and treatment times against one inoculum (249). Cells recovered from test surfaces were grown in a 96 well plate, which necessitated elucidation of the limit of detection of *M. smegmatis* lux (and infer for *M. tuberculosis* lux) growing in this manner. The limit of detection in a 96 well plate was used to inform interpretation of experiments examining the activity of PANI and P3ABA in surfaces. The limit of detection was examined by determining the lowest number of cells added to 180 \(\mu\)l of 7H9 supplemented broth in a 96 well plate that can grow to detectable levels (249). The limit of detection is presented as CFU/ml to relate back to the initial inoculum concentration. The absolute number of cells present in the 180 \(\mu\)l volume in the wells would be roughly 5-fold lower than the CFU/ml value.

After a 24 h incubation of *M. smegmatis* MC\(^2\)155 lux in a 96 well plate, the starting inocula of \(\sim 10^9\) to \(\sim 10^2\) CFU/ml released bioluminescence one log or higher above background light levels indicating bacterial growth (Fig. 6.2). The median bioluminescence level from the starting inoculum of \(\sim 10\) CFU/ml was greater than background light levels; however, cells were only detectable in two of four occasions (Fig. 6.2). The limit of detection of *M. smegmatis* MC\(^2\)155 lux grown in 7H9 supplemented broth in 96 well plate was 100 CFU/ml.
Figure 6.2. The limit of detection of \textit{M. smegmatis lux} when grown in a 96 well plate. \textit{M. smegmatis} MC\textsuperscript{2}155 \textit{lux} was serially diluted in 7H9 supplemented broth from $\sim 10^9$ CFU/ml to $\sim 1$ CFU/ml in a 96 well plate and incubated at 37°C. Light release (RLUs$^{-1}$) was measured from each dilution and detectable light levels above 30 RLUs$^{-1}$ indicates growth. The data presented are from four independent experiments.

6.3.2 PANI in agar and P3ABA in agar are active against \textit{M. smegmatis} MC\textsuperscript{2}155 \textit{lux}

Agar containing 10\% and 8\% PANI reduced bioluminescence measurements from \textit{M. smegmatis} to background levels after 30 min and 120 min exposure times, respectively (Fig. 6.3). Knockdown from both 10\% and 8\% PANI treatment at the 120 min time point was significantly different from untreated cells (Friedman test, P value: less than 0.05, Dunn’s multiple comparison post-hoc test).

Surface incorporated 5\% P3ABA, 3.5\% P3ABA and 2\% P3ABA knocked-down bioluminescence from \textit{M. smegmatis lux} cells to that of uninoculated agar following a 15 min exposure (Fig. 6.4). The activity of 3.5\% P3ABA in agar was statistically significant (Friedman test, P value: less than 0.05, Dunn’s multiple comparison post-hoc test). 1\% P3ABA in agar showed variable activity at the earliest time point (15 min) but there was consistent knockdown of \textit{M. smegmatis} cells occurring after a 30 min exposure (Fig. 6.4). The results presented indicated that the protocol was established and reliable for testing absorbent surfaces against mycobacteria.
Figure 6.3. Sensitivity of *M. smegmatis* lux to PANI in agar. ~10^4 CFU of *M. smegmatis* MC^2^155 lux was exposed to 8% PANI and 10% PANI incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 24 h and light release was measured. The vertical axis shows the bioluminescence measurements (relative light units per second, RLU s^-1^) from the recovered cells with each data point representing an independent experiment and the line representing the median.
Figure 6.4. Sensitivity of M. smegmatis lux to P3ABA in agar. ~10^4 CFU of M. smegmatis MC^{155} lux was exposed to 3.5% and 5% P3ABA (A), and 1% and 2% P3ABA (B) incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 24 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s⁻¹) from the recovered cells with each data point representing an independent experiment and the line representing the median.

6.3.3 PANI in agar is not active while P3ABA in agar is active against M. tuberculosis H37Rv lux

Having optimised the protocol for testing PANI and P3ABA in agar against M. smegmatis lux, the activity against M. tuberculosis was determined. Initial testing was performed with the tagged laboratory reference strain M. tuberculosis H37RV lux(222). Both 10% and 8% PANI
incorporated into an absorbent surface did not mediate knockdown of *M. tuberculosis* H37Rv lux after 120 min exposure (Fig. 6.5). This is in contrast to the action of PANI against *M. smegmatis* MC^2^155 lux (Fig. 6.3). Bioluminescence from *M. tuberculosis* H37Rv lux treated with 5% P3ABA, 3.5% P3ABA and 2% P3ABA in agar for 15 min was reduced to background levels (Fig. 6.6). Knockdown of *M. tuberculosis* H37Rv lux by 3.5% P3ABA and 2% P3ABA in agar after 30 min exposure was statistically significant (Friedman test, P value: less than 0.05, Dunn’s multiple comparison post-hoc test). The action of surface bound 1% P3ABA against *M. tuberculosis* H37Rv lux was variable with most cells being killed after 120 min treatment (Fig. 6.6).

![Figure 6.5](image)

**Figure 6.5. Sensitivity of *M. tuberculosis* H37Rv lux to PANI in agar.** ~ 10^4 CFU of *M. tuberculosis* H37Rv lux was exposed to 8% PANI and 10% PANI incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 21 days and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s\(^{-1}\)) from the recovered cells with each data point representing an independent experiment and the line representing the median.
Figure 6.6. Sensitivity of *M. tuberculosis* H37Rv *lux* to P3ABA in agar. \( \sim 10^4 \) CFU of *M. tuberculosis* H37Rv *lux* was exposed to 3.5% and 5% P3ABA (A), and 1% and 2% P3ABA (B) incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 21 days and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s\(^{-1}\)) from the recovered cells with each data point representing an independent experiment and the line representing the median.

6.3.4 PANI in agar is not active while P3ABA in agar is active against *M. tuberculosis* Rangipo N *lux*

Following on from testing *M. tuberculosis* H37Rv *lux*, the activity of PANI and P3ABA in agar was established against a *lux*-tagged New Zealand clinical isolate, *M. tuberculosis* Rangipo N(222, 426). As for *M. tuberculosis* H37Rv *lux*, both 10% and 8% PANI in agar did not reduce
M. tuberculosis Rangipo N lux bioluminescence levels, even after treatment for 120 min (Fig. 6.7). Similar to the action against M. smegmatis MC\textsuperscript{2}155 lux and M. tuberculosis H37Rv lux, 5% P3SBA, 3.5% P3ABA and 2% P3ABA knocked bioluminescence levels from the lux-tagged clinical isolate down to that of the uninoculated agar (Fig. 6.8). The activity of 3.5% P3ABA against M. tuberculosis H37Rv lux after 15 min treatment was statistically significant (Friedman test, P value: less than 0.05, Dunn’s multiple comparison post-hoc test). 1% P3ABA in agar reduced bioluminescence from M. tuberculosis Rangipo N lux cells to background levels after a 30 min treatment (Fig. 6.8).

![Figure 6.7. Sensitivity of M. tuberculosis Rangipo N lux to PANI in agar. ~ 10\textsuperscript{4} CFU of M. tuberculosis Rangipo N lux was exposed to 8% PANI and 10% PANI incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 21 days and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s\textsuperscript{-1}) from the recovered cells with each data point representing an independent experiment and the line representing the median.](image-url)
Figure 6.8. Sensitivity of *M. tuberculosis* Rangipo N *lux* to P3ABA in agar. ~ 10^{4} CFU of *M. tuberculosis* Rangipo N *lux* was exposed to 3.5% and 5% P3ABA (A), and 1% and 2% P3ABA (B) incorporated into 7H11 supplemented agar. Following treatment for 15 min, 30 min and 120 min, the cells were rescued by washing the agar surface with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 21 days and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s^{-1}) from the recovered cells with each data point representing an independent experiment and the line representing the median.

