MOLECULAR ANALYSIS OF THE ENDOFLAGELLA OF THE PATHOGENIC TREPONEMES.

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Abbreviations

2D-PAGE	two dimensional-polyacrylamide gel electrophoresis
AIDS	acquired immunodeficiency syndrome
A_n	Absorbance at n nm
ATP	adenosine triphosphate
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	dexoycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	(ethylenedinitrilo)tetraacetic acid
FlaA	the mature endoflagellar sheath protein
*fla*A	the gene encoding the endoflagellar sheath protein
FlaB1	a *T. pallidum* endoflagellar core protein of M_r 34.5 kD
*fla*B1	the gene encoding FlaB1
FlaB2	a *T. pallidum* endoflagellar core protein of M_r 33 kD
*fla*B2	the gene encoding FlaB2
FlaB3	a *T. pallidum* endoflagellar core protein of M_r 30 kD
*fla*B3	the gene encoding FlaB3

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kD	kilodalton
min	minute
M_r	relative molecular mass
NP-40	Nonidet 40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG 6000	polyethylene glycol 6000
PMSF	phenyl methyl sulphonyl fluoride
pre-FlaA	the precursor protein of FlaA
RBS	ribosomal binding site
RIP	radioimmunoprecipitation
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
sarkosyl	N-lauroyl sarcosinate
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second
ssDNA	single stranded DNA
subsp.	subspecies
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UV	ultraviolet
vol	volume
wt	weight
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Abstract

1. Motility of *Treponema pallidum* is mediated by endoflagella located within the periplasmic space. The endoflagella comprise a core surrounded by a sheath, with the sheath being composed of a 37-kD antigen (FlaA).

2. Treponemal endoflagella were isolated and component proteins were purified by reverse-phase high pressure liquid chromatography (HPLC). N-terminal amino acid sequence analysis was performed using the sheath protein and two core proteins from *T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue*, and *T. phagedenis*. For all three proteins, the N-terminal sequences found for *T. pallidum* and *T. pertenue* were identical but differed from the sequence of *T. phagedenis*. The sheath proteins from the three treponemes were also compared by HPLC tryptic peptide maps: maps for *T. pallidum* and *T. pertenue* were very similar but differed from *T. phagedenis*.

3. A λgt11 clone, λA34, that expressed a portion of FlaA was selected from a *T. pallidum* genomic library using a murine monoclonal antibody. The insert from λA34 provided a probe with which a chimeric plasmid, pRI4, encoding all but the nine N-terminal amino acids of FlaA was selected from a pBR322 *T. pallidum* genomic library.

4. The nucleotide sequence of *flaA* upstream of amino acid 10 could not be determined by routine cloning strategies.

Instead, the remaining sequence was determined using the inverse- and asymmetric-polymerase chain reaction methods. *fla*A contains a consensus *Escherichia coli* promoter and a ribosome binding site. A single open reading frame encodes a precursor protein, pre-FlaA, of 350 amino acids with a calculated M_r 38,860. A 20 amino acid signal sequence with a typical signal peptidase I cleavage site immediately precedes the known N-terminus of FlaA. Twenty six per cent (91/350) of the DNA-derived amino acid sequence was confirmed by N-terminal sequence analysis of ten tryptic peptides derived from native FlaA.

5. Polymerase chain reaction-derived constructs lacking the native *T. pallidum* promoter were cloned downstream of a T7 promoter. T7 polymerase transcription was under the control of a P_L λ promoter. When the λ repressor was inactivated at 42°C, FlaA was expressed at relatively low levels in *E. coli*. Native and recombinant FlaA were identical as assessed by antibody reactivity and electrophoretic mobility in both sodium dodecyl sulphate- and two dimensional-polyacrylamide gels.

6. Although some pre-FlaA is processed to FlaA in *E. coli*, pre-FlaA is accumulated indicating inefficient processing of pre-FlaA in *E. coli*. Soluble FlaA was not detected in either the cytoplasmic or the periplasmic fractions of *E. coli* transformants. Fractionation of *E. coli* cell envelopes unexpectedly revealed that pre-FlaA and FlaA were associated

with both the inner and outer membranes.

7. Two gene fusions, *cro-lacZ-flaA* and *lacZ'-flaA*, were constructed in an attempt to increase expression of the recombinant antigen; a bovine protease X_a site was engineered into the hybrid proteins immediately upstream of the N-terminus of FlaA to allow for subsequent proteolytic cleavage of the purified hybrid proteins. The LacZ'-FlaA protein was expressed in *E. coli* in increased amounts as compared with FlaA, but like recombinant FlaA, associated with both the inner and outer bacterial membranes. LacZ'-FlaA can be released from the membranes by treatment with 1% [wt/vol] sarkosyl, 6 M urea, or 6 M guanidine hydrochloride.

1. Introduction

Venereal syphilis is a chronic, systemic, sexually transmitted disease caused by the spirochaete *Treponema pallidum* subsp. *pallidum*.[1] Following a variable incubation period, classic untreated syphilis begins as a primary lesion (the chancre) at the site of inoculation. The primary lesion resolves spontaneously, and after a variable period of time is followed by a disseminated secondary stage of infection which is characterized by diverse skin and other systemic manifestations. Following an asymptomatic latent period of many years, tertiary disease is manifest by devastating illness involving the mucocutaneous, cardiovascular, or central nervous systems.

Both the advent of penicillin treatment and public health measures have made a significant impact on the spread of venereal syphilis, but within the past five years there has been a major resurgence of this disease.[2] The recent syphilis epidemic is of additional concern as genital ulcer disease is a risk factor for transmission of the human immunodeficiency virus (HIV), the causative pathogen of the acquired immunodeficiency syndrome (AIDS).[3,4] As a consequence, there has been renewed interest in the development of a vaccine to prevent the spread of syphilis. In order to achieve this, detailed analysis of treponemal antigens is required. A recombinant DNA approach to the study of one treponemal antigen, the endoflagellar sheath protein, is the topic of this thesis.

1.1 Epidemiology of syphilis

The history of syphilis is a fascinating study of world events over the past five centuries as it has affected many rich and powerful figures.[5] Since the advent of simple, effective, safe treatment with penicillin there has been a marked diminution in the number of cases of tertiary syphilis.[2] Active public health measures to control the spread of this disease have been partially successful. For example, the Centres for Disease Control (Atlanta, GA 30333) reported declines in cases of infectious syphilis in the United States during the years 1982 through 1986.[2] However, significant increases in early syphilis have been noted each year since 1987 with syphilis reaching near-epidemic proportions in the United States; the rate of 16.4 cases per 100,000 persons in 1988 is the highest level since the 1950's.[2]

This worsening epidemic has been associated with a change in the socioeconomic pattern of cases: in the years 1965 through 1984, infectious syphilis was most frequently diagnosed in homosexual and bisexual men[6], but in the past decade the greatest increase in incidence has been in heterosexual male and female minorities living in the inner cities.[7] The reasons for the epidemic and for the change in epidemiologic pattern probably are multifactorial. Suggested causes include the following[8]:

- an increase in the incidence of penicillinase-producing *Neisseria gonorrhoeae* has meant that spectinomycin is used commonly for treatment of gonorrhoea. Unlike most of the other

antimicrobials used in the treatment of gonorrhoea, spectinomycin does not effectively treat syphilis during its incubation period.

- a decrease in public health resources set aside for syphilis control as funds have been redistributed to control programmes dealing with either HIV or penicillinase-producing *N. gonorrhoeae* infections.
- an increase in sexually transmitted diseases amongst prostitutes who sell sex for cocaine, usually in the form of "crack".

Syphilis is also a major problem in the third world, accounting for significant morbidity including spontaneous abortion.[9] It may also have a major role in HIV transmission in Africa, along with other diseases which cause genital ulceration.[3]

Public health measures to prevent the spread of AIDS by the widespread use of condoms have decreased the rate of syphilis and other sexually transmitted diseases in homosexual men. Despite this, co-infection with both HIV and *T. pallidum* is very common in homosexual men.[10-14] This is of major concern for a number of reasons[15]:

- an increase in the prevalence of syphilis may result in accelerated heterosexual spread of HIV in populations already at high risk for HIV infection. Genital ulcer disease, including syphilis, has been implicated in the transmission of HIV.[3,4] For example, one United States study suggested that homosexual men were at three to eight times the risk of acquiring HIV if they

had a previous history of either syphilis or genital ulcer disease.[4]

- an epidemic of early syphilis in women, particularly prostitutes abusing intravenous drugs, presages an increased number of cases of congenital syphilis, possibly co-infected with HIV, with resultant fetal death and perinatal morbidity and mortality.

- recent case reports have suggested that HIV co-infection may affect the diagnosis, clinical picture, and treatment outcome for patients with syphilis:

  - the diagnosis of syphilis often relies entirely on the results of serological tests. Delayed seroconversion in an HIV-infected male with histopathological evidence of secondary syphilis[16] has suggested that serodiagnosis may be of lower sensitivity in HIV co-infected patients. As this is a solitary case-report, most authorities feel that current serodiagnostic tests are adequate to diagnose syphilis in HIV-infected patients.[17-19]

  - an altered natural history or accelerated course of syphilis in HIV co-infected patients is suggested by a number of authors. Lues maligna[20], giant primary chancre[21], early gummata[22], pure motor hemiparesis due to meningovascular syphilis[23], syphilitic polyradiculopathy[24], syphilitic uveitis/chorioretinitis[25-27], and otosyphilis[28] have been reported in young HIV infected patients. Although these cases are atypical, similar cases were reported prior to the discovery of penicillin. Further studies are required to see

if there is an interaction between the two diseases.[19]

- the adequacy of current treatment regimens for early syphilis, particularly those utilizing benzathine penicillin, has been questioned following case-reports of relapses of syphilis in HIV-infected patients after presumed adequate treatment.[19,29-31] Invasion of the central nervous system occurs early in a significant proportion of cases of infectious syphilis[32] and is felt to be a *sine quae non* for the later development of clinical neurosyphilis. As benzathine penicillin does not achieve treponemicidal levels in the cerebrospinal fluid[33], it may not prevent neurosyphilis developing, particularly in immunocompromised hosts.

1.2 Biology of *T. pallidum*

The order *Spirochaetales* comprises two families[34,35] (Figure 1): *Spirochaetaceae* and *Leptospiraceae*. The family *Spirochaetaceae* comprises four genera[1]: *Treponema* and *Borrelia*, which are pathogenic for man, and *Spirochaeta* and *Cristispira*, which do not contain human pathogens. All spirochaetes, including leptospires, share common structural features: they are long, thin, coil-shaped, flexible, motile bacteria.[1]

The genus *Treponema* contains two species which are pathogenic for humans[1] (Figure 1): *T. pallidum* and *T. carateum*, the causative bacterium of pinta. *T. pallidum* is speciated[36] further into subspecies *pallidum*, subspecies *pertenue*, and subspecies *endemicum*, which are the causative organisms of venereal

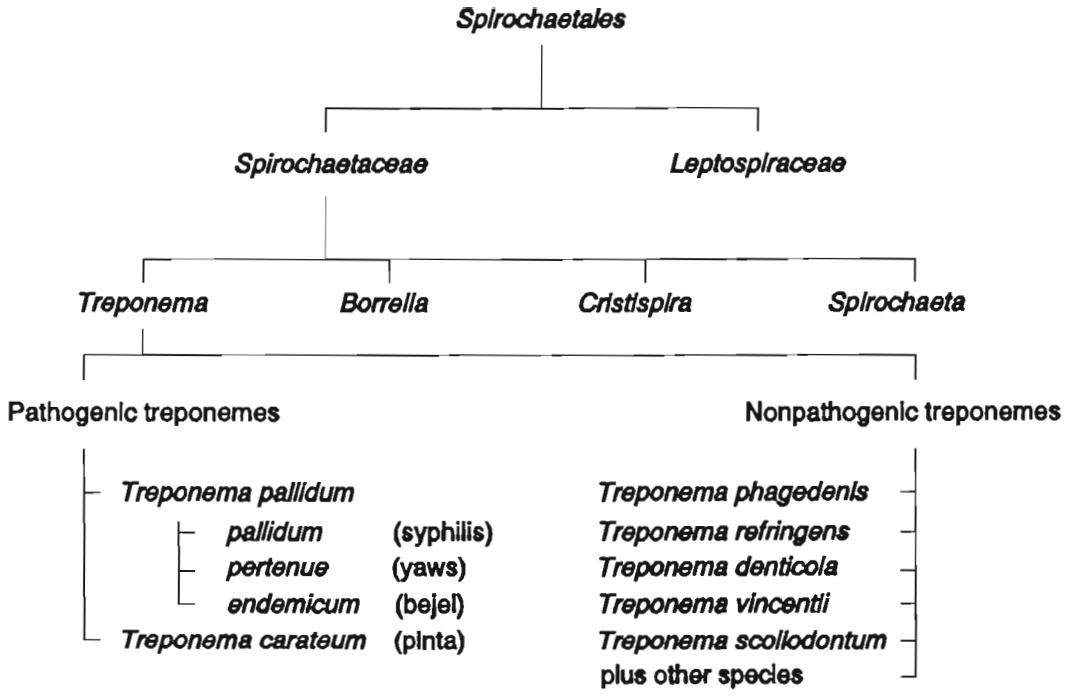

Figure 1. Taxonomy of the genus *Treponema*.

syphilis, yaws, and bejel, respectively.[a] These pathogenic treponemes are indistinguishable from each other on the basis of morphology[1], antigenic structure[37-39], and DNA-DNA homology.[40] They can be differentiated only by epidemiological factors, by their host ranges, and by the distinctive diseases they produce in man and in experimental animals.[1] Furthermore, the pathogenic treponemes can be cultivated only by serial passage in experimental animals. Although significant advances have been made in the past decade, continuous *in vitro* cultivation of *T. pallidum* has not yet been achieved.[41]

The genus *Treponema* also contains numerous treponemes which are not pathogenic for man.[1] They are found, in general, on the mucosal surfaces of the mouth, the genitalia, and the gastrointestinal tract. In contrast to the pathogenic treponemes, these organisms can be readily cultivated *in vitro*. Furthermore individual nonpathogenic treponemes are antigenically, morphologically, and genetically distinct both from other nonpathogens and from the pathogenic treponemes.

Cultivation of *T. pallidum* can be achieved by serial passage through a number of laboratory animals.[42] The usual experimental host is the New Zealand white rabbit. Other experimental hosts include sub-human primates, hamsters and guinea pigs. In

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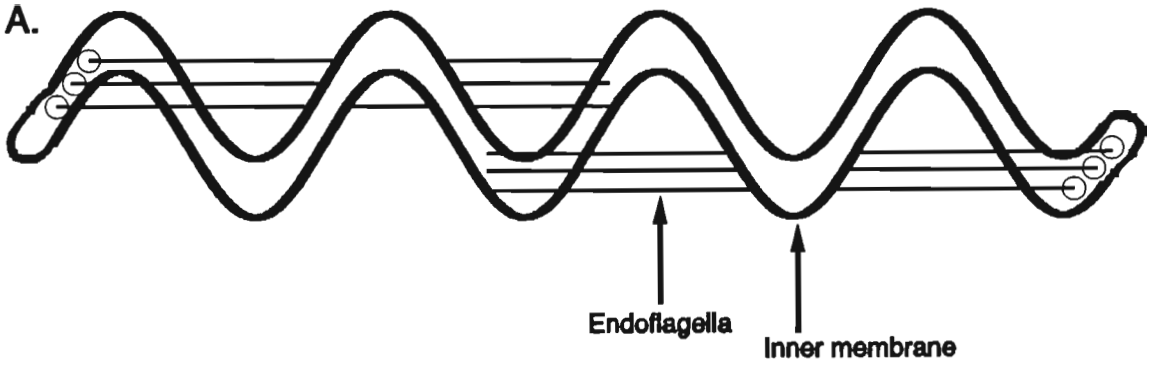
[a] For the remainder of this thesis, *T. pallidum* and *T. pertenue* will be used to refer to *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue*, respectively.

contrast, there are a limited number of animal models of clinical treponemal infection. Intradermal inoculation of rabbits provides a satisfactory model of primary disease as the lesions are identical histologically to human chancres. Infected rabbits also progress to a syndrome similar to secondary syphilis. Hamsters and guinea pigs develop atypical lesions following intradermal inoculation, and infection can only be generated in immunosuppressed mice using large inocula. There is no known parallel in rabbits or subhuman primates for tertiary disease.

