Creation and characterization of *Porphyromonas gingivalis* mutant strains to investigate heme-binding

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Abstract:

The gram-negative anaerobic bacterium *Porphyromonas gingivalis* is the most studied member of the "red complex" which is associated with severe forms of periodontitis. This organism lacks porphyrin-biosynthetic enzymes and acquires heme from external sources for growth enabling it to cause infection. The gingival crevicular fluid contains plasma heme-binding proteins (HBPs): serum albumin (Kd~10-8 M) and hemopexin (Kd ~10-12 M) that scavenge and detoxify heme released from lysed red blood cells. To acquire heme in the presence of these host proteins, the bacterium must either express greater affinity for heme (than do the plasma proteins) or must inactivate the proteins. The mechanism of heme uptake is however, not fully understood. Although several studies have reported heme-binding outer-membrane proteins (OMP) expressed by *P. gingivalis*, the issue of hemin binding to non-specific bacterial surface components (especially the phospholipid membrane) while performing binding assays using free hemin has been largely neglected. Thus, the reported affinities of some documented *P. gingivalis* HBPs such as HmuY and HmuR is very low (Kd ~10-5-10-6 M) placing them in the category of non-specific binding sites. Tompkins et al (1997) performed hemin-binding assays using hemin pre-bound to rabbit serum albumin (RSA) to minimise non-specific binding thereby allowing detection and measurement of specific heme-binding sites. This thesis aimed to expand that approach in order to identify specific (Kd ≤ 10-8 M) HBPs expressed by P. gingivalis.
Two approaches addressed this aim: In the first, mutant *P. gingivalis* strains were created by individually deleting the genes for six reported HBPs (HBP35, IhtB, HmuY, HmuR,Tlr and HusA) by allelic exchange. The mutants grew in heme-supplemented medium and pigmented on blood agar at rates indistinguishable from that of the parent strain implying that the reported HBPs are not essential for heme-uptake. Binding of [55Fe]hemin under stringent conditions (ligand pre-bound to rabbit serum albumin) was slightly (<18%) diminished in ΔhmuY, ΔhmuR, ΔihtB, Δtlr and ΔhusA and binding of the fluorescent heme analogue zinc-protoporphyrin IX (ZPP) under the same conditions was diminished (≤ 25%) in Δhbp35, ΔhmuY, ΔhmuR, ΔihtB, and ΔhusA, suggesting that these proteins may be involved in specific heme binding.

The second (concurrent) approach was to purify and identify HBPs under the assumption that none of the reported HBPs are involved in specific binding. Heme starved wild-type bacteria were incubated with hemin pre-bound to RSA followed by thorough washing to remove unbound ligand and RSA. OMPs were then extracted using n-dodecyl β-D-maltoside (DDM) and resolved by isoelectric focussing (IEF) , SDS-, native- and two-dimensional (2D)-polyacrylamide gel electrophoresis. Heme-complexed proteins were identified by staining with 3,3',5,5'- tetramethylbenzidine. Candidate HBP bands and spots were analyzed by matrix assisted laser desorption/ionization time- of- flight mass spectrometry. RagA, RagB and a fragment (~31 kDa) corresponding to the C-terminal domain of cysteine proteases were identified as candidate HBPs. An additional low molecular weight (15-20kDa) protein, corresponding to an intensely coloured region (due to hemin) on the IEF strip was also observed but could not be identified.

This research suggests RagA and RagB as candidate HBPs involved in specific heme-binding by *P. gingivalis.* Though the first approach showed some evidence that the reported HBPs are involved in specific heme-binding, that was not supported by the second approach that offered other candidate proteins.