6.4 Activity of non-absorbent PANI and P3ABA surfaces against mycobacteria

P3ABA incorporated into an absorbent surface mediated knockdown of the tested mycobacterial strains with a 15 min exposure. PANI incorporated into an absorbent surface
only reduced the bacterial load of *M. smegmatis* MC²155 *lux* after a 120 min exposure. PANI in agar was not active against both *M. tuberculosis* strains tested within the experimental treatment times. Following from this, activity of PANI and P3ABA incorporated into non-absorbent surfaces (SEBS films) against mycobacteria was determined using the micro-JIS assay that was established for testing with *E. coli* and *S. aureus*. The micro-JIS assay was optimised for testing with mycobacteria using *M. smegmatis* MC²155 *lux* before testing with *M. tuberculosis* H37Rv *lux*. Briefly, 10 µl of inoculum in 7H9 supplemented broth was placed between two pieces of film and recovered at particular time points in fresh 7H9 supplemented broth in a 96 well plate (section 2.2.10). Cell viability was determined by measuring bioluminescence following recovery for 24 h (for *M. smegmatis*) and 14 days (for *M. tuberculosis*). Results presented are from three independent experiments performed on different days.

6.4.1 A 24 h treatment of *M. smegmatis* MC²155 *lux* with P3ABA films mediates knockdown while PANI films have no effect

The activity of 5% PANI films and 3% P3ABA films against mycobacteria was first determined for *M. smegmatis* MC²155 *lux*, as was done for testing of absorbent surfaces. Bioluminescence from *M. smegmatis* MC²155 *lux* treated with 5% PANI films for 24 h was comparable to that of untreated cells (Fig. 6.9). In contrast to this, 3% P3ABA facilitated statistically significant knockdown of *M. smegmatis* to background levels during the 24 h exposure (Kruskal-Wallis test, P value: less than 0.05, Dunn’s multiple comparison test). Results presented are from three independent experiments performed on different days.
Figure 6.9. Sensitivity of *M. smegmatis* MC²155 lux to 5% PANI and 3% P3ABA films. ~ $10^4$ CFU of *M. smegmatis* MC²155 lux in 10 µl 7H9 supplemented broth was sandwiched between two pieces of PANI film, P3ABA film or control film for 24 h. The cells were rescued by washing the film samples with 7H9 broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 24 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s⁻¹) from the recovered cells with each data point representing an independent experiment and the line representing the median.

6.4.2 A 2 h treatment with PANI and P3ABA films does not mediate knockdown of low doses of *M. smegmatis* MC²155 lux

24 h treatment of *M. smegmatis* MC²155 lux growing in 7H9 supplemented broth with P3ABA films reduced bacterial load whereas PANI films demonstrated no activity. To determine the minimum CFU dose that PANI and P3ABA films are able to clear, ~ $10^4$ CFU, ~ $10^3$ CFU, ~ $10^2$ CFU and ~ 10 CFU of *M. smegmatis* MC²155 lux in 0.85% saline was challenged with PANI and P3ABA films for 2 h. The lack of nutrients in saline is representative of a real world setting for an antimicrobial surface. Bioluminescence from all CFU doses of *M. smegmatis* lux tested against both PANI and P3ABA films was similar to that from control film, with the exception of the minor reduction in light levels from ~ $10^3$ CFU treated with PANI films (Fig. 6.10).
Figure 6.10. Activity of PANI and P3ABA against a range of CFU doses of *M. smegmatis* lux in saline. 

~ $10^3$ and ~ $10^4$ CFU (A) and, ~ 10 and ~ $10^2$ CFU (B) of *M. smegmatis* MC’155 lux in 10 µl 0.85% saline was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h. The cells were rescued by washing the film samples with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 24 h and light release was measured. The vertical axis shows the bioluminescence measurements (RLUs$^{-1}$) from the recovered cells with each data point representing an independent experiment and the line representing the median.

6.4.3 PANI in films is not active while P3ABA in films is active against *M. tuberculosis* H37Rv lux following a 24 h treatment

Following from testing PANI and P3ABA films against *M. smegmatis*, the ability of PANI and P3ABA films to reduce surface contamination by *M. tuberculosis* H37RV lux with 2 h and 24 h treatment times was investigated. A 2 h exposure to PANI and P3ABA films mediated a slight
reduction in bioluminescence (less than 1 log) from the treated cells (Fig. 6.11). A 24 h treatment of *M. tuberculosis* H37Rv *lux* with PANI film had a very small effect while P3ABA films mediated knockdown of bioluminescence to below background levels (Fig. 6.11).

![Figure 6.11. Sensitivity of *M. tuberculosis* H37Rv *lux* to 5% PANI and 3% P3ABA films.](image)

**Figure 6.11. Sensitivity of *M. tuberculosis* H37Rv *lux* to 5% PANI and 3% P3ABA films.** ~10⁴ CFU of *M. tuberculosis* H37Rv *lux* in 10 µl 7H9 supplemented broth was sandwiched between two pieces of PANI film, P3ABA film or control film for 2 h and 24 h. The cells were rescued by washing the film samples with 7H9 supplemented broth and transferred to a 96 well plate. The rescued cells were incubated at 37°C for 14 days and light release was measured. The vertical axis shows the bioluminescence measurements (RLU s⁻¹) from the recovered cells with each data point representing an independent experiment and the line representing the median.

### 6.5 Discussion

Work in this chapter focussed on the potential application of PANI and P3ABA surfaces to prevent disease transmission by mediating self-disinfection. The approaches developed in chapter 5 for creating and testing the antimicrobial activity of these surfaces were utilised in this chapter to investigate the antimycobacterial bactericidal activity of PANI and P3ABA surfaces. The shortest period of contact tested between bacteria and the surface was 15 min due to the slow nature of laboratory work with *M. tuberculosis* to ensure adequate safety (425). It is debatable how pertinent a contact time of this length is for disrupting infectious disease transmission in hospital settings (89). It is possible that surface disinfection may occur in less than 15 min although that was not able to be experimentally investigated. Effective antimicrobial activity in shorter contact times requires higher concentrations of PANI and P3ABA. For example, 2% P3ABA in agar required only 15 min to achieve surface
sterilisation of *M. smegmatis* while 1% P3ABA in agar needed twice as much time for this (Fig. 6.4).

*M. smegmatis* and *M. tuberculosis* strains constitutively expressing the *lux* operon were used in this work as a semi-quantitative practical alternative to enumeration by plate counts(53, 425). Mycobacteria, particularly *M. tuberculosis*, grow slowly compared to *E. coli* and *S. aureus*. Enumeration by plate counts is resource intensive and requires a longer incubation time to determine bacterial cell numbers compared to measuring bioluminescence(214, 425). The reduced resource and time requirement for enumeration using bioluminescence facilitates high-throughput testing, which allows for more factors and replicates to be tested against the same culture(249, 425). In order to sustain the bioluminescence reaction, aldehyde substrate and FMNH$_2$ must be present at sufficiently high levels(53). The aldehyde substrate is regenerated from tetradecanoic acid, which is removed from the fatty acid biosynthesis pathway(402). Additionally, synthesis of the proteins in the *lux* operon will require energy and metabolites. Therefore, it stands to reason that *lux*-tagged cells need to be sufficiently energised to produce bioluminescence.