T. pallidum measures 6 to 16 μm in length, less than 0.2 μm in diameter, and has 4-14 spirals.[1,43] It is a delicate, corkscrew-shaped organism with uniform, tightly wound, deep spirals. By electron microscopy, *T. pallidum* consists of an outer membrane surrounding a cytoplasmic membrane and a helically shaped protoplasmic cylinder[43] (Figure 2 and Figure 3). Recent studies have confirmed the presence of peptidoglycan as a structural component, and as with other spirochaetal peptidoglycans, ornithine is a major constituent.[44,45] Although the presence of an outer membrane was previously controversial, it has been conclusively demonstrated recently.[46,47] Freeze fracture[46,47] and differential detergent solubilization studies[48] indicate that this membrane contains a paucity of protein elements, an observation which accounts for its lability *in vitro*. Furthermore, the lack of antigenic components in the outer membrane provides a possible explanation for the ability of *T. pallidum* to evade the immune system. The periplasmic space, bounded by the

Figure 2. Schematic diagram demonstrating the ultrastructural features of *T. pallidum*. **Panel A:** Longitudinal section. The outer membrane is not shown. **Panel B:** Cross-section.

A.


Endoflagella
Inner membrane

B.

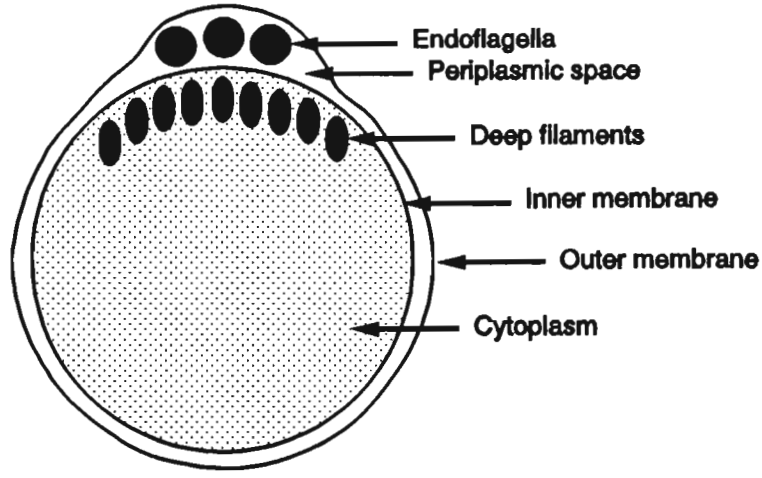
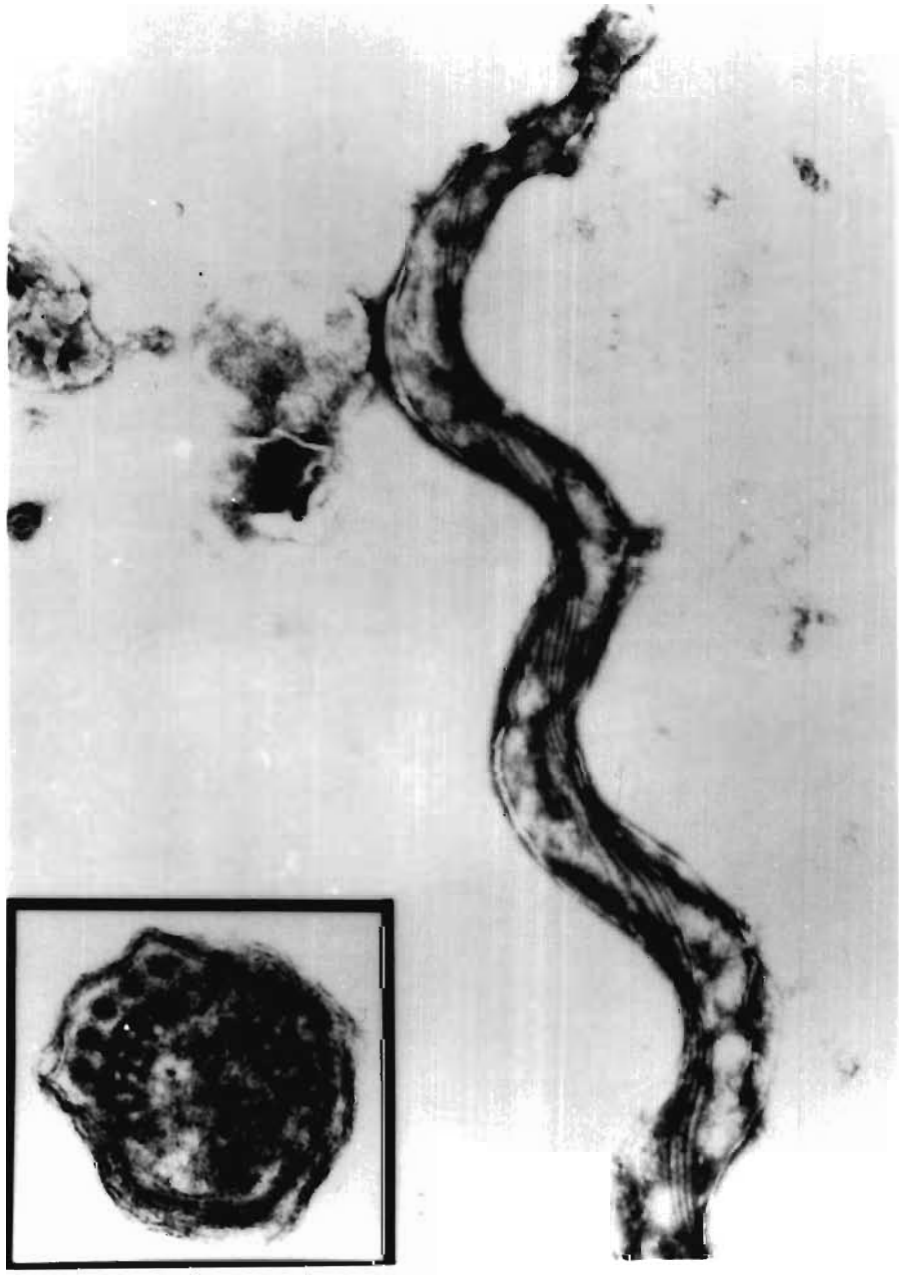

Endoflagella
Periplasmic space
Deep filaments
Inner membrane
Outer membrane
Cytoplasm

Figure 3. Electron micrographs of *T. pallidum* subsp. *pallidum*. Whole *T. pallidum* negatively stained with uranyl acetate. Inset shows transmission electron micrograph of cross-section of *T. pallidum*.



inner and outer membranes, contains six endoflagella[43]; three originate from each end of the organism, and they overlap slightly at the centre. *T. pallidum* is believed to contain a conventional super-coiled circular genome, unlike the linear DNA chromosome of *B. burgdorferi*.[49,50] DNA hybridization suggests that the *T. pallidum* genome has a double-stranded molecular weight of 9.05×10^6 kD (equivalent to 1.37×10^4 kilobase pairs (kb)), approximately four times larger than the *E. coli* genome (double stranded molecular weight 2.5×10^6 kD; equivalent to 0.38×10^4 kb).[51] Extrachromosomal DNA elements have been identified only once from *T. pallidum* and their presence needs further confirmation.[52]

1.3 Treponemal motility and pathogenicity

Motility is a characteristic feature of all spirochaetes, including the pathogenic treponemes.[53] Unlike other flagellated bacteria, motile spirochaetes have the ability to translate through environments of relatively high viscosity.[53] *T. pallidum* exhibits three characteristic motions[54,55]:

- *translation*, or slow movements backwards and forwards,
- corkscrew *rotation* about the long axis, and
- *flexion*, or soft bending, particularly about the mid-point,

with twisting and undulation from side to side.

The coil appearance is maintained despite the motility, except when the organism is attached to, or obstructed by, larger objects -- in which case the coils may be distorted.[54] In

contrast[54-56], many commensal treponemes (e.g. *T. refringens*) are clearly larger (8-20 µm long, 0.4-0.75 µm wide), more loosely coiled, thicker and coarser, and have a different pattern of motility; they demonstrate rapid translation with a writhing motion, active rotation, and marked flexion with frequent relaxation of the coils.

The relationship between motility and host invasion has been clearly demonstrated for several enteric pathogens, including *Salmonella typhi*[57], *S. typhimurium*[58], and *Vibrio cholerae*.[59,60] It is probable that motility is a critical component of the haematogenous dissemination of *T. pallidum* which occurs during the course of syphilis.[61] This invasive potential has been investigated both *in vivo* and *in vitro*:

- Mahoney and Bryant[62] demonstrated that viable *T. pallidum* applied to the surface of intact rabbit mucosa are capable of moving through the epithelium into the connective tissues below the site of application.
- after intratesticular inoculation in rabbits, *T. pallidum* can be demonstrated to enter the blood stream within five minutes.[63]
- *T. pallidum* spreads from the site of inoculation to distant anatomical locations within the host.[64]
- after direct application of *T. pallidum* onto mucous membrane surfaces, treponemes disseminate into deeper tissues within three hours.[65]
- *T. pallidum* can penetrate living rabbit cells both *in*

vitro[66] and *in vivo*[67-70]; these intracellular treponemes can travel between the vascular and extravascular compartments inside mobile host cells.

- *T. pallidum* can pass through a monolayer of endothelial cells *in vitro* by actively moving between cells with tight intracellular junctions.[71]
- this ability of *T. pallidum* to penetrate host tissue has been further investigated using double-sided culture chambers constructed by mounting abdominal walls excised from mice between two sides of small dialysis cells.[72] *T. pallidum* could pass through the abdominal walls within ten hours, whereas the non-pathogenic *T. phagedenis* could not. The abdominal walls only have an epithelial surface on the peritoneal side; *T. pallidum* only passed through the walls if they were exposed to this epithelial surface.

These data indicate that *T. pallidum* is capable of crossing intact epithelial surfaces. Although infection with *T. pallidum* appears to be mainly extracellular, intracellular treponemes have been reported in biopsied chancres as well as in tissue culture systems.[66-70,73-75] Thus *T. pallidum* can disseminate in hosts either by intracellular penetration or by intracellular migration, or a combination of both.[72] Whether the presence of intracellular treponemes represents a significant aspect of either the pathogenesis of syphilis or its natural history is unknown.[71]

1.4 Spirochaetal motility and the endoflagella

It is postulated that the endoflagella located within the periplasmic space are the organelles responsible for spirochaetal motility. This conclusion is supported by three experimental studies:

- Paster and Canale-Parola[76] identified four types of immotile mutants following ultraviolet and chemical mutagenesis of *S. halophila* RS1 and *S. aurantia*:
  - type 1 had normal ultrastructure
  - type 2 lacked endoflagella and its associated hook structure
  - type 3 had a proximal hook-like structure with normal morphology and no endoflagella
  - type 4 had a proximal hook-like structure with abnormal morphology and no endoflagella.

Revertants of types 2 and 3 had normal ultrastructure and motility.

- immotile mutants generated by chemical mutagenesis of *L. interrogans* serovar illini all had endoflagella of abnormal morphology.[77]
- Limberger and Charon performed chemical mutagenesis on *T. phagedenis* Kazan 5.[78] Immotile mutants lacked periplasmic endoflagella as assessed by electron microscopy and showed sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles consistent with an absence of the endoflagellar proteins. Revertants were all morphologically normal and motile.

Collectively, these studies indicate that an intact, morphologically correct, hook-endoflagellar structure is required for spirochaetal motility. Because similar *in vitro* mutagenesis experiments cannot be performed with *T. pallidum*, it is assumed that *T. pallidum* endoflagella serve an similar role.

1.5 Antigenic analysis of *T. pallidum* and its endoflagella

In the last decade, significant advances have been made in the delineation of the antigenic make-up of *T. pallidum* using Western blot analysis of both one-[37,48,79-91] and two-dimensional[39,79,92-98] polyacrylamide gels, and by radio-immunoprecipitation experiments.[39,79,99,100] Using polyvalent antisera, a clear profile of the major antigenic proteins has emerged: in a one-dimensional gel system 23 antigens are routinely identified, and in a two-dimensional system approximately 67 antigens are identified. Attempts have been made to standardize the molecular weights assigned to these antigens.[79] Similar antigenic analyses have been performed for the non-pathogenic treponemes. By comparing the antigenic profiles of *T. pallidum* with that of the non-pathogens, epitopes specific to *T. pallidum* have been identified.[48,78,80,81,84,86,88,95,96,101,102] Murine monoclonal antibodies which have been generated against *T. pallidum* antigens using a variety of immunization strategies and preparations[39,103-115] will enable more detailed antigenic analyses to be performed.

Endoflagella have been purified from a number of spirochaetes, including *Leptospira* B16[116], *S. aurantia*[117], *S. stenostrepta*, *S.*

zuelzerae, and *S. litoralis*[118], *T. zuelzerae*[119], *T. hyodysenteriae*[120], *T. phagedenis* biotype Reiter[102,121], *T. pallidum*[122,123], and *Borrelia burgdorferi*.[124,125] The different procedures share common steps:

- *removal of the outer membrane*: incubation in n-butanol, repetitive washing in phosphate buffers, or incubation in either ionic or nonionic detergents.
- *cell lysis and release of endoflagella*: incubation in detergents, repetitive freeze/thaw cycles, blending in an omnimixer, or ultrasonic disruption of endoflagella into fragments.
- *purification of the endoflagellar fraction*: isopycnic density gradient centrifugation using either potassium bromide, cesium chloride or rubidium chloride gradients.

Ultrastructurally, treponemal endoflagella are comprised of a shaft or core which is covered by a sheath.[126] *T. pallidum* endoflagella have a diameter of 16 to 22 nm[43]; the core has a diameter of 10-16 nm and is composed of a longitudinal row of globular subunits.[43] An insertion apparatus at the base of the endoflagellum is differentiated into a terminal knob composed of a single insertion disk and a proximal hook.[43] SDS-PAGE and two dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses indicate that *T. pallidum* endoflagella are composed of a number of proteins[100,122,123]; at least 37-kD, 34.5-kD, 33-kD, and 30-kD species have been identified.

The disposition of these protein elements within the endoflagella is clearly of interest. The sheath is composed of the 37-kD antigen and the remaining protein elements comprise the core. There are three lines of evidence which support this conclusion:

- Cockayne *et al.*[127], using monoclonal antibodies, demonstrated that removal of the sheath with urea and trypsin results in the loss of binding of a monoclonal antibody directed against the 37-kD antigen.

- epitope bridging studies were reported by Blanco *et al.*[123] This technique is a modification of Western blotting.[128] Whole *T. pallidum*, separated by SDS-PAGE, is transferred to nitrocellulose and is incubated sequentially in polyclonal sera directed against purified endoflagella and radio-iodinated purified intact endoflagella. The authors argue that antibodies directed against the sheath protein(s) will bind both to the blotted proteins and to the radio-iodinated endoflagella. As only the 37-kD antigen was identified by autoradiography, they infer that the sheath is composed only of this protein.

- a monoclonal antibody directed against an epitope of the 39-kD protein of *T. phagedenis* immunoprecipitates all of the major endoflagellar proteins[78] (the 39-kD protein is analogous structurally to the 37-kD protein of *T. pallidum*).

N-terminal sequence analysis indicate that the 37-kD protein is divergent from the other endoflagellar proteins.[100] On the basis of these studies, the endoflagellar proteins can be divided into two families[100]:

- the "A" family contains a single member, the 37-kD sheath protein (gene designation *fla*A), and
- the "B" family contains the core proteins (gene designations *fla*B1, *fla*B2, *fla*B3).