The mycobacterial cultures used in this work were grown and incubated in a rich media, 7H9 supplemented broth, to reduce time required to establish a turbid inoculum and to ensure that the cells would be able to sustain the bioluminescence reaction. Bacteria persisting on surfaces that have no contamination with organic matter would have less energy and be less metabolically active compared to bacteria growing in rich laboratory media, such as 7H9 supplemented broth. Experimental high nutrient conditions used in this work may not reflect the conditions found on surfaces contaminated with bacteria and therefore might not represent surface activity in application conditions. Further testing is required to confirm antimycobacterial activity in low nutrient conditions, which may be achieved by growing cells in minimal media.

*M. smegmatis* was more susceptible to the action of PANI in agar compared to *M. tuberculosis*. *M. smegmatis* MC$^\text{2}155$ *lux* was reduced to background levels following a 2 h treatment with 8% PANI in agar (Fig. 6.3). Both *M. tuberculosis* H37Rv *lux* and *M. tuberculosis* Rangipo N *lux* were not reduced in numbers following a 2 h treatment with 8% PANI in agar (Fig. 6.5; Fig. 6.7). Overall, the mycobacteria were less sensitive to PANI action compared to *E. coli* 25922 *lux*,

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which was completely knocked-down after a 1 h exposure (Fig. 5.9, section 5.3.2). It is hypothesised that PANI action involves induction of hydrogen peroxide based oxidative stress. *M. tuberculosis* is able to successfully withstand oxidative environments to cause infections (427–429). Mechanisms underlying this reduced sensitivity to oxidative stress include control of *katG* expression by two regulators, constitutive expression of SodA that increases in response to oxidative stress, linking of AhpC peroxidase activity to the cellular metabolic state, and altering metabolism and redox balance (416, 428). *M. tuberculosis* is unique in that it has redox buffers, mycothiol and ergothioneine, with overlapping functions, which permit the cell to protect itself against a wide range of free radicals (419, 428). *M. smegmatis* is an environmental, non-pathogenic mycobacterium that is not exposed to highly oxidative environments that *M. tuberculosis* is privy to (428). For this reason, *M. smegmatis* is not adapted to such an environment and is more susceptible to oxidative stress (428, 429). For example, knockout of both methionine sulfoxide reductases (MsrA and MsrB), which function to repair oxidation of methionine in *M. tuberculosis* did not alter susceptibility to hydrogen peroxide (430). In contrast to this, knockout of only MsrA in *M. smegmatis* increased sensitivity to ROS (431). Additionally, mycobacteria have a thick cell envelope made of lipoarabinomannan and phenolic glycolipid I, which are oxygen radical scavengers that add a level of protection against oxidative stress (414, 428). *M. tuberculosis* cyclopropanates cell wall mycolic acids conferring resistance to lipid peroxidation while the cell wall of *M. smegmatis* contains oxygen-sensitive olefinic bonds (414, 432). The reduced sensitivity of *M. smegmatis* and the lack of sensitivity of *M. tuberculosis* supports the hypothesis that the mode of action of PANI involves induction of oxidative stress.

Overall, mycobacteria are more susceptible to P3ABA action than *E. coli* or *S. aureus* (chapter 5). 1% P3ABA in agar was able to mediate knockdown of *M. smegmatis MC²155 lux* (Fig. 6.4) and *M. tuberculosis* Rangipo N *lux* (Fig. 6.8) within a 30 min exposure whereas 1 h and 4 h treatments were required for knockdown of *E. coli* 25922 *lux* (Fig. 5.10, section 5.3.2) and *S. aureus* 6538 (Fig. 5.12, section 5.3.3), respectively. The greater susceptibility of *M. smegmatis* and *M. tuberculosis* to P3ABA may reflect the mode of action. P3ABA is hypothesised to target metabolic and respiratory machinery, including ATP synthase, based on results obtained in chapters 3 and 4. Unlike *E. coli* and many other bacteria, mycobacteria cannot grow in the absence of oxidative phosphorylation as the ATP made through substrate
level phosphorylation is insufficient to sustain growth of bacteria (433, 434). The essentiality of electron transport and ATP synthase function in mycobacteria results in these bacteria being highly sensitive to antimicrobial agents that target respiratory machinery. Therefore, putative targeting of oxidative phosphorylation by P3ABA would explain the increased sensitivity of *M. smegmatis* and *M. tuberculosis* to P3ABA. P3ABA in surfaces demonstrated greater activity against mycobacteria than PANI in surfaces. For example, 10% PANI in agar was unable to reduce the numbers of *M. tuberculosis* H37Rv lux cells following a 2 h treatment (Fig. 6.5) while 2% P3ABA in agar reduced cell number to background levels after only a 15 min treatment (Fig. 6.6). A 24 h exposure to 5% PANI in film was unable to reduce the load of *M. smegmatis* MC²155 lux while 3% P3ABA in film was able to mediate complete knockdown (Fig. 6.9). The sensitivity of *M. tuberculosis* to P3ABA containing surfaces supports the use of such surfaces in hospitals to prevent the spread of infection.

PANI and P3ABA in absorbent surfaces had greater activity than when incorporated into non-absorbent surfaces against mycobacteria. The same trend was demonstrated for activity against *E. coli* and *S. aureus* (section 5.3; section 5.4). For example, 2% P3ABA in agar was able to knockdown *M. tuberculosis* H37Rv lux with a 15 min exposure time (Fig. 6.6) while 3% P3ABA in film demonstrated no activity following a 2 h treatment (Fig. 6.11). The differences seen in the activity of PANI and P3ABA incorporated into absorbent and non-absorbent surfaces is likely due to the properties of the surface matrix (53). Absorbent surfaces made of agar allow for the absorption of the drop of liquid inoculum into the surface facilitating more contact between the bacteria and the antimicrobial agent. On non-absorbent surfaces made of SEBS films the drop of liquid inoculum sits on the surface resulting in less contact between the bacteria and the active agent. PANI and P3ABA require contact with bacteria to exert antimicrobial activity so the greater contact that occurs with absorbent surfaces would facilitate the greater activity of these surfaces. For non-absorbent surfaces a higher concentration of the antimicrobial agent or a longer contact time is needed, as demonstrated by the bactericidal activity of 3% P3ABA in films against *M. tuberculosis* H37Rv lux in a 24 h treatment compared to the absence of bacterial knockdown in a 2 h treatment (Fig. 6.11). The efficacious antimycobacterial activity of P3ABA in agar against *M. smegmatis* and *M. tuberculosis* supports the use of P3ABA impregnated surfaces to reduce bacterial surface contamination in hospital settings.
P3ABA incorporated into an absorbent surface was shown to be very active against *M. tuberculosis* in the shortest time frame tested (15 min) including a clinical isolate, *M. tuberculosis* Rangipo *N lux* (Fig. 6.8). This time interval was the shortest that could be tested due to the time constraints involved in the local Standard Operating Procedures for *M. tuberculosis* laboratory work. It is possible that P3ABA can decontaminate mycobacteria laden surfaces in less than a 15 min exposure. The results presented support the incorporation of P3ABA into materials to create anti-tubercular surfaces.

HCWs in low- and middle-income countries have an increased risk of TB infection due to contact with patients and lack of proper room ventilation (435, 436). *M. tuberculosis* is transmitted via infectious aerosols expelled by an infected person, which desiccate forming small droplet nuclei that can remain suspended in the air for days after expulsion and can travel substantial distances from the patient (435, 437–439). The aerodynamic characteristics of droplet nuclei means that they are readily inhaled and deposited in lung alveoli to establish infection (435, 436, 438). Aerosols produced from patients with pulmonary TB following 10 min of coughing were demonstrated to contain a median 16 CFU per droplet (range 1-701 CFU) (438). TB bacilli can remain viable for several days to months post-expulsion (400, 439). The infectious dose for *M. tuberculosis* has been estimated at less than 10 bacilli although this is influenced by biological factors such as virulence of the bacteria and susceptibility of the person (440). The contamination of environmental surfaces that occurs following expulsion of aerosols from an infected person combined with the persistence of *M. tuberculosis* in the air and on surfaces supports use of an antimicrobial surface to reduce surface contamination and decrease the risk of TB transmission in a hospital setting.

The WHO recommends that HCWs in high incidence TB areas wear respirators to protect against TB transmission (406). More than 50% of new MDR-TB cases occur within hospitals and communities among people that haven’t been previously treated for TB, which highlights the lack of adequate infection control measures in high MDR-TB incidence areas (407, 441, 442). Improved TB infection control is essential to curtail the development and spread of drug resistance (407). There are not many studies that address the efficacy of infection control measures on reducing transmission; however, there is limited evidence which suggests that the incidence of TB infection decreases after execution of control measures (443).
Respirators are intended to protect the wearer from inhaling infectious particles by filtering small infectious droplets (435, 437). This type of PPE is effective at preventing inhalation of infectious particles; however, the cost of the product renders respirators unobtainable for healthcare facilities in LMICs, which decreases the level of protection offered (407, 435). Efforts to curtail the spread of MDR-TB is hampered by insufficient funding (406). The funding gaps associated with a full response to the global TB epidemic in LMICs has been steadily increasing, amounting to US$ 1.4 billion in 2015 (406).

Facemasks are worn over nose and mouth to establish a barrier between the respiratory tract and splashes and droplets in the external environment (435, 437). Facemasks offer less protection against M. tuberculosis transmission as they have a lower filtration efficiency and a higher degree of face seal leakage than respirators (435, 437, 444). In a resource-limited setting where there are few/no respirators available, wearing of facemasks may help decrease transmission even if each individual HCW is not completely protected (407, 436, 444). Use of a facemask that reduces inhalation of particles by 50% has been estimated to give the same level of protection as a doubling of room ventilation, but at a much lower cost (407). The greater affordability of facemasks is associated with fewer availability issues than respirators (407).

Facemasks provide important protection in resource-limited settings where recommended infection control measures are not feasible. However, healthcare facilities in these areas are not able to reliably provide HCWs with sterile facemasks, increasing the risk of TB transmission in high bacterial load situations, such as an infectious patient in a poorly ventilated room (445–447). Used contaminated facemasks can be a source of infection either due to penetration of particles through the facemask into the respiratory tract of the wearer or release of aerosols back into the air (445, 446). Used facemasks isolated from a hospital have been shown to have bacterial contamination on the outside of the mask (445). Bacterial counts as high as 166 CFU (per ml of recovery broth) have been isolated from the outside of the used facemasks worn by healthcare workers in a hospital (445). M. abscessus, used as a surrogate for M. tuberculosis, is able to survive on respiratory PPE for 5 days and can be transferred to gloves during handling (446). Decontamination of facemasks by HCWs may also cause reaerosolisation of TB bacilli (447). A facemask impregnated with an antimicrobial agent would ameliorate the risk of TB transmission associated with these non-recommended practises.
Bacteria that come into contact with the facemask surface would be killed, facilitating safer reuse and disposal as well as eliminating the need to decontaminate (448).

There are several commercially available decontaminating facemasks; however, there is limited published work on the efficacy of these facemasks. Two notable examples include silver-based facemasks and quaternary ammonium compound (QAC) based facemasks (449, 450). The cell wall of *M. tuberculosis* is protective against penetration of silver nanoparticles into the cytoplasm as disruption with chloroform was required for inhibitory activity (451). *Mycobacterium avium*, a pathogenic mycobacterium, was reduced slightly in number after a 24-48 h treatment with silver nanoparticles, which suggests that the bactericidal activity of silver nanoparticles against *M. tuberculosis* in a relevant time frame (15 min) would be negligible (452, 453). Furthermore, the potential for development of resistance to silver and cross-resistance to antibiotics reduces the suitability of silver as an antimycobacterial agent. Following a single exposure to silver nanoparticles, *M. smegmatis* developed resistance to silver nanoparticles, silver nitrate and the antibiotic isoniazid (454). QACs have been demonstrated to be active against hydrophilic bacteria that have a negatively charged cell surface, including LPS-expressing Gram-negative bacteria and teichoic-acid containing Gram-positive bacteria; however, the efficacy against hydrophobic mycobacteria is limited (455, 456). The lack of an antimicrobial facemask with reasonable activity against *M. tuberculosis* supports the development of a P3ABA antimicrobial facemask.

The findings presented here identify P3ABA as a suitable candidate for the production of antimicrobial surfaces in the patient environment, including facemask materials due to the low cost of synthesis, environmental and thermal stability, and lack of mammalian cytotoxicity (158, 397). The broad spectrum activity of fPANI may confer protection against other respiratory pathogens that also have a high burden in LMICs, such as bacterially derived pneumonia (444).

Challenges remain for researchers; the first is to demonstrate tuberculocidal activity of P3ABA in facemask material. We believe this is likely as melt blends of PANI in low density polyethylene (457) and P3ABA electrospun in nanofibers of poly(lactic acid) (165, 397) retain antimicrobial activity. Assuming an effective facemask can be fabricated it will be important to establish that it is effective in reducing TB transmission and to determine the expected usage.
lifetime to give guidelines as to when to discard a used mask. As users may be tempted to reuse masks, irrespective of mask age, for the same reasons that they currently reuse infected masks; thought will still need to be given to removing the reasons for extended use and to enforcing expiration guidelines.

6.6 Conclusion

Agar containing PANI and P3ABA are active against *M. smegmatis* while only the latter was active against *M. tuberculosis*. PANI in films does not reduce cell viability of mycobacteria whereas P3ABA in films decontaminates surface contamination of *M. smegmatis* and *M. tuberculosis*. Surfaces containing PANI and P3ABA have potential for use in hospitals to reduce transmission of *M. tuberculosis*. 
Chapter 7: Discussion

It is important to elucidate the mechanism(s) of action of an antimicrobial agent as an understanding of the mode of action would facilitate interpretation of results derived from antimicrobial susceptibility testing, encompassing the effects of media, pH, RH and temperature\(^{(53, 183)}\). Knowledge of mechanism of action is also crucial for rational improvement of an antimicrobial agent as well as informing potential resistance mechanisms and the possibility of antibiotic cross-resistance\(^{(47, 183, 184)}\). In terms of application of antimicrobial surfaces, determination of the antimicrobial mechanism can inform cleaning procedures and suitability for potential settings\(^{(47, 184)}\). Thus, the antimicrobial mechanisms of PANI and P3ABA were investigated.