Furthermore, FlaB1, FlaB2, and FlaB3 share major N-terminal sequence homologies with other bacterial flagellins whereas the FlaA protein shows no such homologies.[100,129-135]

1.6 Is a syphilis vaccine possible?

The increasing epidemic of syphilis and the role of genital ulcer disease in HIV transmission have added impetus to the development of vaccines to prevent infection with *T. pallidum*. Vaccine research has been complicated by the inability to continuously cultivate *T. pallidum in vitro*, which has severely limited detailed antigenic analysis. Further, any organisms obtained are invariably contaminated with host tissues. In an attempt to overcome these difficulties a number of investigators have utilized recombinant DNA technology to clone treponemal genes and to express treponemal proteins in *E. coli*.[61,107,109,112,115,128,136-159] This has enabled researchers to start characterising individual treponemal antigens and it is hoped that these studies will facilitate vaccine development by allowing detailed studies of the pathogenetic mechanisms of *T. pallidum*.

Several studies suggest a vaccine against syphilis may be possible. Two successful animal immunization studies using whole

organisms as the immunogen have been reported:

- Miller[160] demonstrated complete resistance to intradermal challenge with the homologous *T. pallidum* strain in New Zealand white rabbits which had received intravenous immunization weekly over a 37 week period with γ-irradiated, living *T. pallidum* Nichols.
- Metzger[161] reported 41% protection to intradermal challenge with the homologous *T. pallidum* strain in rabbits immunized intravenously over an 11 week period with organisms that had been aged at 4°C.

These immunization strategies are clearly impractical in man. Complete protection against homologous challenge has not been achieved in rabbits immunized with either recombinant or native *T. pallidum* antigens. Studies include:

- Champion *et al.*[162] immunized New Zealand white rabbits subcutaneously for 32 weeks with a total of 450 µg purified *T. pallidum* endoflagella mixed with either complete or incomplete Freund's adjuvant. Following intradermal challenge with *T. pallidum*, lesions developed in both immunized and control animals, but they developed earlier and were atypical in the immunized animals.
- Borenstein *et al.*[163], using purified recombinant *T. pallidum* antigen 4D as immunogen, also produced accelerated lesions in the immunized animals.
- Lovett and co-workers[162] produced accelerated lesions in New Zealand white rabbits using as immunogen the hydrophobic

immunogenic proteins extracted by phase-partitioning of whole *T. pallidum* antigens in the detergent Triton X-114.

- Strugnell *et al.*[164,165] expressed *T. pallidum* antigens TmpA, TmpB, TmpC, TpE, TpF1, and TpF2 in *aro*A *S. typhimurium*. Rabbits immunized with *S. typhimurium* expressing TmpA, TmpB, and TmpC had accelerated lesion development.

Champion speculates that the early development of atypical lesions in immunized animals indicates an enhancement of the cellular immune response against *T. pallidum* as a consequence of immunization.[162]

1.7 Experimental aims of this study

The endoflagellar apparatus is the putative mediator of treponemal motility. In addition, antibodies directed against endoflagellar epitopes cross-reactive between commensal treponemes and *T. pallidum* have been implicated in immunity to syphilitic infection[166], suggesting that humoral response to the endoflagellar proteins is part of the development of protective immunity to *T. pallidum* during syphilis. Further, early, atypical chancres develop in rabbits immunized with isolated endoflagella following intradermal challenge with *T. pallidum* (*q.v.*).[162] These data provide compelling reasons to investigate further the endoflagella apparatus.

The studies reported in this thesis were carried out to further characterize the endoflagellar antigens of *T. pallidum*. This was

achieved through antigenic and genetic analysis of the sheath protein of *T. pallidum* endoflagella. The sheath protein was chosen for these studies for several reasons:

- it is the most exposed of the endoflagellar antigens.[78,123,126,127]
- comparative studies between pathogenic and non-pathogenic treponemes indicate that the sheath proteins are the more antigenically divergent, whereas the core proteins share significant homologies with other core proteins and other bacterial flagellins.[100,123,167]
- development of antibody against the 37-kD antigen occurs early in the host humoral response in both experimental[87,89,93] and natural syphilitic infection.[82,85,96,168]
- in preliminary studies, the endoflagellar sheath protein causes a proliferative response in splenic lymphocytes from rabbits with experimental syphilis.[169]
- as the endoflagellar sheath protein is one of the few proteins of *T. pallidum* which has been unequivocally localized within the organism, it will be extremely useful as an ultrastructural marker to delineate the ultrastructural locations of the major treponemal antigens.

The research plan adopted is outlined in Figure 4. Both protein purification and recombinant DNA techniques were used to determine the nucleotide sequence of *flaA*. Using these data, recombinant FlaA was expressed in *Escherichia coli*. In addition, comparative analysis of endoflagella from several treponemes was

also performed as part of the work undertaken in these studies.

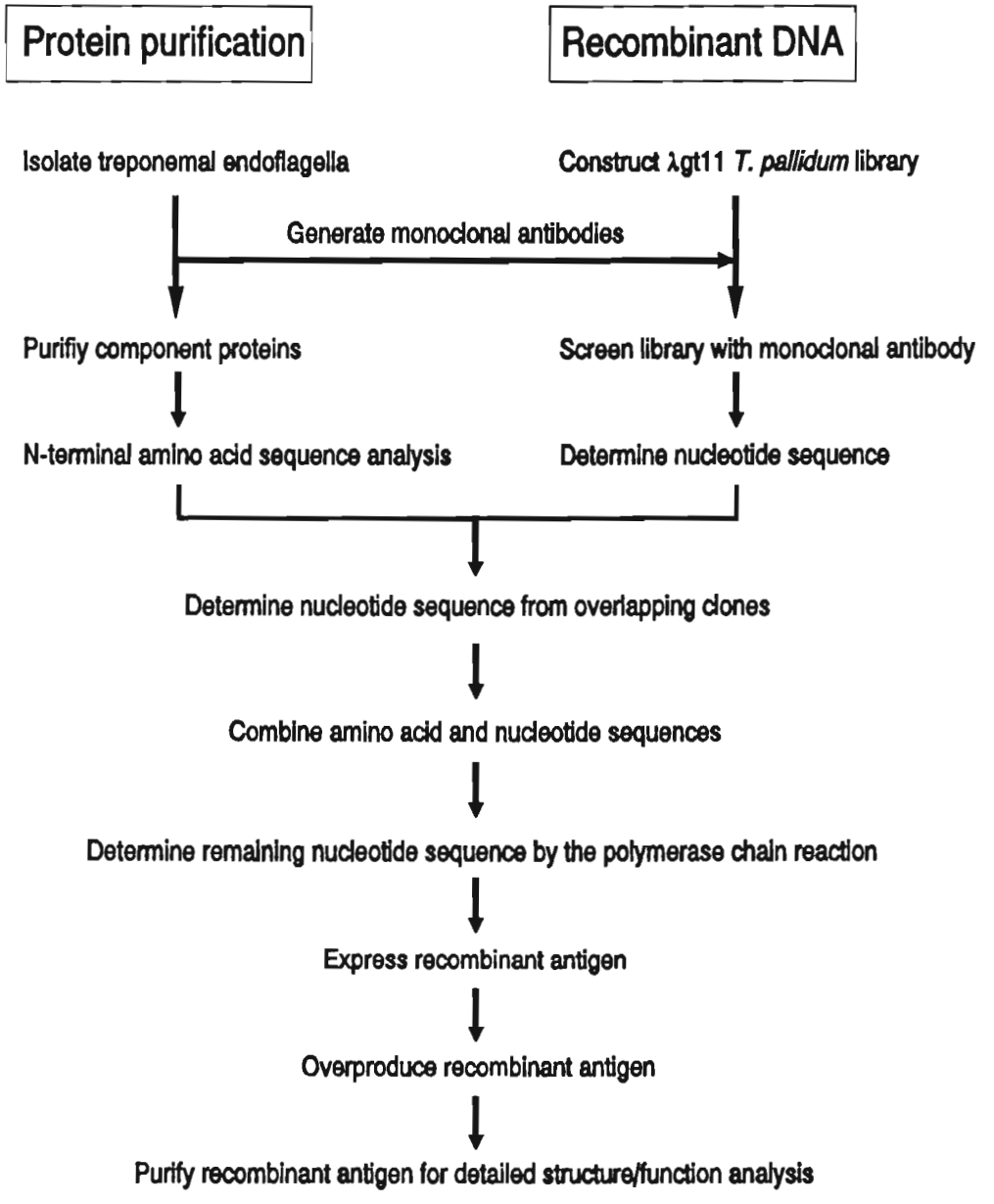


Figure 4. Outline of the studies detailed in this thesis.

2. Materials and Methods

2.1 Culture media. Culture media used in these studies include:

- *LB medium*. 1% [wt/vol] NaCl, 1% [wt/vol] Bacto yeast extract (Difco Laboratories, Detroit, MI), 0.5% [wt/vol] Bacto tryptone (Difco Laboratories).
- *CY medium*. 0.2% [wt/vol] KCl, 0.3% [wt/vol] NaCl, 0.5% [wt/vol] Bacto yeast extract, 1% [wt/vol] casamino acids (Difco Laboratories).
- *NZY medium*. 0.5% [wt/vol] NaCl, 0.2% [wt/vol] $MgSO_4.7H_2O$, 0.5% [wt/vol] Bacto yeast extract, 1% [wt/vol] NZ amine (casein hydrosylate, ICN Biomedicals, Inc., Lisle, IL).
- *M9 medium (with glucose and thiamine)*. M9 salts (1 x M9 salts is 0.6% [wt/vol] Na_2HPO_4, 0.3% [wt/vol] KH_2PO_4, 0.05% [wt/vol] NaCl, 0.1% [wt/vol] NH_4Cl), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% [wt/vol] glucose, 5 mg/l Vitamin B_1.
- *2TY medium*. 1.6% [wt/vol] Bacto tryptone, 1% [wt/vol] Bacto yeast extract, 0.5% [wt/vol] NaCl.

In general, *E. coli* strains were grown in LB medium supplemented with appropriate antibiotics: ampicillin 50 mg/l, tetracycline 20 mg/l, or kanamycin 40 mg/l. *Col*E1-derived plasmids with medium copy number were amplified by the addition of chloramphenicol, 100 mg/l, at mid-log growth. Solid phase growth of *E. coli* was supported on LB agar 1.5% [wt/vol] plates supplemented with appropriate antibiotics. *E. coli* strain DH5αF′ was grown on M9 (with glucose and thiamine) agar 1.5% [wt/vol] plates to preserve the F′ sex plasmid. *E. coli* strain XL1-Blue was grown on LB agar 1.5% [wt/vol] supplemented with tetracycline as a tetracycline

resistance gene had been introduced into the F' plasmid. *E. coli* clones containing recombinant pUC or pBluescript plasmids were grown on media containing 0.5 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) and 0.004% [vol/vol] 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) to allow for blue/white screening.

E. coli clones containing recombinant M13 were isolated on LB soft agar 0.7% [wt/vol] overlays containing 0.5 mM IPTG, 0.004% [vol/vol] X-Gal, and either *E. coli* DH5αF' or XL1-Blue. M13 phage was recovered from these clones by growth in 2TY medium. λgt11 and λ2001 phage clones were isolated on NZY soft agarose 0.7% [wt/vol] overlays containing appropriate *E. coli* cells; recipient *E. coli* cells were grown in CY media supplemented with 0.2% [wt/vol] maltose and 10 mM $MgSO_4$. Stocks of λ phage were stored in λ diluent (λ diluent is 100 mM NaCl-10 mM $MgSO_4.7H_2O$-50 mM Tris-HCl [pH 7.5] containing 0.01% [wt/vol] gelatin) over chloroform.

2.2 Bacterial strains. Bacterial strains used in these studies are listed in Table 1.

T. pallidum (Nichols) and *T. pertenue* (Haiti B) were passaged by intratesticular inoculation in New Zealand white rabbits without the use of hydrocortisone.[42,48] Briefly, one ml of a suspension containing approximately 5 x 10^7 treponemes per ml in phosphate buffered saline (PBS) (PBS is 0.01 M phosphate buffer [pH 7.4]-0.15 M NaCl) was injected into each testicle using sterile

Table 1. Bacterial strains used in these studies.

Bacteria	Strain	Genotype	Source/Reference
Escherichia coli K-12	Y1088	Δ(*lac*)U169 *sup*E *sup*F *hsd*R *met*B *trp*R *ton*A21 *pro*C::Tn5, {pMC9}	170
Escherichia coli K-12	Y1089	Δ(*lac*)U169 Δ(*lon*) *ara*D139 *str*A *hfl*A150::Tn10, {pMC9}	170
Escherichia coli K-12	Y1090	Δ(*lac*)U169 Δ(*lon*) *hsd*R *ara*D139 *str*A *sup*F *mcr*A *trp*C22::Tn10, {pMC9}	170
Escherichia coli K-12	JM83	*ara* Δ(*lac-pro*AB) *rsp*l F80 *lac*ZΔM15	171
Escherichia coli K-12	JM109	*rec*A1 *end*A1 *gyr*A96 *thi* *hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB), [F', *tra*D36 *pro*A^+B^+ *lac*$I^qZ\Delta M15$]	171
Escherichia coli K-12	DH5α	F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-,m_K^+) *sup*E44 λ^- *thi*-1 *gyr*A *rel*A1	Bethesda Research Laboratories Life Technologies, Inc.

Bacteria	Strain	Genotype	Source/Reference
Escherichia coli K-12	DH5αF′	DH5α F′	Bethesda Research Laboratories Life Technologies, Inc.
Escherichia coli K-12	XL1-Blue	*end*A1 *hsd*R17 (r_K^- ,m_K^+) *sup*E44 *thi*-1 λ^-*rec*A1 *gyr*A96 *rel*A1 (*lac*$^-$) [F′, *pro*AB, *lac*$I^qZ\Delta M15$, Tn10, (tet^r)]	Stratagene
Escherichia coli K-12	HB101	F^- *mcr*B *mrr* *hsd*S20(r_B^-,m_B^-) *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 *leu* *mtl*1 λ^-	172
Escherichia coli K-12	RR1	F^- *hsd*S20(r_B^-,m_B^-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 *leu* *mtl*1 λ^-	173
Escherichia coli K-12	ORN103	*thr* *leu* *min*A *min*B *gal* *thi* *str* *rec*A *lac*U169	174-176
Escherichia coli K-12	LE392	*hsd*R514 *hsd*M *sup*E44 *sup*F58 *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55	177,178
Escherichia coli K-12	P2LE392	LE392 (P2 lysogen)	177,178

Bacteria	Strain	Genotype	Source/Reference
Treponema pallidum subsp. *pallidum*	Nichols		179
Treponema pallidum subsp. *pertenue*	Haiti B		180
Treponema phagedenis biotype Reiter*			181
*Treponema vincentii**			106
*Treponema scoliodontum**			106
*Treponema denticola**			106
Spirochaeta aurantia M1†			182

* Cultivatable treponemes were kindly provided by J.N. Miller, University of California, Los Angeles, CA

† *S. aurantia* was kindly provided by E.P. Greenberg, University of Iowa, Des Moines, IA

technique. When the orchitis was clinically at a maximum (approximately 10 days later for *T. pallidum* and 14 days later for *T. pertenue*) the rabbits were sacrificed and the treponemes were extracted by agitating the excised testicles in PBS. The organisms were enumerated by darkfield microscopy using the 400 x objective; ten fields were averaged using a conversion factor of 5×10^5 for each organism seen per high dry darkfield. Large testicular debris was removed by two consecutive centrifugations for 7 min each in a clinical centrifuge.

If no further purification was planned, the treponemes were collected by centrifugation at 30,000 x g for 20 min at 4°C. Otherwise, the treponemes were purified further by Percoll density gradient centrifugation.[183] Briefly, 10 ml of treponemal suspension containing approximately 2×10^8 organisms per ml was overlaid upon 20 ml of 43% [vol/vol] Percoll (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) in PBS. Following centrifugation at 34,800 x g for 60 min at 4°C, the band containing treponemes was recovered and contaminating Percoll was removed by ultracentrifugation at 100,000 x g for 60 min at 4°C.

T. phagedenis biotype Reiter was cultivated in 2.9% [wt/vol] Spirolate broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% [vol/vol] heat inactivated normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) at 33°C.

E. coli strains Y1088, Y1089 and Y1090 were used for λgt11

library construction[170] and LE392 and P2LE392 were used for λ2001 library construction.[177] *E. coli* strains JM83[171], JM109[171], HB101[172], RR1[173], and DH5α (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD) were used as recipients for plasmid transformations. *E. coli* strains DH5αF′ (Bethesda Research Laboratories Life Technologies) and XL1-Blue (Stratagene, La Jolla, CA) were used as host strains for M13 phage.[171] *E. coli* ORN103 was used for minicell analyses.[176] The procedure of Hanahan[184] was used to transform *E. coli* K-12 strains with plasmid DNA.

2.3 Plasmid and oligonucleotide DNAs. Plasmid DNAs used in these studies are listed in Table 2. Oligonucleotides synthesized for these studies are listed in Table 3 and their location within the *T. pallidum* genome is shown in Figure 5.

2.4 Determination of protein concentration. Protein concentrations were determined by the BCA protein assay micromethod (Pierce, Rockford, IL) using bovine serum albumin as a standard.

2.5 Immunologic reagents. The production of murine monoclonal antibody H9-2 (immunoglobulin G_1 (IgG_1)) directed against a 37-kD antigen of *T. pallidum* has been described previously.[105] Murine monoclonal antibody 4H4-20 (IgG_1) was directed against the 39-kD endoflagellar sheath protein of *T. phagedenis* and cross-reacts with the equivalent *T. pallidum* protein. It was generated by immunizing Balb/c mice with endoflagella of *T. phagedenis* biotype

Table 2. Plasmid DNAs used in these studies.

Plasmid	Description*	Source/Reference
pBR322	Tc^r, Ap^r; replicon pMB1	173
pUC19	Ap^r; replicon pMB1	171
pUC18	Ap^r; replicon pMB1	171
M13mp18 RF	Replicative form of phage M13mp18	171
M13mp19 RF	Replicative form of phage M13mp19	171
pTTQ19	Ap^r; replicon pMB1	185
pBluescript II KS-	Ap^r; replicon pMB1; polylinker contains T7 promoter transcribing in opposite orientation to *lac* promoter	Stratagene
pT7-3†	Ap^r; replicon pMB1; *amp* is transcribed from T7 promoter	186
pGP1-2†	Km^r; replicon p15A; encodes for T7 DNA-dependent RNA polymerase under control of λ P_L promoter; temperature-sensitive λ repressor	186
pEX1	Ap^r; replicon pMB1; encodes for a *cro-lacZ* gene fusion with a 3′-located polylinker for insertion of foreign gene sequence; hybrid protein transcription is under control of λ P_L promoter; temperature-sensitive λ repressor	187
pRI1	pUC19 with 0.13 kb λA34 *Eco*R1-*Eco*R1 insert	This study

Plasmid	Description*	Source/Reference
pRI2	pBR322 with 6.6 kb *Treponema pallidum* subsp. *pallidum* chromosomal DNA fragment encoding amino acids 215 through 350 of pre-FlaA	This study
pRI3	pBR322 with 1.6 kb *T. pallidum* chromosomal DNA fragment encoding amino acids 215 through 350 of FlaA	This study
pRI3.1	pUC19 with 1.6 kb *Pst*I-*Pst*I fragment from pRI3 insert	This study
pRI3.3	pUC19 with 1.3 kb *Sal*I-*Pst*I fragment from pRI3 insert	This study
pRI3.4	pUC18 with 0.6 kb *Pst*I-*Hin*dIII fragment from pRI3 insert	This study
pRI3.5	pUC18 with 1.0 kb *Pst*I-*Hin*dIII fragment from pRI3 insert	This study
pRI3.6	pUC19 with 1.6 kb *Pst*I-*Pst*I fragment from pRI3 insert in opposite orientation to pRI3.1	This study
pRI4	pBR322 with a 1.7 kb *T. pallidum* chromosomal DNA fragment encoding amino acids 30 through 350 of pre-FlaA	This study
pRI4.1	pUC19 with 1.7 kb *Pst*I-*Pst*I fragment from pRI4 insert	This study
pRI4.2	pUC19 with 0.2 kb *Eco*RI-*Pst*I fragment from pRI4 insert	This study
pRI4.3	pUC19 with 1.5 kb *Eco*RI-*Pst*I fragment from pRI4 insert	This study

Plasmid	Description*	Source/Reference
pRI4.5	pUC19 with 1.0 kb *Hin*dIII-*Pst*I fragment from pRI4 insert	This study
pRI4.6	pUC18 with 0.8 kb *Eco*RI-*Hin*dIII fragment from pRI4 insert	This study
pRI5	pBR322 with 2.0 kb *T. pallidum* chromosomal DNA fragment encoding amino acids 215 through 350 of pre-FlaA	This study
pRI6	pBR322 with 2.2 kb *T. pallidum* chromosomal DNA fragment encoding amino acids 215 through 350 of pre-FlaA	This study
pRI7	pBR322 with 2.4 kb *T. pallidum* chromosomal DNA fragment encoding amino acids 215 through 350 of pre-FlaA	This study
pRI9	M13mp19 RF containing a *Sau*3A I-*Sau*3A I fragment derived from an inverse-PCR product generated from *T. pallidum* chromosomal DNA using primers oRI9 and oRI10 and identified by colony blot DNA-DNA hybridization with the intact inverse-PCR product	This study

Plasmid	Description*	Source/Reference
pRI10.1	0.26 kb portion of PCR product from *T. pallidum* chromosomal DNA amplified with primers oRI16 and oRI11. Product digested with *Eco*RI and cloned into pUC19 digested with *Sma*I and *Eco*RI	This study
pRI11	0.27 kb portion of PCR product from *T. pallidum* chromosomal DNA amplified with primers oRI11 and oRI1. Product digested with *Taq*I and *Eco*RI and cloned into pUC18 digested with *Acc*I and *Eco*RI	This study
pRI17	PCR product from *T. pallidum* chromosomal DNA amplified with primers oRI17 and oRI7. Product digested with *Pst*I and cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI18	PCR product from *T. pallidum* chromosomal DNA amplified with primers oRI18 and oRI7. Product digested with *Pst*I and cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI19	pBluescript II KS- digested with *Bam*HI and *Pst*I and containing 0.4kb *Bam*HI-*Eco*RI fragment of pRI18 and the 1.5 kb *Eco*RI-*Pst*I fragment from pRI4.3	This study

Plasmid	Description*	Source/Reference
pRI22.2	PCR product from *T. pallidum* chromosomal DNA amplified with primers oRI23 and oRI7. Product digested with *Pst*I and cloned into pUC18 digested with *Sma*I and *Pst*I	This study
pRI23	2.8 kb fragment of pRI22.2 digested with *Eco*RV and *Pst*I containing the 1.7 kb *Eco*RV-*Pst*I fragment of pRI4.	This study
pRI24	pRI23 is digested with *Kpn*I and the 3′ overhang filled with T4 DNA polymerase. Following digestion with *Pst*I, the 1.9 kb fragment is cloned into pEX1 digested with *Pst*I and *Sma*I	This study

*Abbreviations: Tc^r, Ap^r, and Km^r, resistance to tetracycline, ampicillin and kanamycin, respectively; kb, kilobase pairs; PCR, polymerase chain reaction

†S. Tabor and C. Richardson, Harvard University, supplied plasmids pT7-3 and pGP1-2.

Table 3. Oligonucleotides used in these studies.

Designation	Sequence	Coding strand*	Location†
flaA-related primers			
oRI1	5'-CCCATCTTCAAAACGCCCCCT-3'	-	590-609
oRI3	5'-AAGCTGAACGCAAAACACCGC-3'	+	184-203
oRI4	5'-AAATAACCAACATAGTC-3'	-	904-920
oRI5	5'-CACAGAGGAGGAGAAGG-3'	+	564-580
oRI7‡	5'-CCCTGCAGAACGCCAAAACACCGC-3'	-	1234-1257
oRI9	5'-TCATACCTCCACTCTTA-3'	-	210-226
oRI10	5'-TAAGAGAGTTGAGGTTG-3'	+	1023-1039
oRI11	5'-TCATTATGACGCGGAAA-3'	+	NK
oRI12	5'-TTTCCGCGTCATAATGA-3'	-	NK
oRI16	5'-ATGAAGAAAGCGGTTGT-3'	+	97-113
oRI17	5'-GAAAGGAGCGTTTGAATGAA-3'	+	82-101
oRI18**	5'-TAGTCGACGAGTGGTTATCTTATT-3'	+	54-77
oRI23††	5'-GTGATCGAGGGTAGAGATGAGTCAGTGCTC-3'	+	142-171
λgt11 sequencing primers			
oBG1	5'-GGTGGCGACGACTCCTGGAGCCCG-3'		
oBG2	5'-TTGACACCAGACCAACTGGTAATG-3'		

Designation	Sequence	Coding strand*	Location†
M13/pUC sequencing primers			
Forward primer	5'-TGACCGGCAGCAAAATG-3'		
Reverse primer	5'-CAGGAAACAGCTATGAC-3'		
-40 primer	5'-GTTTTCCCAGTCACGAC-3'		

* Oligonucleotides derived from the coding strand of *fla*A are shown as '+'; oligonucleotides derived from the non-coding strand of *fla*A are shown as '-'

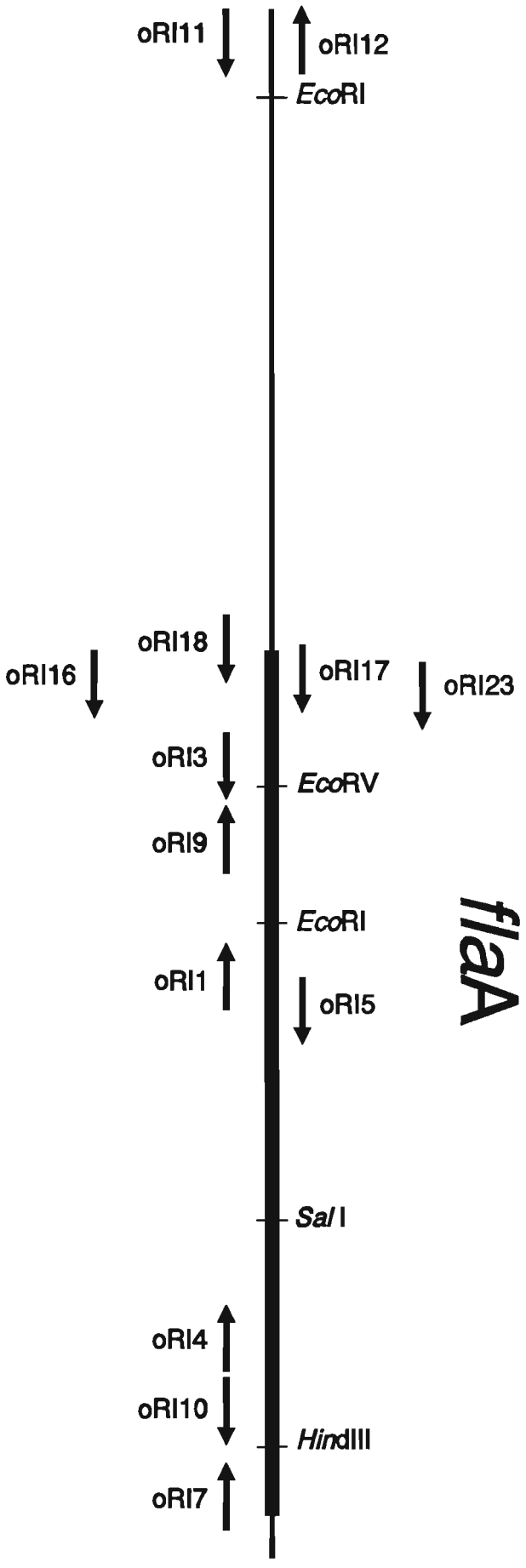
† The location of the oligonucleotides in *fla*A. Nucleotide position 1 is the first nucleotide of the sequence shown in Figure 19. The exact location of oRI11 and oRI12 are not known (NK).

‡ The 3' end of the primer has been altered to include a *Pst*I restriction endonuclease site

** The 5' end of the primer has been altered to include a *Sal*I restriction endonuclease site; in addition, this alters the putative '-10' promoter box of *fla*A.

†† The 5' end of the primer has been altered to encode for a Bovine Factor X_a protease site (Ile-Glu-Gly-Arg) immediately upstream of the first amino acid of mature FlaA.

Figure 5. Location of oligonucleotides used in these studies. The region of the *T. pallidum* subsp. *pallidum* genome in the vicinity of *flaA* (thick line) is shown. The 5′ to 3′ orientation of the oligonucleotide sequences is indicated by the direction of the arrow. The exact location of oRI11 and oRI12 is not known as their sequences were derived from a randomly selected fragment of an inverse-PCR product (see text).


oRI11
oRI12
EcoRI
oRI18
oRI16
oRI17
oRI23
oRI3
EcoRV
oRI9
EcoRI
flaA
oRI1
oRI5
SalI
oRI4
oRI10
HindIII
oRI7

Reiter and was identified by a solid-phase radioimmunoassay using sonicated *T. pallidum* as the antigen. Murine monoclonal antibody 11E3 (IgG_{2a}) directed against the 47-kD detergent-phase protein of *T. pallidum* has been described previously.[95]

Murine monoclonal antibody H9-2 was purified from hybridoma supernatants using recombinant Protein G-agarose (Gammabind G-agarose, Genex Corp., Gaithersburg, MD) according to the manufacturer's instructions. Rabbit antisera directed against isolated *T. pallidum* endoflagella were raised in female New Zealand white rabbits by priming with 100 µg isolated endoflagella in complete Freund's adjuvant (Difco Laboratories) intramuscularly followed by two intramuscular boosts of 50 µg in incomplete Freund's adjuvant (Difco Laboratories) given four and six weeks later. Rabbit antisera directed against *T. phagedenis* endoflagella, acetone-precipitated *T. pallidum* detergent-phase proteins, and β-galactosidase, respectively, were generated using the same protocol.

2.6 SDS-PAGE, 2D-PAGE, and immunoblotting. For SDS-PAGE, samples were suspended in SDS-PAGE gel loading buffer (62.5 mM Tris-HCl [pH 6.8] containing 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, and 0.001% [wt/vol] bromophenol blue), boiled for 10 min and then separated by electrophoresis using the discontinuous buffer system of Laemmli.[188] The stacking gel was 2.5% [wt/vol] acrylamide and the acrylamide concentration of the separating gel was varied depending upon the molecular

weight range resolution required. Both stacking and separating gels used 0.8% bis-acrylamide crosslinking.

Two dimensional polyacrylamide gel electrophoresis was performed using the procedure of O'Farrell[189] with modifications as described by Norris *et al.*[79] Samples, solubilized by repeated freeze-thawing in 2D-PAGE lysis buffer (9.5 M urea containing 4% [vol/vol] Nonidet 40 (NP-40, Sigma Chemical Co., St. Louis, MO), 3.2% [vol/vol] [pH 5-7] ampholines (LKB, Bromma, Sweden), 0.8% [vol/vol] [pH 3.5-10] ampholines (LKB), and 1% [vol/vol] 2-mercaptoethanol), were separated in the first dimension by isoelectric focusing (IEF) using 2.5 mm x 11 cm (4% acrylamide with 0.8% bis-acrylamide crosslinking) tube gels containing 3.2% [vol/vol] [pH 5-7] and 0.8% [vol/vol] [pH 3.5-10] ampholines, 2% [vol/vol] NP-40, and 9.5 M urea. The second dimension consisted of SDS-PAGE using an 8-20% polyacrylamide gradient gel; the tube gel was soaked in SDS-PAGE gel loading buffer (without glycerol or bromophenol blue) for 5 min prior to being loaded on and secured to the stacking gel in 1.5% [wt/vol] agarose in SDS-PAGE gel loading buffer (without glycerol).

SDS- and 2D-polyacrylamide gels were either stained or transferred to nitrocellulose sheets (0.2μm pore size, Schleicher & Schuell, Inc., Keene, NH) for immunoblotting.[48]

- *Stained gels*. Either Coomassie brilliant blue or silver were used to stain the polyacrylamide gels.
    - Coomassie brilliant blue. Gels were incubated in stain

(1.7 M acetic acid containing 50% [vol/vol] ethanol and 0.05% [wt/vol] Coomassie brilliant blue R-250 (Sigma Chemical Co.)) for at least 30 min and then placed in destain (1.7 M acetic acid containing 10% [vol/vol] methanol and 2% [vol/vol] glycerol) until background staining had cleared.