7.1 Antimicrobial mechanism of PANI

The mechanism of action of PANI was initially explored in this thesis by examining the relative sensitivities of *E. coli* deletion mutants in rich and minimal media (chapter 3). The resulting sensitivity profiles for PANI reinforced the hypothesis that PANI action involves production of ROS. In rich media, the supersensitivity of *E. coli* ΔkatG, which is unable to scavenge exogenous \(\text{H}_2\text{O}_2\), and the decreased sensitivity of *E. coli* ΔahpC (section 3.2.1), which is postulated to be preadapted to oxidative stress, supported this hypothesis. There was also evidence to suggest perturbation of iron homeostasis in PANI treated cells in iron rich media as demonstrated by the reduced sensitivity of *E. coli* ΔtonB, which has reduced internal iron levels (section 3.2.4). Iron homeostasis is intimately related to oxidative stress as free iron can propagate \(\text{H}_2\text{O}_2\) stress by participating in Fenton reaction producing highly damaging hydroxyl radicals\(^{(197)}\). The increased susceptibility of *E. coli* ΔiscS (section 2.3.4) to PANI treatment may be due to an inability to modify DNA to protect against oxidation, suggesting involvement of oxidative stress\(^{(197, 238, 296–298, 306, 376)}\). There was also evidence of periplasmic stress as indicated by the supersensitivity of *E. coli* Δspy to PANI suspension (section 3.2.6).

The role of induction of oxidative stress in the mode of action of PANI was further investigated in *E. coli* 25922 and *S. aureus* 6538 by examining the activity of the antimicrobial agent in aerobic and anaerobic conditions (section 4.2). PANI was able to exert greater lethal activity against both bacterial strains when oxygen was present compared to anaerobic conditions.
Endogenous ROS are generated at increased rates when the concentration of oxygen is high and cannot be produced when oxygen is absent (197, 225, 244). Therefore, it was concluded that the greater activity of PANI in aerobic conditions reflects an oxidative stress mechanism involving increased production of ROS in treated bacterial cells, which may mediate bactericidal action (228). PANI demonstrated the most efficacious activity against *E. coli* in minimal media in aerobic conditions compared to rich media, while similar activity of PANI in rich and minimal media was observed in anaerobic conditions (section 4.2.1). The increased susceptibility to PANI action in aerobic, low nutrient conditions may reflect induction of oxidative stress in exposed *E. coli* cells, which may be less able to mount an oxidative stress response (compared to cells incubated in rich media) due to the requirement of synthesis of precursor building blocks necessary for the protective response. Furthermore, treatment of *E. coli* cells in aerobic conditions in minimal media with succinate as the carbon source was associated with decreased efficacy relative to minimal media with glycerol (section 4.2.1). The anaerobic activity of PANI against *E. coli* in the presence of succinate was comparable to that observed with glycerol. The difference in aerobic susceptibility to PANI could be indicative of the high concentration of succinate (~15 mM) reducing endogenous hydrogen peroxide formation by preventing fumarate reductase autoxidation and thus protecting against the putative PANI antimicrobial action (375). The antimicrobial activity of PANI is more affected by the presence of oxygen compared to P3ABA, which supports the hypothesis that PANI mediates induction of oxidative stress (section 4.2.3).

Mycobacteria were demonstrated to be less susceptible to the mode of action of PANI compared to *E. coli* (section 5.3; section 6.3). The thick mycobacterial cell envelope contains oxygen radical scavengers, lipoarabinomannan and phenolic glycolipid I, that primarily function to protect against exogenous oxidative stress (414, 428). Protection against endogenously produced ROS, including that generated following PANI exposure, may also be afforded by the cell envelope as lipoarabinomannan and phenolic glycolipid I can help reduce ROS levels and thus contribute to defence against PANI action (414, 428). The reduced susceptibility of mycobacteria to PANI treatment is supportive of induction of oxidative stress as a mode of action of PANI. Furthermore, 8% PANI in agar was not active against *M. tuberculosis* (section 6.3), a pathogen that can successfully withstand oxidative environments to cause infections (427–429). *M. tuberculosis* cyclopropanates cell wall mycolic acids conferring further
resistance to lipid peroxidation and has a unique manner of dealing with oxidative stress (414, 416, 428, 432). The MIC of hydrogen peroxide has been found to be 26-fold higher for *M. tuberculosis* compared to *M. smegmatis* (458). *M. tuberculosis* is resistant to low (0.5 mM) and intermediate (5-10 mM) levels of hydrogen peroxide with a reduction in viability only observed at very high (50-200 mM) level of hydrogen peroxide (419). In comparison, 7 mM of hydrogen peroxide is lethal to *M. smegmatis* (458). The inability of PANI to reduce viability of *M. tuberculosis* adds extra support to the oxidative stress hypothesis.

The mechanistic basis of PANI antimicrobial activity is hypothesised to be electrical conductivity, which can mediate contact with the negatively charged bacterial cell surface through electrostatic adherence and may be responsible for increased production of hydrogen peroxide (163, 178, 181). PANI may perturb the flow of electrons through the ETC (potentially mediated through electron acceptor capabilities), which would result in accumulation of reducing equivalents leading to accelerated side reactions with oxygen, generating ROS (Fig. 7.1) (459). Hydrogen peroxide is formed inside aerobically growing *E. coli* is 10-15 μM per second but is effectively scavenged by alkyl hydroperoxide reductase and catalase (covered in section 3.2.1) (197). If hydrogen peroxide levels increase past the threshold that can be scavenged, then potentially lethal damage will occur. Many metabolic and respiratory enzymes are inactivated following oxidation by hydrogen peroxide within minutes (see section 3.2.5) (203, 262, 291, 293). Susceptible enzymes have solvent exposed iron atoms and include those containing [4Fe-4S]^{2+} clusters (dehydratase enzymes) and mononuclear iron enzymes (203, 291, 293). Oxidation of the iron atom promotes its dissociation from the enzyme, causing loss of function and an increase in free iron levels (203–205). Hydrogen peroxide has been shown to inactivate the [4Fe-4S]^{2+} cluster containing dehydratases, 6-phosphogluconate dehydratase, isopropylmalate isomerase, fumarase A and fumarase B in *E. coli* (291). Similarly, it has been demonstrated that hydrogen peroxide inactivates the iron mononuclear enzymes, ribulose-5-phosphate 3-epimerase and 3-deoxy-D-arabinofuranosyl-7-phosphate synthase (DAHP synthase), involved in the pentose-phosphate pathway and aromatic compound biosynthesis, respectively (460, 461).

Increased free iron released from oxidised enzymes can participate in Fenton reaction producing hydroxyl radicals (Fig. 7.1) (197, 328). Fur acts to control intracellular free iron levels by suppressing iron uptake and promoting iron utilisation (see section 3.2.4). Iron bound Fur
can be oxidised by hydrogen peroxide inactivating the repressor (Fig. 7.1)(462). OxyR increases the expression of the fur gene in response to this; however, if the hydrogen peroxide levels are excessively high, most Fur would be inactivated leading to derepression of iron acquisition systems and the deleterious import of more iron(462). Inactivation of Fur would release RyhB from Fur-mediated repression preventing synthesis of proteins involved in iron utilisation and storage, including sdhCDAB(286, 463, 464). Thus, hydrogen peroxide can cause free iron levels to increase due to targeting of oxidation susceptible iron containing enzymes and deregulation of iron homeostasis involving uncontrolled iron import and a decreased capacity for incorporation of iron into proteins. Upregulation of ryhB expression was found to occur in response to fPANI treatment, which supports de-repression of ryhB expression due to oxidative inactivation of Fur(163). Further increase in intracellular free iron levels would result in the production of more hydroxyl radicals and exacerbate the oxidative stress(197, 462).

Hydroxyl radicals are potent oxidants that can damage biomolecules, including DNA, proteins and lipids(328).