• Silver stain. A modification of the procedure of Wray *et al.*[190] was performed. Briefly, the polyacrylamide gel was incubated in 50% [vol/vol] methanol with several changes of methanol. The gel was incubated in the stain (0.16 M NH_4OH containing 0.06% [wt/vol] NaOH and 0.8% [wt/vol] $AgNO_3$), washed three times in water, and then incubated in developer (0.005% [wt/vol] citric acid, 0.002% [vol/vol] formaldehyde) in the dark until nearly developed. The gel was then washed several times in 50% [vol/vol] methanol, with the first wash being performed in the dark.

• *Immunoblotting*. When horse-radish peroxidase-conjugated probes were used for Western blotting, blots were blocked in PBS containing 0.05% [vol/vol] Tween-20 (Sigma Chemical Co.) for at least 15 min at room temperature. The blots were incubated sequentially in the primary, secondary, and if necessary, tertiary antibody. For each antibody, the blot was incubated at room temperature for at least one hour and then washed repeatedly in a large volume of PBS-Tween-20. Finally, the blot was rinsed twice with water prior to development in 50 mM Tris-HCl [pH 7.6]-200 mM NaCl containing 0.06% [wt/vol] 4-chloro-1-naphthol and 0.03% [vol/vol] hydrogen peroxide.

When [^{125}I]-labelled probes were used, Western blots were performed using TSA (50 mM Tris-HCl [pH 7.4]-150 mM NaCl containing 0.2% [wt/vol] sodium azide) for washes. Prior to the addition of each antibody, blots were blocked in 5% [wt/vol] milk powder in TSA for 30 min and then the antibody was added. Blots were washed repeatedly in large volumes of TSA after incubation in each antibody. Autoradiography was performed with enhancing screens at -70°C using Kodak XAR-5 film.

Antibodies were used in the following dilutions for Western blotting:

- H9-2 murine ascites was used in a dilution of 1:100.
- murine monoclonal antibody clone supernatants were used either undiluted or in a dilution of 1:10.
- rabbit antisera raised against isolated endoflagella were used in a dilution of 1:250.
- horseradish peroxidase conjugates of goat anti-mouse IgG, rabbit anti-goat IgG (Cappel-Worthington, Malvern, PA), rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA) and staphylococcal Protein A (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used in dilutions of 1:1,000.
- five to 10 µg of purified monoclonal antibody was used for each blot.

Goat anti-mouse IgG radiolabelled with ^{125}I was kindly provided by E. Vitetta (University of Texas Southwestern Medical Centre, Dallas, TX 75235).

2.7 Extrinsic radiolabelling of *T. pallidum*. Percoll-purified *T. pallidum* were radiolabelled with $Na^{125}I$ using the lactoperoxidase method.[92,106] Briefly, 2 x 10^8 *T. pallidum* suspended in PBS were mixed with lactoperoxidase in PBS and $Na^{125}I$ (100 µCi/µl, ICN Biomedicals, Inc.). Hydrogen peroxide was added with vortexing and the mixture incubated at room temperature for 15 min. An additional aliquot of hydrogen peroxide was added with vortexing and the mixture incubated for a further 15 min. Radiolabelled *T. pallidum* were recovered by centrifugation in a microfuge at room temperature for 10 min. Unincorporated radiolabel was removed by repeated washing in PBS.

2.8 Radioimmunoprecipitation (RIP).[106] [^{125}I]-labelled treponemes (approx. 10^7 cpm; 3 to 5 cpm per treponeme) were incubated with agitation in RIP-solubilization buffer (10 mM Tris-HCl [pH 7.8]-150 mM NaCl-10 mM EDTA containing 0.2% [wt/vol] Zwittergent 3-12 (Calbiochem-Behring, La Jolla, CA)) for 60 min at 37°C. Prewashed protein A-bearing *Staphylococcus aureus* (Cowan I) was added (200 µl) and the mixture incubated with agitation for a further 60 min at 4°C. Insoluble material, including nonsolubilized antigens and material which nonspecifically binds to protein A-bearing *S. aureus*, was removed by centrifugation at 13,000 x g for 10 min at room temperature. Antibody (10-20 µg of purified monoclonal antibody, 1 ml of clone supernatant, or 50 µl of polyclonal sera) was then added to the supernatant and the mixture incubated with agitation overnight at 4°C. A further 200 µl of protein A-bearing *S. aureus* was added and the mixture

incubated with agitation for 60 min at 4°C. *S. aureus*-protein A-antibody-antigen complexes were recovered by centrifugation at 13,000 x g for 10 min, and washed several times in PBS. The washed pellet was suspended in SDS-PAGE gel loading buffer, heated at 100°C for 10 min and the supernatant recovered by centrifugation at 13,000 x g for 10 min. The supernatant containing the immunoprecipitated radiolabelled antigens was analyzed by SDS-PAGE gel electrophoresis. Gels were processed in fluorographic enhancer (Autofluor, National Diagnostics, Sommerville, NJ) prior to drying down, and radiolabelled antigens were detected by autoradiography with enhancing screens at -70°C using Kodak XAR-5 film.

2.9 Radio-immunocolony blot assay to detect expressed antigens.

A simple solid-phase radioimmunoassay for the detection of *T. pallidum* antigens being expressed by individual *E. coli* colonies has been described previously.[146] Briefly, the pBR322 *T. pallidum* gene library in *E. coli* RR1 was plated to a density of 500 to 1000 colony forming units per 100 mm petri dish onto LB agar supplemented with tetracycline and incubated at 37°C for 14 hours. Colonies were lifted gently onto sterile, dry, Whatman no. 42 filter paper and lysed in 20 mM Tris-HCl [pH 8.0]-10 mM EDTA containing lysozyme 10 mg/ml for 30 min at room temperature. After an equal volume of 0.2% [vol/vol] Triton X-100 (Sigma Chemical Co.) was added, the filters were incubated a further 30 min at room temperature with gentle agitation. Filters were gently rinsed in 0.1 x PBS, air dried, and then fixed by brief

immersion in 10% [vol/vol] ethanol followed by air drying. Filters were blocked for 3 hr at 4°C in PBS containing 2% [vol/vol] fetal calf serum and 1 mM NaI and then incubated overnight at 4°C in the same buffer containing a 1:10 dilution of monoclonal antibody supernatant. After four washes in PBS, the filters were blocked in PBS-fetal calf serum-NaI and then incubated overnight at 4°C in the same buffer containing goat anti-mouse [^{125}I]IgG. Antibody-reactive colonies were identified by autoradiography with Kodak XAR-5 film at -70°C. Isolated clones were identified by repeated radio-immunocolony blot.

2.10 Isolation of treponemal endoflagella. *T. pallidum* was collected by centrifugation at 13,000 x g for 15 min at 4°C and washed twice by repeated centrifugation in PBS. The pellet was suspended in PBS-5 mM EDTA containing 0.5% [wt/vol] sarkosyl (N-lauroyl sarconsinate, Sigma Chemical Co.) and incubated overnight with mixing at 4°C; 10 ml of detergent solution was used per 1 x 10^{10} organisms. The insoluble material was collected by centrifugation at 30,000 x g for 20 min at 4°C and washed twice with ice-cold PBS by repeated centrifugation. The washed pellet then was suspended in 10 ml of PBS and sheared in a VirTis '23' blender (The VirTis Co. Inc., Gardiner, NY) at medium speed for 10 min. The cytoplasmic bodies and sheared endoflagella were separated by centrifugation at 20,000 x g for 20 min at 4°C. The supernatant, containing sheared endoflagella, was decanted and retained. The pellet was suspended in 10 ml of PBS, sheared as above for 15 min, and then combined with the retained

supernatant. The combined supernatants were centrifuged at 100,000 x g for 60 min at 4°C in a SW40Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The insoluble material was suspended in 26% [wt/wt] CsCl in PBS-5 mM EDTA containing 0.02% [wt/vol] sarkosyl and then subjected to isopycnic density gradient centrifugation at 160,000 x g for 48 hours at 4°C. The endoflagella band was collected and dialysed against distilled water, and the endoflagella were recovered by centrifugation at 100,000 x g for 60 min at 4°C.

Endoflagella of *T. phagedenis* biotype Reiter were isolated as above except that the sarkosyl concentration used during isopycnic centrifugation was 0.05% [wt/vol]. Endoflagella were isolated from radio-labelled *T. phagedenis* using this procedure; as no endoflagellar band was visible following isopycnic density gradient centrifugation, the gradient was removed in 500µl aliquots and the location of the endoflagella band was determined by scintillation counting (Figure 6).

2.11 Electron microscopy of isolated endoflagella. Specimens were prepared for whole-mount electron microscopy by the single droplet method using Parlodion (Ted Pella, Inc., Tustin, CA) and carbon-coated copper grids (400 mesh; Ted Pella, Inc.) which were glow discharged immediately before use.[48] Isolated endoflagella were floated on grids at 4°C, and the grids were washed with ice-cold PBS before negative staining was carried out at room temperature with 1% [wt/vol] uranyl acetate (Sigma Chemical Co.).

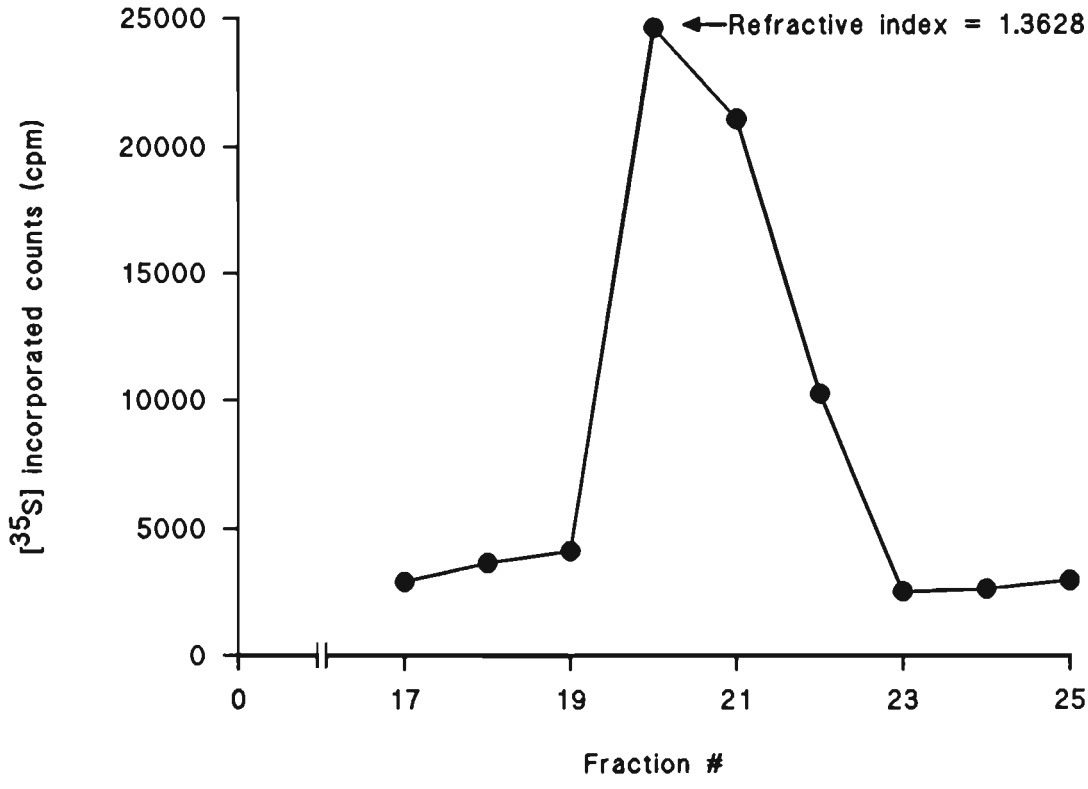


Figure 6. Isopycnic density gradient centrifugation of radiolabelled *T. phagedenis* endoflagella. Twenty five fractions were collected starting from the top of the gradient. The fractions containing radiolabelled endoflagella (Fraction # 20, 21 and 22) were identified by scintillation counting. The refractive index was estimated from parallel analysis.

Electron micrographs were taken at 80kV of accelerating voltage on a JEOL 100C electron microscope.

2.12 Radiolabelling of *T. phagedenis*. *T. phagedenis* were radiolabelled with Trans-[^{35}S]-label (an approximate 80:20 mixture of [^{35}S]-methionine and [^{35}S]-cysteine, ICN Biomedicals, Inc.) and [2,3,4,5-^{3}H]-leucine (1 mCi/ml, 115 mCi/mmol, ICN Biomedicals, Inc.) using a modification of the procedure of Stamm and Bassford.[141] 1 x 10^{10} *T. phagedenis* were recovered from spirolate broth by centrifugation at 13,000 x g for 15 min at 4°C and suspended aseptically in 1 ml of filter-sterilized (0.2µm) labelling media without radiolabel (0.5 x M9 salts containing 0.6% [wt/vol] glucose, 0.002% [wt/vol] thiamine, 0.1 mM $CaCl_2$, 7% [vol/vol] normal rabbit serum which has been heat inactivated at 50°C for 30 min, threonine 50 µg/ml, proline 50 µg/ml, aspartate 50 µg/ml, glycine 50 µg/ml, glutamate 50 µg/ml, histidine 50 µg/ml, isoleucine 50 µg/ml, lysine 50 µg/ml, phenylalanine 50 µg/ml, tryptophan 50 µg/ml, tyrosine 50 µg/ml, valine 50 µg/ml, arginine 100 µg/ml, cysteine 100 µg/ml, and thioglycolic acid 50 µg/ml). Nineteen ml of filter-sterilized labelling media containing 1.5 mCi of Trans-[^{35}S]-label and 1 mCi of [^{3}H]-leucine were added and the suspension incubated at 33°C for 20 hours without agitation. Treponemes were recovered by centrifugation at 13,000 x g for 15 min at 4°C and washed twice with cold PBS.

2.13 Purification of the endoflagellar proteins. Isolated endoflagella were suspended in guanidine hydrochloride (3 M final concentration) and heated for 30 min at 60°C prior to being loaded onto a 2.1 mm x 30 mm diameter RP300 reverse-phase column (Brownlee Laboratory, Santa Clara, CA) in 0.1% [vol/vol] trifluoroacetic acid (Pierce) in water for separation by high pressure liquid chromatography (HPLC) using the Applied Biosystems Model 130A separation system (Foster City, CA). The endoflagellar antigens were eluted sequentially in a gradient of increasing concentrations of 72% [vol/vol] acetonitrile (Mallinckdrodt, Paris, KY) and 0.085% [vol/vol] trifluoroacetic acid in water. Elution profiles were monitored by absorbance at 214 nm (A_{214}).

2.14 N-terminal amino acid sequence analysis. N-terminal amino acid sequence analyses were performed by J. Hanke (University of Texas Southwestern Medical Centre, Dallas, TX 75235). Samples containing either the purified 37-kD antigen or eluted peptides were applied directly onto an Applied Biosystems Model 477A protein sequencer for automated N-terminal amino acid sequence determination.

2.15 Tryptic peptide analysis. HPLC fractions containing purified endoflagellar proteins (5-10 µg) were dried under nitrogen and suspended in 50 µl of 0.1 M ammonium bicarbonate for overnight digestion with 50-100 ng of trypsin-L-1-chloro-3-(4-tosylamido-4-phenyl-2-butanone (Worthington Biochemical Corp., Freehold, NJ)

at 37°C. If N-terminal amino acid sequence analysis was planned, the tryptic peptides were separated as described above using a 2.1 mm x 100 mm RP-300 reverse-phase column.