It is postulated that the lethality of hydrogen peroxide is due to ROS-mediated DNA double-strand breaks, particularly due to oxidation of guanine and thymine nucleotides(197, 298, 380). DNA repair systems function to detect and repair damage; however, if these systems are overwhelmed then damage is not repaired(197). Increased hydroxyl radicals derived from elevated free iron levels would result in increased rates of DNA damage(197, 328). Thus, oxidative stress propagated by free iron can cause lethal DNA damage.

The involvement of DNA in the antimicrobial mechanism of PANI was investigated by challenging DNA repair mutants, E. coli ΔrecA and E. coli Δnth. The decreased sensitivities of these mutant strains to PANI may reflect mode-2 hydrogen peroxide mediated killing as opposed to mode-1 killing, which involves DNA damage (see section 3.2.7). Mode-2 killing occurs at higher hydrogen peroxide concentrations (more than 10 mM), involves Fenton reaction and hydroxyl radicals, and reflects general damage to cellular biomolecules (326, 328, 329). Mode-2 killing does not require active growth, which is supported by the efficacious activity of PANI in minimal media against E. coli (see section 4.2.1). This type of hydrogen peroxide killing is reduced in anoxic conditions, which is consistent with the reduction in activity observed for PANI in anaerobic conditions (see section 4.2) (328, 465). Bimodal action of hydrogen peroxide has also been demonstrated in M. smegmatis and
Therefore PANI may mediate production of a high concentration of hydrogen peroxide resulting in mode-2 killing characterised by damage to proteins and/or the cell membrane. Oxidation of amino acids in proteins can cause a range of outcomes, from total degradation of the protein backbone to minor side-chain modification of individual residues. Loss of essential protein function may cause bacterial cell death.

There is a range of evidence to suggest that PANI mode of action includes production of hydrogen peroxide and the consequences of this; however, it is likely that there are additional mechanisms involved. PANI activity was reduced in anaerobic conditions compared to aerobic conditions (section 4.2) but bactericidal activity was not completely abolished. Thus, in anaerobic conditions there must be other unidentified mechanisms occurring to cause cell death, the impact of which would likely vary relative to environmental conditions.

The antimicrobial mechanism of PANI has been partially characterised in this thesis, which necessitates further investigation in the future. The involvement of ROS in this mechanism supports the utilisation of approaches optimised to examine the role of ROS in antibiotic action (section 4.3). For example, fluorescent dyes, including HPF, may be applied to bacterial cells sublethally treated with PANI to detect the hypothesised presence of hydroxyl radicals. Utilisation of a range of dyes to increase sensitivity of the assay and reduce the potential for false positive results would be most beneficial.

Another useful approach would be to perform metabolomics on PANI treated cells as the metabolic products in these cells will reflect the interaction between the genome and PANI exposure. Thus, global metabolite profiling can provide deeper insight into cellular physiology, which can be used to infer antimicrobial mechanism. The effect of ROS exposure on *E. coli* cells has been examined using a metabolome approach. A metabolome approach would be complimented by a transcriptomic analysis of PANI treated bacterial cells, such as by using quantitative polymerase chain reaction (qPCR). The hypothesised changes in gene expression that occur following PANI exposure, including upregulation of genes in the OxyR regulon, can be examined using qPCR. Taken together, detection of hydroxyl radicals and examination of the transcriptomic and metabolomic responses of *E. coli* cells sublethally treated with PANI will contribute to elucidation of the antimicrobial mechanism of PANI.
Following contact with a bacterial cell, PANI is hypothesised to disrupt electron (e-) flow through the ETC resulting in hydrogen peroxide production. Hydrogen peroxide oxidises iron atoms in enzymes resulting in free iron release. Hydrogen peroxide also inactivates Fur causing deregulation of iron import. The increased levels of free iron can participate in Fenton reaction producing hydroxyl radicals, which can damage proteins, possibly leading to cell death.
7.2 Antimicrobial mechanism of P3ABA

The mechanism of action of P3ABA was initially explored in this thesis by examining the relative sensitivities of *E. coli* deletion mutants in rich and minimal media (chapter 3). The resulting sensitivity profiles for P3ABA led to the generation of new hypotheses pertaining to the mode of action, which were distinct from those relating to PANI mechanism. It was apparent that ATP synthase is a target of P3ABA action, akin to the action of bedaquiline, as *E. coli* Δ*atpE* had reduced sensitivity to P3ABA treatment compared to the parent strain (Fig. 3.10, section 3.2.5), inferring that P3ABA acts as an uncoupler (226).

There was some evidence based on the P3ABA sensitivity profiles of the deletion mutants in minimal media (Fig. 3.26, section 3.3) to suggest oxidative damage (*E. coli* Δ*grxA*) to metabolic and respiratory enzymes (*E. coli* ΔiscS) occurs leading to perturbation of iron homeostasis (*E. coli* Δ*fur* and *E. coli* Δ*tonB*) (253, 273, 293, 333, 334). P3ABA is known to cause an increase in intracellular free iron in *E. coli* cells (200). Furthermore, the decreased sensitivity of *E. coli* Δ*sdhB* in LB broth to P3ABA at the earlier time point is suggestive of oxidation of [4Fe-4S]^{2+} clusters in metabolic and respiratory enzymes. The increased sensitivity of *E. coli* Δ*asr* to P3ABA in minimal media, but not rich media, at the later time point indicates involvement of acid stress as a downstream effect (189, 336). Damage to periplasmic proteins was inferred to occur as a later effect in P3ABA treated cells as *E. coli* Δ*spy* in LB broth had decreased sensitivity to P3ABA, reflecting potential upregulation of an additional extracytoplasmic stress response resulting in pre-adaption to P3ABA action (section 3.2.6).

The activity of P3ABA in aerobic and anaerobic conditions against *E. coli* 25922 was examined (chapter 4) to determine the involvement of ROS in the mechanism of P3ABA. *E. coli* 25922 was challenged with P3ABA in both rich and minimal media (section 4.2.1.2), the latter with glycolytic and non-glycolytic carbon sources, allowing for inferences to be made relating to the effect of the energy state of the cell on the susceptibility to P3ABA action. Involvement of oxidative stress, indicated by greater activity in aerobic conditions, was only associated with *E. coli* challenged in rich media and not in minimal media. Production of ROS has been shown to be a downstream effect of futile cycling caused by uncoupling activity (226, 322). P3ABA was more active against *E. coli* in rich media compared to minimal media in aerobic conditions (section 4.2.1.2; section 5.2.3), which is consistent with the involvement of ROS in antimicrobial action on rich media as well as the increased susceptibility to futile cycling and
bactericidal action associated with high respiration rates (245, 376). There was no difference in the activity of P3ABA when the E. coli cells were challenged in minimal media with a glycolytic carbon source (glycerol) compared to a non-glycolytic carbon source (succinate), from which ATP can only be made by oxidative phosphorylation, signifying that the ability to synthesise ATP by alternate methods does not prevent P3ABA bactericidal action (226, 330, 379). A similar trend has been observed for M. smegmatis treated with bedaquiline, an inhibitor of ATP synthase supporting the hypothesis of P3ABA acting as an uncoupler (226).

Further support towards the hypothesis that P3ABA mode of action involves targeting of ATP synthase resulting in uncoupling of ATP synthesis from electron transport was the sensitivity of mycobacteria to P3ABA (section 6.3). P3ABA in agar cleared ~ $10^4$ CFU of M. smegmatis and M. tuberculosis from the agar surface within the shortest time frame tested. P3ABA antimicrobial action is hypothesised involve targeting of ATP synthase. ATP synthase is an essential cellular component in mycobacteria as substrate level phosphorylation is insufficient to sustain growth of bacteria (370, 433, 434). The notable sensitivity of M. smegmatis and M. tuberculosis to P3ABA action may reflect the essentiality of ATP synthase, supporting the hypothesis that P3ABA uncouples ATP synthesis from electron transport leading to cell death.