2.16 DNA modifying reagents. Restriction enzymes and other DNA modifying enzymes were obtained from commercial sources and were used according to the manufacturer's instructions unless otherwise stated. Restriction endonuclease digestions were performed in a buffer containing 33 mM Tris-acetate [pH 7.9]-66 mM potassium acetate-10 mM magnesium acetate-3 mM spermidine-2.5 mM DTT containing bovine serum albumin 100 µg/ml. DNA-DNA ligations were performed in 1 x ligation buffer (50 mM Tris-HCl [pH 7.8]-10 mM $MgCl_2$-20 mM DTT-1 mM ATP containing bovine serum albumin 50 µg/ml) overnight at room temperature using T4 DNA ligase. Oligonucleotide probes and polymerase chain reaction-products were phosphorlyated in 1 x kinase buffer (50 mM Tris-HCl [pH 7.6]-10 mM $MgCl_2$-5 mM DTT containing bovine serum albumin 50 µg/ml) at 37°C for 30 min using T4 polynucleotide kinase (New England Biolabs, Beverly, MA); subsequently, the enzyme was heat-inactivated at 65°C for 20 min.

2.17 General DNA procedures.[191]

- *Gel electrophoresis*. Restriction endonuclease digestions and polymerase chain reaction (PCR) DNA amplification products were analysed by either ethidium bromide-agarose gel electrophoresis or polyacrylamide gel electrophoresis. The concentration of agarose or acrylamide, respectively, was varied depending on

the size of the DNA fragments of interest. Either TAE (0.4 M Tris-acetate-0.001 M EDTA) or TBE (0.089 M Tris-borate-0.089 M boric acid-0.002 M EDTA) was used as the buffer system. The agarose gels contained 0.00002% [wt/vol] ethidium bromide while the polyacrylamide gels were stained with ethidium bromide for 20 min following electrophoresis. Samples were loaded onto gels in DNA gel loading buffer (2 mM Tris-HCl [pH 8.0]-0.02 mM EDTA containing 5% [wt/vol] sucrose and 0.02% [wt/vol] bromophenol blue).

• *Electroelution*. The DNA sample was electrophoresed through either an agarose or a polyacrylamide gel. The DNA band of interest, identified under UV illumination, was cut from the gel, placed in a dialysis bag, and then submerged in a submarine gel rig containing either 0.05 x TBE or 0.05 x TAE. Electroelution was performed until no further ethidium bromide-stained material was present in the gel fragment. The DNA was recovered by ethanol precipitation.

• *Purification from low-melting point agarose gels*.[192] The DNA was electrophoresed through a low melting point Sea-Plaque (FMC Corp., Rockland, ME) agarose gel in the dark in a 4°C cold room. The DNA band of interest, identified under UV illumination, was cut from the gel and liquified in an eppendorf tube in a 37°C bath. An Elutip-d column (Schleicher & Schuell, Inc.) was washed with high salt buffer (0.02 M Tris-HCl [pH 7.5]-1 mM EDTA-1 M NaCl) and then equilibrated with low salt buffer (0.02 M Tris-HCl [pH 7.5]-1 mM EDTA-0.2 M NaCl). The liquid DNA-agarose was diluted in warm low salt buffer and applied to the Elutip-d

column. Contaminants were removed by washing with low salt buffer, and then the retained DNA was eluted using high salt buffer. The DNA was recovered by ethanol precipitation.

DNA samples were purified from protein contaminants by a combination of buffer saturated phenol, buffered saturated phenol/chloroform/isoamyl alcohol (25:24:1 [vol:vol:vol]), or chloroform/isoamyl alcohol (24:1 [vol:vol]) extractions.

2.18 Purification of plasmids and phage DNAs. Plasmid DNAs were purified by a number of methods:

- an alkaline lysis procedure was used for small-scale (1.5 to 5 ml cultures) plasmid DNA isolation.[193] Briefly, *E. coli* cells, recovered by centrifugation in a microfuge, were sequentially treated with 25 mM Tris-HCl [pH 8.0]-10 mM EDTA-50 mM glucose, 0.2 M NaOH containing 1% [wt/vol] SDS, and 5 M potassium acetate [pH 4.8]. Genomic DNA and bacterial debris were removed by centrifugation in a microfuge, and the DNA recovered from the supernatant by isopropanol precipitation.

- a boiling lysis procedure was used also for small-scale plasmid DNA isolation.[194] Briefly, *E. coli* cells, recovered by centrifugation in a microfuge, were suspended in 10 mM Tris-HCl [pH 8.0]-100 mM NaCl-0.1 mM EDTA containing 0.5% [vol/vol] Triton X-100 and lysozyme 2 mg/ml and then placed in a boiling water bath for 40 sec. Following centrifugation in a microfuge, the genomic DNA and bacterial cell debris which formed a tight, viscous pellet was removed using a sterile toothpick. The DNA was

recovered by isopropanol precipitation.

• plasmid DNA for double stranded sequencing was extracted from *E. coli* in late-log growth (5 ml cultures) using an alkaline lysis procedure. Prior to isopropanol precipitation, the DNA solution was phenol extracted. The DNA pellet was suspended in 10 mM Tris-HCl [pH 8.0]-1 mM EDTA containing 50 µg/ml DNase-free RNase and incubated at 37°C for 30 min. Following phenol extraction and several water saturated-ether extractions, the DNA was recovered by ethanol precipitation. For the sequencing reactions, plasmid DNA was denatured in NaOH followed by ethanol precipitation using ammonium acetate.

• medium scale plasmid DNA preparations from 100 ml cultures grown overnight used an alkaline lysis procedure followed by RNase treatment.

• ultra-pure, closed circular plasmid DNA was purified from large scale overnight cultures (500 to 1,000 ml) using an alkaline lysis procedure followed by two sequential CsCl-ethidium bromide isopycnic density gradient separations. Briefly, the isopropanol precipitated-DNA was dissolved in 10 mM Tris-HCl [pH 8.0]-1 mM EDTA and then mixed gently with solid CsCl (1 g per ml of DNA solution). Ethidium bromide (0.8 mg/ml of DNA solution) was added and the gradient formed by ultracentrifugation in a Ti70 rotor (Beckman Instruments, Inc.) at 42,000 rpm for 36 hours at 20°C. The band containing the closed circular plasmid DNA was recovered and further purified on a second CsCl-ethidium bromide density gradient. The ethidium bromide was removed by repeated phase-separation with water saturated 1-butanol, and the DNA

recovered by ethanol precipitation.

Single stranded DNA (ssDNA) for sequencing reactions was recovered from M13 phage clones obtained from the supernatant of 4 to 6 hour cultures grown in 2TY broth.[195] Briefly, the *E. coli* cells were removed by two sequential centrifugations in a microfuge. M13 phage, precipitated from the supernatant by the addition of an equal volume of 2.5 M NaCl containing 20% [wt/vol] polyethylene glycol 6000 (PEG 6000, Aldrich Chemical Co., Inc., Milwaukee, WI), were recovered by centrifugation in a microfuge at room temperature. The phage pellet was suspended in 10 mM Tris-HCl [pH 8.0]-1 mM EDTA and ssDNA isolated by phenol extraction followed by ethanol precipitation. This ssDNA was suspended in 10 mM Tris-HCl [pH 8.0]-0.1 mM EDTA.

For the purification of λA34 phage from an *E. coli* Y1089 lysogen, cells were grown to mid-log phase at 30°C with vigorous shaking in LB broth containing 10 mM $MgSO_4$. Following induction at 42°C for 30 min, the cultures were incubated at 37°C for a further two hours. Bacteria were collected by centrifugation at 5,000 x g for 10 min at 4°C, suspended in 10 mM Tris-HCl [pH 7.4]-50 mM NaCl-5 mM $MgCl_2$, and lysed by the addition of an equal volume of chloroform. Phase separation was achieved by centrifugation at 11,000 x g for 10 min at 4°C and the aqueous phase retained. *E. coli* DNA and RNA was removed by treatment with DNase and RNase. NaCl was added to a final concentration of 1 M and the insoluble debris removed by centrifugation at 11,000 x g for 10 min at 4°C.

Phage were precipitated from the supernatant by the addition of PEG 6000 (10% [wt/vol]), recovered by centrifugation at 11,000 x g for 10 min at 4°C, and suspended in 10 mM Tris hydrochloride [pH 7.7]-100 mM NaCl-10 mM $MgSO_4$.

The phage were purified further on a glycerol gradient using a modification of the procedure of vande Woude *et al.*[196] The bacteriophage suspension was layered onto to a glycerol step gradient composed of 5% [vol/vol] glycerol and 40% [vol/vol] glycerol steps (from top to bottom) in 10 mM Tris-HCl [pH 7.7]-100 mM NaCl-10 mM $MgSO_4$ and then ultracentrifuged in a SW40Ti rotor at 35,000 rpm for 60 min at 4°C. The pellet of bacteriophage was suspended in 10 mM Tris-HCl [pH 7.7]-100 mM NaCl-10 mM $MgSO_4$ containing DNase and RNase at a final concentration of 5 µg/ml and 1 µg/ml, respectively, and incubated at 37°C for 30 min. Following sequential phenol, phenol/chloroform/isoamyl alcohol, and chloroform/isoamyl alcohol extractions, the DNA was recovered by ethanol precipitation.

2.19 Purification of treponemal DNA. A number of protocols were used to purify treponemal chromosomal DNA. For all procedures, freshly extracted treponemes were harvested in PBS by centrifugation at 39,000 x g for 20 min at 4°C. The procedures used included:

A. Treponemes were washed twice in 0.14 M NaCl-75 mM sodium citrate [pH 7.0] by differential centrifugation and then incubated in 10 mM Tris-HCl [pH 8.0]-10 mM EDTA containing

50 µg/ml DNase-free RNase, 2 mg/ml lysozyme and 1% [wt/vol] tri-isopropylnaphthalene sulfonate (Eastman Kodak Co., Rochester, NY) for 5 min at 37°C. The solution was made to 1 M NaCl, incubated at 37°C for a further 5 min, then extracted with phenol/chloroform/isoamyl alcohol. The DNA was recovered by two sequential ethanol precipitations.

B. A modification of the method described by Hsu *et al.*[145] was used. Briefly, the treponemes were suspended in 10 mM Tris-HCl [pH 8.0]-5 mM EDTA containing 1 mg/ml lysozyme, 50 µg/ml proteinase K, 0.5% [wt/vol] SDS and incubated for 2 hours at 37°C. The solution was made to 1 M NaCl, extracted in phenol/chloroform/isoamyl alcohol, and the DNA recovered by ethanol precipitation. The DNA was suspended in 10 mM Tris-HCl [pH 8.0]-1 mM EDTA, containing 100 µg/ml DNase-free RNase and incubated at 37°C for 30 min. The DNA was recovered by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation.

C. A modification of the method described by Peterson *et al.*[150] was used. Briefly, the treponemes were suspended in 50 mM Tris-HCl [pH 8.0]-50 mM EDTA containing 1 mg/ml lysozyme and incubated on ice for 30 min. Proteinase K and SDS were added to a final concentration of 50 µg/ml and 0.5% [wt/vol], respectively, and the solution incubated at 50°C for 60 min. Following two phenol/chloroform/isoamyl alcohol extractions, the DNA was ethanol precipitated, then suspended in 10 mM Tris-HCl [pH 8.0]-1 mM EDTA containing 10 µg/ml DNase-free RNase. Following incubation at 37°C for 30 min the DNA was recovered by

phenol/chloroform/isoamyl alcohol extraction and subsequent ethanol precipitation.

D. The treponemes were suspended gently in 5 ml of 50 mM Tris-HCl [pH 8.0]-50 mM EDTA. Proteinase K (Boehringer Mannheim Biochemicals) was added to a final concentration of 100 µg/ml and the mixture incubated at 50°C for 5 min. SDS was then added (0.5% [wt/vol] final concentration), followed by incubation for two to four hours at 50°C. Sequential gentle extractions were performed with equal volumes of phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, respectively. After the addition of 1.25 g CsCl per ml to the aqueous phase, isopycnic density gradient centrifugation was performed in a V65Ti rotor (Beckman Instruments, Inc.) at 240,000 x g for 10 hours at 20°C. The gradient was collected in 500 µl aliquots and DNA-bearing fractions were identified by ethidium bromide-agarose gel electrophoresis prior to dialysis against 10 mM Tris-HCl [pH 8.0]-1 mM EDTA.

2.20 DNA-DNA hybridizations. Double stranded DNA probes for use in Southern blot analyses were purified either by electroelution of the individual DNA fragments from SeaKem GTG (FMC Corp.) agarose gels, or by extraction from low melting point agarose gels using an Elutip-d column. They were labelled with [α-^{32}P]dCTP (ICN Biomedicals, Inc.) using random-primer labelling (Boehringer Mannheim Biochemicals).[197] Dephosphorylated oligonucleotide probes were labelled with [γ-^{32}P]ATP (Du Pont Co. Biotechnology Systems, Wilmington, DE) using T4 polynucleotide

kinase.

DNA transfers to nylon membranes (Zetaprobe, Bio-Rad Laboratories, Richmond, CA; Hybond-N, Amersham Corp., Arlington Heights, IL) were performed by passive transfer.[198] When double stranded DNA probes were used, membranes were prehybridized in 1 M NaCl containing 10% [wt/vol] dextran sulphate (Pharmacia LKB Biotechnology, Inc.) and 1% [wt/vol] SDS at 65°C. Radiolabelled probe (2-5 x 10^5 cpm/ml final concentration) and heat denatured salmon sperm DNA (100 µg/ml final concentration; Sigma Chemical Co.) were added and hybridization was performed at 65°C for 14 hours. After two brief washes in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) at room temperature, the following higher stringency washes were performed: two washes in 2 x SSC containing 0.1% SDS [wt/vol] at 65°C for 30 minutes, followed by two washes in 0.1 x SSC containing 0.1% SDS [wt/vol] at 65°C for 10 minutes.

When oligonucleotide probes were used, prehybridization and hybridization were performed at 42°C in 6 x SSC-50 mM sodium phosphate buffer [pH 6.8] containing 10% [wt/vol] dextran sulphate, 5 x Denhardts solution (1 x Denhardts solution is 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, 0.02% [wt/vol] bovine serum albumin), and 100 µg/ml of denatured, sheared, salmon sperm DNA[199]; three washes in 6 x SSC at room temperature for 10 min each were performed prior to a stringency wash at 45°C in 6 x SSC containing 0.1% [wt/vol] SDS for 30 min.

Colony blot DNA-DNA hybridization studies were performed using nitrocellulose filters (Millipore Corp., Bedford, MA) as described above. Prior to prehybridization, filters were treated sequentially with 0.5 M NaOH, 1 M Tris-HCl [pH 7.6], and 0.5 M Tris-HCl [pH 7.6]-1 M NaCl, followed by baking at 80°C under vacuum for 2 hours.

DNA-DNA hybridization studies to identify recombinant phage plaques were performed using nitrocellulose filters (Millipore Corp.) as described above. Prior to prehybridization, filters were treated sequentially for 30-60 sec at each step with 0.1 M NaOH-1.5 M NaCl, 0.2 M Tris-HCl [pH 7.5], 0.2 M Tris-HCl [pH 7.5], and 2 x SSC, followed by baking at 80°C under vacuum for 2 hours.

Autoradiography was performed with enhancing screens at -70°C using Kodak XAR-5 film. In order to re-use membranes used for Southern transfers or colony blots, bound probe was removed by sequential washes in 0.5 M NaOH, 0.5 M NaOH, 1 M Tris-HCl [pH 7.6], and 0.5 M Tris-HCl [pH 7.6]-1 M NaCl.