P3ABA is postulated to target, and probably damage, ATP synthase. This targeting is likely to be due to the functionalisation of P3ABA as there was no evidence to suggest that PANI targets ATP synthase. Damage to this biomolecule may permit H$^+$ ions to move from the periplasmic space into the cytoplasm in an uncontrolled manner that is uncoupled from ATP synthesis (226). The cell would continue to respire and the ETC would continue to pump H$^+$ into the periplasm as electrons are transported along the chain, resulting in a futile proton cycle (Fig. 7.2) (226). In response to the futile cycling, respiration and oxygen consumption would increase potentially resulting in elevated production of ROS (226, 308, 322, 347). The effects of increased ROS production in a bacterial cell are outlined in Figure 7.1 and include dysregulation of iron homoeostasis. P3ABA targeting of ATP synthase may also cause acid stress as a downstream effect as indicated by the susceptibility of E. coli Δasr to P3ABA (section 3.3.5). ATP synthase and other respiratory machinery components are implicated in the regulation of internal pH, which implies that damage to ATP synthase and perturbation of respiration would sensitise the cells to acid stress (303, 474).
The exact events that directly lead to P3ABA-mediated cell death are not known. The uncontrolled leakage of protons may cause dissipation of proton motive force (PMF; the sum of the transmembrane pH gradient and the electrical potential), which is lethal to all living cells(226, 371, 377, 475). Supportive of this is the rapid loss of membrane potential in *E. coli* that occurs following P3ABA exposure(200). Collapse of PMF in *E. coli* and *S. aureus* has been shown to trigger the process of autolysis(476, 477). A proposed model for this, as described for *B. subtilis*, involves repression of murein hydrolase activity in cells with an intact PMF mediated by the localised reduction in pH within the cell wall(478–480). Following dissipation of membrane potential, the cell wall pH increases resulting in derepression of the murein hydrolases(476, 478). Deregulated murein hydrolases degrade peptidoglycan in the bacterial cell wall, disrupting cell wall integrity and resulting in cell lysis(476, 478). It remains to be investigated whether autolysis occurs in P3ABA treated cells. Bactericidal action of bedaquiline, which seems to act in a similar manner to P3ABA, does not involve dissipation of PMF, attributed to the coupled exchange of other cations maintaining the electrical potential(226). Elucidation of the lethal events in bedaquiline action is still ongoing and may inform potential events in P3ABA bactericidal action(475).

While it is apparent that the antimicrobial mechanism of P3ABA it involves targeting of ATP synthase, the events that occur following this are not fully characterised and require further investigation. The involvement of ROS, as a downstream effect of respiratory uncoupling, can be determined using fluorescent dyes, as for PANI, to detect the presence of hydroxyl radicals(344, 382). Examining the global transcription response of *E. coli* cells exposed to P3ABA for a range of times would illuminate downstream events, as reflected by the genomic response of the cells, which has been done for *M. tuberculosis* exposed to bedaquiline(370). Gene expression changes in response to P3ABA exposure can be verified using qPCR. Furthermore, as discussed for PANI (section 7.1), metabolomic analysis of sublethally treated *E. coli* cells would complement the transcriptomic approach(370).
Figure 7.2. Putative uncoupling mechanism of P3ABA. (A) Oxidative phosphorylation in *E. coli* cells involves transport of electrons through the ETC permitting pumping of H\(^+\) ions into the periplasm. The H\(^+\) ion gradient is used by ATP synthase to synthesise ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). Thus, electron transport and the H\(^+\) gradient is coupled to ATP synthesis. (B) P3ABA is hypothesised to target ATP synthase, which may result in an uncontrolled proton leak. The movement of H\(^+\) ions back into the cytoplasm would be uncoupled from ATP synthesis resulting in a futile proton cycle.
7.3 Conclusions

PANI and P3ABA are broad spectrum antimicrobial agents with potential for use in surface coatings or for incorporation into materials to generate surfaces resistant to contamination, as a means of reducing bacterial spread in hospitals, food processing plants and farms. Surfaces containing PANI and P3ABA are non-leaching, which is suggestive of longer-term activity compared to leaching surfaces. Based on this property, absorbent surfaces containing PANI or P3ABA, such as facemasks, would have more efficacious surface activity compared to the equivalent non-absorbent surfaces, such as metal or plastic surfaces, due to the requirement of bacterial contact with the surface for PANI or P3ABA to exert bactericidal activity. PANI and P3ABA are suited to surface applications due to their low cost and simple synthetic procedures, environmental stability and demonstrable biocompatibility with mammalian cells(481, 457). Both PANI and P3ABA containing surfaces show reduced activity in the presence of organic matter, which is a common outcome for antimicrobial surfaces, especially those with immobilised antimicrobial agents. The negative effect of organic soiling supports investigation of a cleaning regime that would not affect activity of the surface.

PANI is postulated to exert antimicrobial activity through increasing hydrogen peroxide levels leading to oxidative stress characterised by perturbation of iron homeostasis, Fenton reaction and damage by hydroxyl radicals. Therefore, PANI is best suited to aerobic applications including walls, door handles and fabric in hospitals, and conveyer belts and equipment in food processing plants. Following from this, PANI containing surfaces would not be appropriate for settings associated with lower oxygen levels, such as inside pipes in food processing plants. P3ABA is proposed to reduce bacterial cell viability by targeting ATP synthase causing uncontrolled proton leak into the cytoplasm. The resulting futile cycling may cause dissipation of PMF as well as acid stress as downstream effect. Collapse of PMF likely underpins loss of cell viability, potentially mediated through deregulation of murein hydrolases causing cell lysis. Knockdown of *E. coli*, *S. aureus* and *M. tuberculosis* on P3ABA containing surfaces in the shortest time tested supports the use of such surfaces in hospitals to prevent the spread of infection.
7.4 PANI and P3ABA containing surfaces

In this thesis, PANI and P3ABA were investigated for their potential as antimicrobial additives to materials to create contamination resistance surfaces. As discussed in section 1.2, properties of antimicrobial surfaces influence the overall efficacy of the surface in the particular application. The activity of the antimicrobial agent against target bacteria relevant to the application is an important consideration (section 1.2.1, section 1.2.2). The activity of PANI and P3ABA was determined against *E. coli* and *S. aureus*, representing important pathogens found in hospitals and food processing plants. Both polymers demonstrated activity in suspension (Fig. 5.3, Fig. 5.4) and as part of absorbent surfaces (Figs. 5.9-5.12) and non-absorbent surfaces (Fig. 5.13, Fig. 5.14). The most efficacious activity was observed in suspension while the lowest activity was observed for non-absorbent surfaces, which demonstrates how surface incorporation can influence antimicrobial activity. Efficacy of antimicrobial surfaces now needs to be determined in appropriate environmental conditions (section 1.2.3), particularly in terms of temperature and RH. Typically, indoor environments, such as hospitals, have ~ 24% RH and are ~ 20°C. Experimental investigation of the activity of PANI and P3ABA containing surfaces (chapter 5, chapter 6) was performed at 37°C and more than 90% RH, conditions that are both easy to control in the laboratory and support the growth of test bacteria. Future work will focus on testing these surfaces in more relevant conditions, including at 20°C with 24% RH.