2.21 Construction and screening of *T. pallidum* chromosomal DNA libraries. Many genomic DNA libraries, both random and directed, were constructed during these studies:

- *Directed libraries*. Chromosomal DNA was digested to completion with restriction endonucleases. In some instances the digested DNA was size-fractionated using either a SeaPlaque low-

melting point agarose gel and purified using an Elutip-d column or by electroelution from SeaKem GTG agarose gels. The chromosomal DNA fragments were ligated into either plasmid (i.e. pUC18, pUC19) or phage (i.e. λgt11, λ2001) vectors digested with compatible restriction endonucleases and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals). Libraries constructed in plasmids were grown on LB agar containing ampicillin to a density of approximately 5,000 colonies per 150 mm petri dish and were screened in duplicate by colony blot DNA-DNA hybridization (see above). Libraries constructed in phage vectors were packaged with Gigapack Gold packaging extracts (Stratagene) according to the manufacturer's instructions and were screened in duplicate using plaque transfer and DNA-DNA hybridization (see above) at a density of approximately 50,000 plaques per 150 mm petri dish. λ2001 libraries were initially plated onto both *E. coli* LE392 and P2LE392 in order to assess the efficiency of library construction, but were screened using *E. coli* LE392 only.[200,201]

- *Random libraries*. Two random *T. pallidum* chromosomal DNA libraries were used in these studies:

  - a λgt11 *T. pallidum* genomic library containing inserts ranging in size from 0.25 to 1.1 kb was constructed and screened with monoclonal antibody H9-2 in the laboratory of N. Agabian (Harborview Medical Centre, Seattle, WA 98104). The λA34 lysogen was kindly provided by Dr Agabian.

  - a pBR322 library was constructed by M.V. Norgard (University of Texas Southwestern Medical Centre, Dallas, TX

75235). *T. pallidum* chromosomal DNA was partially digested with *Hae*III and *Alu*I, size-fractionated by sucrose density gradient centrifugation, and cloned into the *Pst*I site of pBR322 after G-C tailing.[146] In this work, I screened this library both by radio-immunocolony blot assay with monoclonal antibodies (see above) and by colony blot DNA-DNA hybridization (see above).

2.22 DNA sequence analysis. DNA sequence analysis was performed using Richardson's modification[202] of the dideoxy termination method of Sanger[203] utilizing modified-T7 DNA polymerase (Sequenase 2.0, United States Biochemicals Corp., Cleveland, OH). Single stranded DNA isolated from recombinant M13 phage clones and double stranded plasmid DNA were used for sequencing reactions.[171] Both standard M13 sequencing primers and oligonucleotides derived from the nucleotide sequence of *fla*A were utilized to sequence both strands of *fla*A (Table 3). Sequencing reactions were analyzed on 6% polyacrylamide TBE gels containing 8 M urea at 35 W constant power. Areas of compression were resolved by either using 7-deaza-dGTP (United States Biochemicals Corp.) in the sequencing reactions or by the addition of 25% [vol/vol] formamide (Bethesda Research Laboratories Life Technologies, Inc.) to the sequencing gels. The University of Wisconsin Genetics Computer Group sequence analysis software package[204] was used in conjunction with Microgenie software (Beckman Instruments, Inc.) for DNA sequence analysis.

2.23 DNA amplification by the polymerase chain reaction. DNA amplification was performed using 2.5 units *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT) and a Perkin Elmer Cetus thermocycler.[205] The PCR was carried out in a volume of 100 µl in the presence of 1 x PCR buffer (10 mM Tris-HCl [pH 8.3]-50 mM KCl-1.5 mM $MgCl_2$ containing 0.01% [wt/vol] gelatin), 200 µM of each deoxynucleoside and 1 µg of each primer; the template DNA was either 1 µg of chromosomal DNA or 1 ng of plasmid or phage DNA. PCR conditions for each pair of oligonucleotide primers are outlined in Table 4. Inverse-PCR[206] was performed as shown in Figure 7.

PCR products were analyzed by ethidium bromide-agarose gel electrophoresis and purified in one of the following manners: 1) from low melting point agarose gels using an Elutip-d column, 2) by electroelution from SeaKem GTG agarose gels, or 3) by using Centricon-30 microconcentrators (W.R. Grace & Co., Danvers, MA).[207]

2.24 Expression of the 37-kD sheath protein. Bacteria were grown at 30°C to an A_{590} of 1.5 in LB broth containing ampicillin (40 µg/ml) and kanamycin (40 µg/ml). The temperature was shifted to 42°C for 25 min and then rifampin was added to a final concentration of 200 µg/ml. The culture was incubated for 2 hours at 30°C and the cells were recovered by centrifugation.

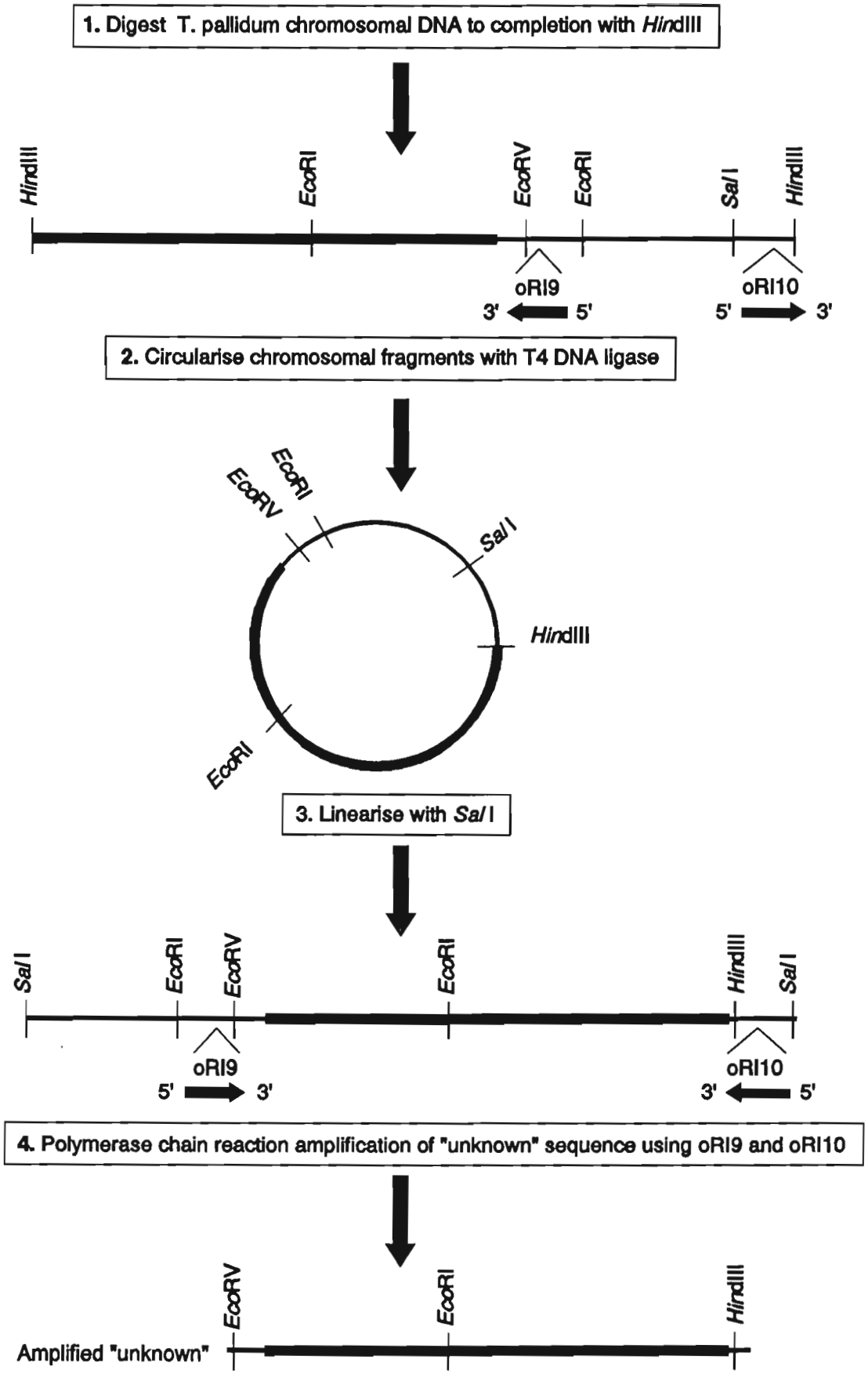
Table 4. Polymerase chain reaction conditions*.

Primers	n	Denaturation		Annealing		Extension		Comments
		T	t	T	t	T	t	
oBG1, oBG2	50	94	1	50	1	72	1	Standard
oRI9, oRI10	25	94	2	48	2	72	3	Inverse
oRI1, oRI11	40	94	2	48	2	72	3	Asymmetric
oRI1, oRI11	25	94	2	48	2	72	3	Standard
oRI1, oRI12	25	94	2	48	2	72	3	Standard
oRI7, oRI17	30	94	2	66	2	72	2	Standard
oRI7, oRI18	30	94	2	66	2	72	2	Standard
oRI7, oRI23	30	94	2	72	5			Standard†

*Abbreviations: n, number of cycles; T, temperature in °C; t, time in min; Standard, standard PCR; Inverse, inverse PCR; Asymmetric, asymmetric PCR

†temperatures for annealing and extension are the same

Figure 7. Outline of the inverse-PCR procedure used to amplify the upstream regions of *fla*A. The region of unknown nucleotide sequence containing the control regions of *fla*A is indicated by the thick line (**Box 1**). Initially, oligonucleotides oRI9 and oRI10 cannot be used for amplification of DNA by the polymerase chain reaction as they are orientated away from each other (**Box 1**). However, following circularisation of the *Hin*dIII chromosomal fragment (**Box 2**) and subsequent linearisation by *Sal*I restriction endonuclease digestion (**Box 3**), the region of unknown nucleotide sequence is bounded by regions of known sequence. As oRI9 and oRI10 are now correctly orientated for PCR (**Box 3**), the region of unknown nucleotide sequence can be amplified (**Box 4**).


1. Digest T. pallidum chromosomal DNA to completion with HindIII
HindIII
EcoRI
EcoRV
EcoRI
SalI
HindIII
oRI9
3' 5'
oRI10
5' 3'
2. Circularise chromosomal fragments with T4 DNA ligase
EcoRV
EcoRI
SalI
HindIII
EcoRI
3. Linearise with SalI
SalI
EcoRI
EcoRV
EcoRI
HindIII
SalI
oRI9
5' 3'
oRI10
3' 5'
4. Polymerase chain reaction amplification of "unknown" sequence using oRI9 and oRI10
EcoRV
EcoRI
HindIII
Amplified "unknown"

2.25 Pulse-chase experiments. Bacteria were grown at 30°C to an A_{590} of 0.5 in LB broth containing ampicillin and kanamycin. Cells were recovered by centrifugation, washed in ice-cold M9 medium (without glucose and thiamine) and then suspended in M9 medium supplemented with 0.2% [wt/vol] glucose, 20 µg/ml of thiamine and 0.01% [wt/vol] amino acids (without methionine and cysteine). The cultures were incubated at 30°C for 30 min and then at 42°C for 15 min. Rifampin was added to a final concentration of 200 µg/ml and the culture incubated for 10 min more at 42°C. After 30 min of incubation at 30°C, Trans-[^{35}S]-label was added; this was incubated at 30°C for 1 min. The culture was then chased with 0.1% methionine in M9 medium. Samples were removed at various times, immediately mixed with one half the volume of ice-cold stop solution (0.4 M sodium azide-0.02 M 2,4-dinitrophenol containing 0.04% [wt/vol] chloramphenicol)[208], and the cells were recovered by centrifugation.

2.26 Minicell analyses.[109] Bacteria were grown at 30°C with vigorous shaking for 18 hours to late log phase in LB broth containing ampicillin and kanamycin. Cells were harvested by centrifugation at 5,500 x g for 15 min at 4°C, suspended in 12 ml of LB broth, and layered onto 0 to 30% [wt/vol] linear sucrose gradients prepared in M9 medium (without glucose or thiamine). Following centrifugation at 5,000 x g for 20 min at 4°C in a JS13.1 swinging bucket rotor (Beckman Instruments, Inc.), the minicells were concentrated by centrifugation at 27,000 x g for 30 min at 4°C and further purified by a second sucrose gradient

centrifugation. Minicells were suspended in M9 medium without glucose and thiamine (approximately 1 x 10^8 minicells/ml) containing D-cycloserine (20 µg/ml) and incubated at 30°C for 15 min followed by 42°C for 5 min. Rifampin was added to a final concentration of 100 µg/ml and the minicells were first incubated at 42°C for an additional 5 min, then for 15 min at 30°C. Ice-cold 95% ethanol was added to each sample so that the final ethanol concentration was either 0%, 1%, 3%, or 5% [vol/vol]. Following the addition of Trans-[^{35}S]-label, each sample was incubated at 30°C for 30 min and the cells then were recovered by centrifugation.

2.27 Selective release of *E. coli* soluble periplasmic proteins. Bacteria were recovered by centrifugation at 4°C, washed in ice-cold 0.2 M Tris-HCl [pH 8.0], and then suspended in ice-cold 0.2 M Tris-HCl [pH 8.0]-1 M sucrose; EDTA and lysozyme were added sequentially to a final concentration of 0.5 mM and 60 µg/ml, respectively. An equal volume of ice-cold 0.2 M Tris-HCl [pH 8.0] was added and the mixture was incubated on ice for 30 min. The cell pellet and the supernatant containing the soluble periplasmic fraction were separated by centrifugation at 4°C. The presence of β-lactamase in each fraction was assessed visually using the colorimetric substrate [1-(thienyl-2-acetamido)]-3-[2-(4-N,N-dimethylaminophenylazo)pyridium methyl]-3-cepem-4-carboxylic acid.[209,210]

2.28 Membrane fractionation experiments. Bacteria suspended in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) [pH 7.4] containing RNase (4 µg/ml) and DNase (4 µg/ml) were disrupted by French press and intact cell bodies were removed by centrifugation at 16,000 x g for 10 min at 4°C. *E. coli* inner and outer membranes were either collected by centrifugation at 240,000 x g for 60 min at 4°C or fractionated using a modification of the procedure of Osborn and co-workers[211] described by Munford *et al.*[212] Briefly, for membrane fractionation studies, the preparation was brought to a final concentration of 20% [wt/wt] sucrose and 5 mM EDTA in 10 mM HEPES [pH 7.4] and layered onto a 60% [wt/wt] sucrose cushion. Following centrifugation at 100,000 x g for three hours at 4°C, the membrane bands were recovered and diluted with 10 mM HEPES [pH 7.4] to a sucrose concentration of 25 to 30%. The membranes were fractionated on a 30 to 55% sucrose gradient in an AH650 swinging bucket rotor (Du Pont Co. Biotechnology Systems, Wilmington, Del.) by ultracentrifugation at 240,000 x g for 22 hours at 4°C. The sucrose gradient was harvested from the top in 250 µl aliquots.

2.29 Extraction and phase-partitioning with Triton X-114. Extraction and phase-partitioning of hydrophobic proteins with Triton X-114 was performed essentially as described by Bordier[213] except that the precondensation step was omitted.[48] Briefly, extractions were performed at 4°C in 1% [vol/vol] Triton X-114 (Sigma Chemical Co.) for at least 60 min. Insoluble material was

recovered by centrifugation at 20,000 x g for 20 min at 4°C, and subsequently washed several times in PBS by differential centrifugation. The supernatant was phase-separated by incubation at 37°C, followed by centrifugation at 12,600 x g for 10 min at room temperature. The aqueous (top) and detergent (bottom) phases were separated by using a pipette. Each phase was washed extensively by repeated phase separations: the aqueous phase by the addition of Triton X-114 to 1% [vol/vol] and the detergent phase by the addition of excess aqueous buffer. Proteins in the detergent phase were recovered by acetone precipitation: following the addition of 10 volumes of ice-cold acetone, samples were incubated at -70°C for 60 min and then the precipitated proteins recovered by centrifugation at 12,600 x g for 10 min at room temperature. To remove any traces of detergent, the precipitate was washed repeatedly in ice cold acetone.

2.30 Purification of Cro-LacZ-FlaA fusion protein. The Cro-LacZ-FlaA fusion protein was purified from DH5α(pRI24) using a commercially available anti-β-galactosidase immunoaffinity column (1 ml bed volume; Protosorb LacZ immunoaffinity adsorbent, Promega Corp., Madison, WI) which had been pre-equilibrated with 10 mM HEPES [pH 7.4]. DH5α(pRI24) were grown to late log phase at 37°C and cells were recovered by centrifugation at 1600 x g for 10 min at 4°C. The cells were suspended in 10 mM HEPES [pH 7.4]-10 mM EDTA-1 mM phenyl methyl sulphonyl fluoride (PMSF), prior to disruption in a French press. Intact cells were removed by centrifugation at 16,000 x g for 10 min at 4°C. The

supernatant, containing cytosolic, periplasmic and membrane fractions, was applied to the immunoaffinity column for at least 60 min, followed by extensive washing with 10 mM HEPES [pH 7.4] containing 0.2% [vol/vol] NP-40. Purified protein, eluted using 100 mM Na_2CO_3 [pH 10.8], was collected in 1 ml fractions.