Organic soiling of antimicrobial surfaces is a known cause of loss of activity. The effect of soiling on the activity of PANI and P3ABA containing surfaces against *E. coli* was determined. Both types of antimicrobial surface showed reduced activity in the presence of organic matter (Fig. 5.17). While this is not ideal, loss of activity upon soiling is common (table 1.1) and the effect of organic soiling can be reduced by regular cleaning. Antimicrobial surfaces need to be able to withstand any adverse environmental conditions relating to their application (section 1.2.4) including extreme temperatures, acidic and alkaline conditions, mechanical abrasion and cleaning. PANI and P3ABA have thermal stability up to 300 °C and environmental stability in the conducting form. Future work will include examining the influence of acidic and alkaline conditions on the activity of surface incorporated PANI and P3ABA. An fPANI containing surface was demonstrated to retain activity against *E. coli* and *S. aureus* after 10 repeated challenges if H₂O₂, but not bleach, was the cleaning
agent (482). PANI and P3ABA have simple and inexpensive synthetic procedures (163, 182, 158, 173), which means they are suitable for large scale production, and have been demonstrated to be biocompatible with mammalian cells (208–211), both of which increases their commercial viability (section 1.2.6). Surfaces containing PANI and fPANI are non-leaching (163, 181, 185), which promotes activity over a longer period of time and reduces both personal and environmental safety concerns (52) (section 1.2.5).

P3ABA containing surfaces demonstrated potential as contamination resistant surfaces for applications; however, the opposite was found for PANI containing surfaces. P3ABA as part of a non-absorbent surface reduced *E. coli* by 2 log after a 24 h incubation at more than 90% RH (Fig. 5.13). The P3ABA containing surfaces described in Chapter 5 indicate a superior performance than has been reported for triclosan, a popular additive claiming antimicrobial activity, which had no effect on the viable cell count of *E. coli* following a 24 h exposure at more than 90% RH (483). Similarly, triclosan-incorporated plastic only inhibited *E. coli* O157:H7 after a 24 h incubation (111) and triclosan melt-mixed with 4.5% polystyrene inhibited *E. coli* Y1090 for 5 h after which viable cell number increased (110). P3ABA containing surfaces have similar activity against *E. coli* compared to copper containing surfaces; however, copper surface properties, such as concentration and surface type, can influence activity, making exact comparisons of surface activity difficult (57, 52, 121). Coupons containing 95% copper knocked *E. coli* down within 70 mins while 93% copper coupons required 4 h and 78% copper coupons only reduced *E. coli* by 1 log in 6 h (57). P3ABA in a non-absorbent surface achieved knockdown of *E. coli* after a 24 h treatment but not a 2 h treatment (Fig. 5.13). Therefore, the copper surfaces containing more than 93% copper are more active against *E. coli* than P3ABA films while surfaces tested with lower copper content do not work as well. Materials containing P3ABA may therefore have a future as a cost-effective antimicrobial surface to prevent or at least reduce the undesirable spread of micro-organisms.
Appendix 1

The tables that follow were published as supplementary material to Gizdavic-Nikolaidis MR, Bennett JR, Swift S, Eastal AJ, Ambrose M. 2011. Broad spectrum antimicrobial activity of functionalized polyanilines. Acta Biomater. 7:4204-9. doi: 10.1016/j.actbio.2011.07.018. The tables contain lists of the genes up- and down-regulated in E. coli following challenge with fPANI. The tables have been annotated further to identify the genes knocked-out in mutant studies found in Chapter 3. Yellow highlights identify genes where knockout mutants were tested. Green highlights regulatory genes that although not tested directly in this thesis, did suggest other genes from their respective regulons to include in the mutant testing panel. The broad findings are summarised in the text below.

The following are involved in the response to oxidative stress.

Catalases, Superoxide dismutases
OxyS, SoxS and SoxR induce oxidative stress responses and include KatG, KatE, AhpC, SodA, SodB and SodC as products of the regulated genes. Mutants in these enzymes were chosen to dissect the important enzyme activities in the oxidative stress responses.

Oxidoreductases
Fpr, YqhD, YgiD

Uptake systems for metal ions that co-factor, or participate in, oxidative stress responses.
CopA (copper uptake); MntH (manganese uptake)

Redox damage repair
GrxA, Gst, TrxC. For the repair of redox damage to proteins especially. Mutants unable to produce either GrxA or TrxC were chosen for testing.

Repair and/or assembly of FeS clusters
IscS, IscR, IscU, SufA, SufB, SufC. IscS was chosen for testing as a key functional enzyme in the repair of FeS clusters. FumC is an alternative enzyme in energy metabolism that does not possess an FeS cluster and is induced in times of oxidative stress.
The following are involved in other stress responses, e.g. acid stress, periplasmic stress.

Asr (acid stress), CpxP, Spy (periplasmic and envelope stress), DnaK, HtpX, IbpA, IbpB, YecG, YiiT (heat shock, UV and other stresses) and YeaY and YjiY (carbon starvation stress). Mutants lacking Asr and Spy were chosen as highly induced representatives to probe the importance of these responses. Mutants lacking RecA and Nth, required for DNA repair activity following oxidative and UV DNA damage were also included in the mutant testing panel.

The following are involved in iron homeostasis: iron uptake, regulation of iron, storage of iron, production and repair of FeS centres.

Bfd (iron storage)

FecA, Fes, FhuF (iron uptake). Mutants lacking TonB, a protein that energises most iron uptake systems, was chosen to study the effect of disabling iron uptake.

RyhB (Regulation of use of iron in the cell during iron starvation). A mutant in RyhB was not available, a mutant lacking Fur, the global iron regulator, was chosen for study.

As noted with regard to oxidative stress: IscS, IscR, IscU, SufA, SufB, SufC. IscS was chosen for testing as a key functional enzyme in the repair of FeS clusters. FumC is an alternative enzyme in energy metabolism that does not possess an FeS cluster and is induced in times of oxidative stress.

The cultures used for the transcriptomic study were not grown in iron restricted conditions. Although not iron restricted, the cells are behaving as if they iron restricted (iron uptake, RyhB induction) and have a potentially dangerous excess of iron (Bfd).

The following are involved in energy metabolism.

FumC, GlpD, LdhA, Ndh and YfiD are upregulated. Sdh components are down-regulated. Overall the changes in energy metabolism suggested a shift to alternative pathways avoiding enzymes containing FeS clusters and enzymes/pathways associated with ROS production. A
mutant in ATPE (essential for Proton translocation in ATP synthesis) was chosen for the test panel to test the requirement for this activity as part of the killing mechanism.

\(^a\) Functional group descriptions are taken from [http://ecocyc.org/](http://ecocyc.org/) and [http://genprotec.mbl.edu/](http://genprotec.mbl.edu/)

\(^b,c\) GeneChip *E. coli* Genome 2.0 Array ([http://www.affymetrix.com/](http://www.affymetrix.com/))


**Table 1a. Genes whose expression is up-regulated in response to PolySO\(_3\)H**

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<th>Gene product(^c)</th>
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Vitamins, nucleotides and cofactors

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Table 1b. Genes whose expression is down-regulated in response to PolySO$_3$H

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<td>kgtP alpha-ketoglutarate transporter</td>
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<td>dctA C4-dicarboxylic acid, orotate and citrate transporter</td>
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<td>dcp dipeptidyl carboxypeptidase II</td>
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<td>fbaB fructose-bisphosphate aldolase</td>
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References


30. Otter JA, Yezli S, French GL. 2011. The role played by contaminated surfaces in the


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