2.31 Purification of LacZ'-FlaA fusion protein. As the LacZ'-FlaA fusion protein contains only the first nine N-terminal amino acids of β-galactosidase (designated LacZ'),[171] it does not react with the monoclonal antibody used in the Protosorb LacZ immunoaffinity adsorbent. An H9-2 immunoaffinity column was therefore constructed using Aminolink gel (Pierce). Aminolink gel, pre-equilibrated with phosphate buffer, was coupled to the purified monoclonal antibody H9-2 in the presence of sodium cyanoborohydride as reducing agent for 2 hours at room temperature. The gel was washed with phosphate buffer and then equilibrated with 1 M Tris-HCl [pH 7.4]. In order to block free binding sites not coupled to immunoglobulin, the gel was incubated with 1 M Tris-HCl [pH 7.4] in the presence of reducing agent for 30 min at room temperature. Following extensive washing with 1 M NaCl, the gel was equilibrated with 10 mM HEPES [pH 7.4] prior to use.

DH5α(pRI23), expressing the LacZ'-FlaA fusion protein, were grown to late log phase in the absence of IPTG; cells were recovered by centrifugation at 1600 x g for 10 min at 4°C and suspended in 10 mM HEPES [pH 7.4]-1 mM PMSF prior to disruption in a French press. Intact cells were recovered by centrifugation at

16,000 x g for 10 min at 4°C. Membranes were recovered from the supernatant by ultracentrifugation in an AH650 swinging bucket rotor at 240,000 x g for 60 min at 4°C and then suspended in 10 mM HEPES [pH 7.4]. Urea was added to a final concentration of 6 M and the suspension was gently mixed for 60 min at room temperature. Following extensive dialysis against 10 mM HEPES [pH 7.4], insoluble aggregates were removed by ultracentrifugation in an AH650 rotor at 40,000 x g for 60 min at 4°C. The supernatant then was applied to the immunoaffinity column for at least 60 min, followed by extensive washing with 10 mM HEPES [pH 7.4]. Purified protein, eluted with 100 mM glycine [pH 2.5], was collected in 1 ml fractions into tubes containing 50 µl 2 M Tris-HCl [pH 7.6].

2.32 Purification of the 47-kD antigen. The *T. pallidum* 47-kD antigen was purified by immunoaffinity chromatography using murine monoclonal antibody 11E3 conjugated to Reactigel 6x (Pierce).[137] The Reactigel was washed extensively with cold 0.1 M borate buffer [pH 8.5], combined with purified 11E3 in a final volume of 40 ml 0.1 M borate buffer [pH 8.5]), then gently agitated for at least 30 hours at 4°C. The beads, separated from the supernatant by gravity, were suspended in 40 ml of 1 M ethanolamine and gently agitated for a further four hours at room temperature to block free binding sites not coupled to immunoglobulin. All subsequent reactions were performed at 4°C. The 11E3-Reactigel was washed sequentially with 10 mM Tris-HCl [pH 8.0]-5 mM NaCl containing 1% [vol/vol] Triton X-114, 3 M

guanidine hydrochloride containing 1% [vol/vol] Triton X-114, and 10 mM Tris-HCl [pH 8.0]-5 mM NaCl containing 1% [vol/vol] Triton X-114, prior to use.

The Triton X-114 detergent phase prepared from whole *T. pallidum* was phase-separated five times in 10 mM Tris-HCl [pH 8.0]-5 mM NaCl, and then diluted with the same buffer to a final Triton X-114 concentration of 1% [vol/vol]; the detergent phase is approximately 20% [vol/vol] Triton X-114. The combined dilute detergent phase and the 11E3-Reactigel were gently agitated for at least 60 min at 4°C and then the supernatant was separated from the beads by gravity; this process was repeated on four separate occasions using fresh 11E3-Reactigel each time. All the 11E3-Reactigel-bound protein were combined, washed extensively with 10 mM Tris-HCl [pH 8.0]-5 mM NaCl containing 1% [vol/vol] Triton X-114, and then the purified protein was eluted using 3 M guanidine hydrochloride containing 1% [vol/vol] Triton X-114. The 11E3-Reactigel was immediately washed extensively with 10 mM Tris-HCl [pH 8.0]-5 mM NaCl containing 1% [vol/vol] Triton X-114, prior to storage at 4°C. Fractions containing purified 47-kD protein were dialysed against 10 mM Tris-HCl [pH 8.0]-5 mM NaCl, and the detergent phase containing the purified protein recovered by phase-separation at 37°C.

3. RESULTS

3.1 Purification of treponemal endoflagellar proteins

3.1.1 Isolation of treponemal endoflagella. The procedure for isolation of intact *T. pallidum* endoflagella was based upon the observation that extraction of treponemes in sarkosyl and EDTA produces an insoluble residue consisting of endoflagella attached to the cytoskeletal matrix (Figure 8). The endoflagella were separated from the cytoskeletal matrix by shearing in a blender and purified on a CsCl gradient where they formed a distinct band at a refractive index of 1.3628. Electron microscopy confirmed that the fractions collected from this band contained isolated endoflagella (Figure 9). The electron microscopic appearance[122] and the SDS-PAGE profile of the isolated *T. pallidum* endoflagella were identical to those previously reported.[100,122,123] On average, one milligram of endoflagella was isolated from 2 x 10^{11} organisms. Endoflagella isolated from *T. pertenue* had an identical SDS-PAGE profile to *T. pallidum* while *T. phagedenis* endoflagella showed that the 37-kD protein was replaced by a 39-kD protein as previously reported (Figure 10).

The purity of the endoflagella preparations was assessed as follows:

- Coomassie brilliant blue stained-2D-PAGE analyses were identical to those published by Norris *et al.*[100] and did not show any additional proteins.
- 47- and 17-kD antigens were detected in addition to the endoflagellar antigens when Western blots of *T. pallidum* endoflagella were probed with immune rabbit sera. When rabbit

Figure 8. Isolation of *T. pallidum* subsp. *pallidum* endoflagella. *T. pallidum* whole cell lysate (**Tp**), insoluble material after extraction in sarkosyl and EDTA (**SE**), and isolated endoflagella (**Ef**). 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Molecular weight in kD is indicated on the left.

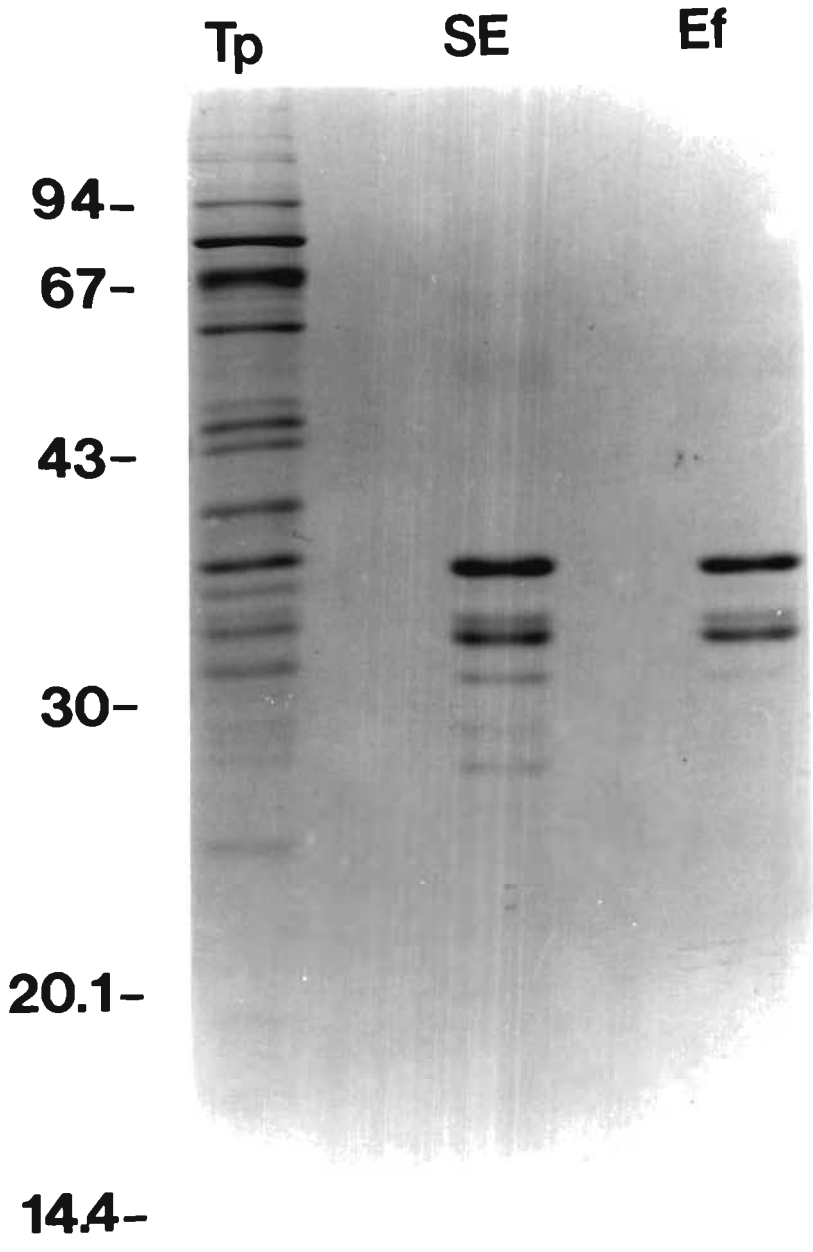

Tp
SE
Ef
94-
67-
43-
30-
20.1-
14.4-

Figure 9. Negative contrast electron micrograph of *T. pallidum* subsp. *pallidum* endoflagella counterstained with uranyl acetate. The bar represents 100 nm.

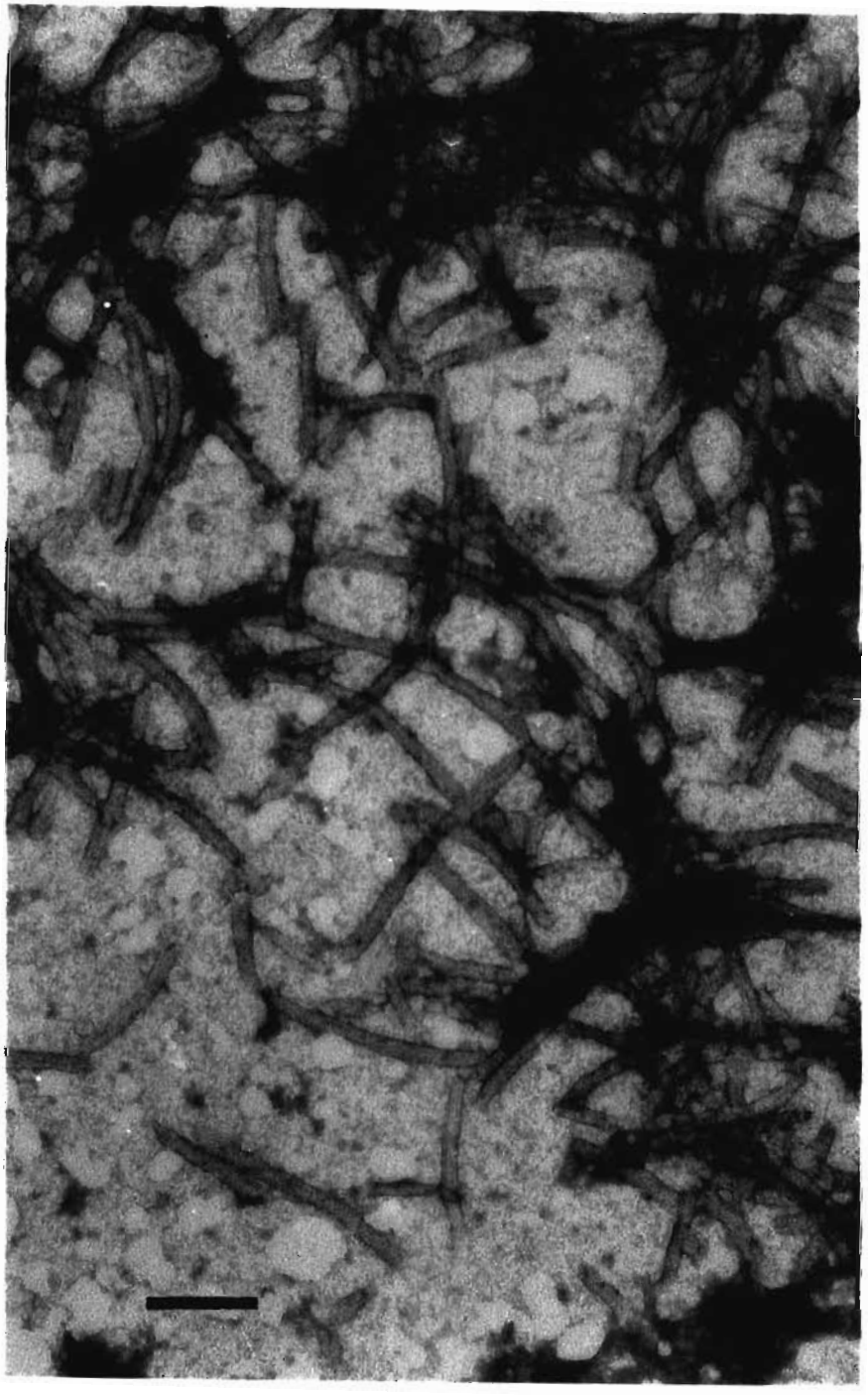
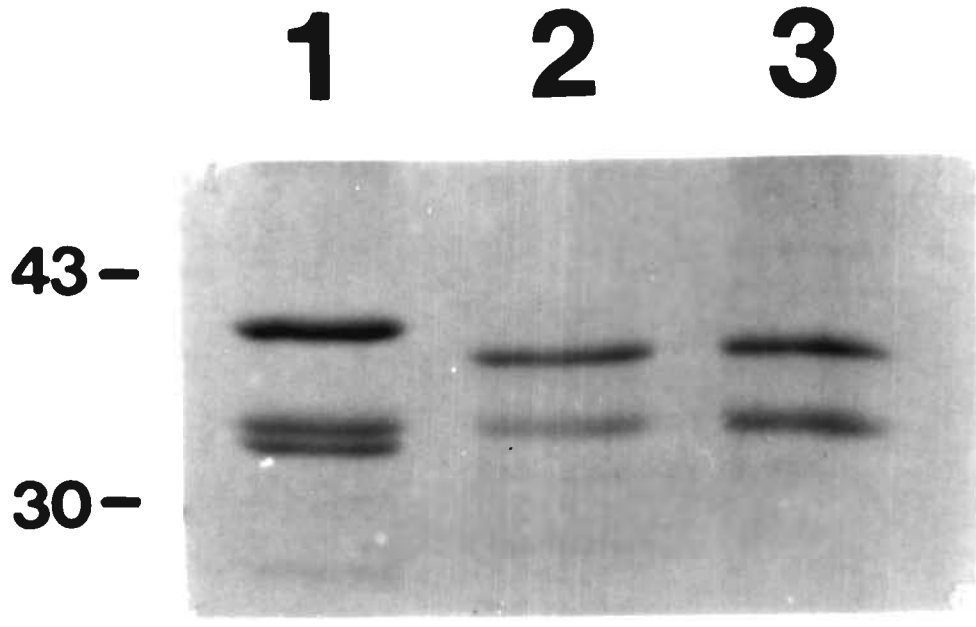


Figure 10. Comparison of the SDS-PAGE profile of endoflagella from three different treponemes. **Lane 1:** *T. phagedenis* biotype Reiter; **Lane 2:** *T. pallidum* subsp. *pallidum*; **Lane 3:** *T. pallidum* subsp. *pertenue*. 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Only the region of the gel containing protein bands is shown. Molecular weight in kD is indicated on the left.


1
2
3
43-
30-

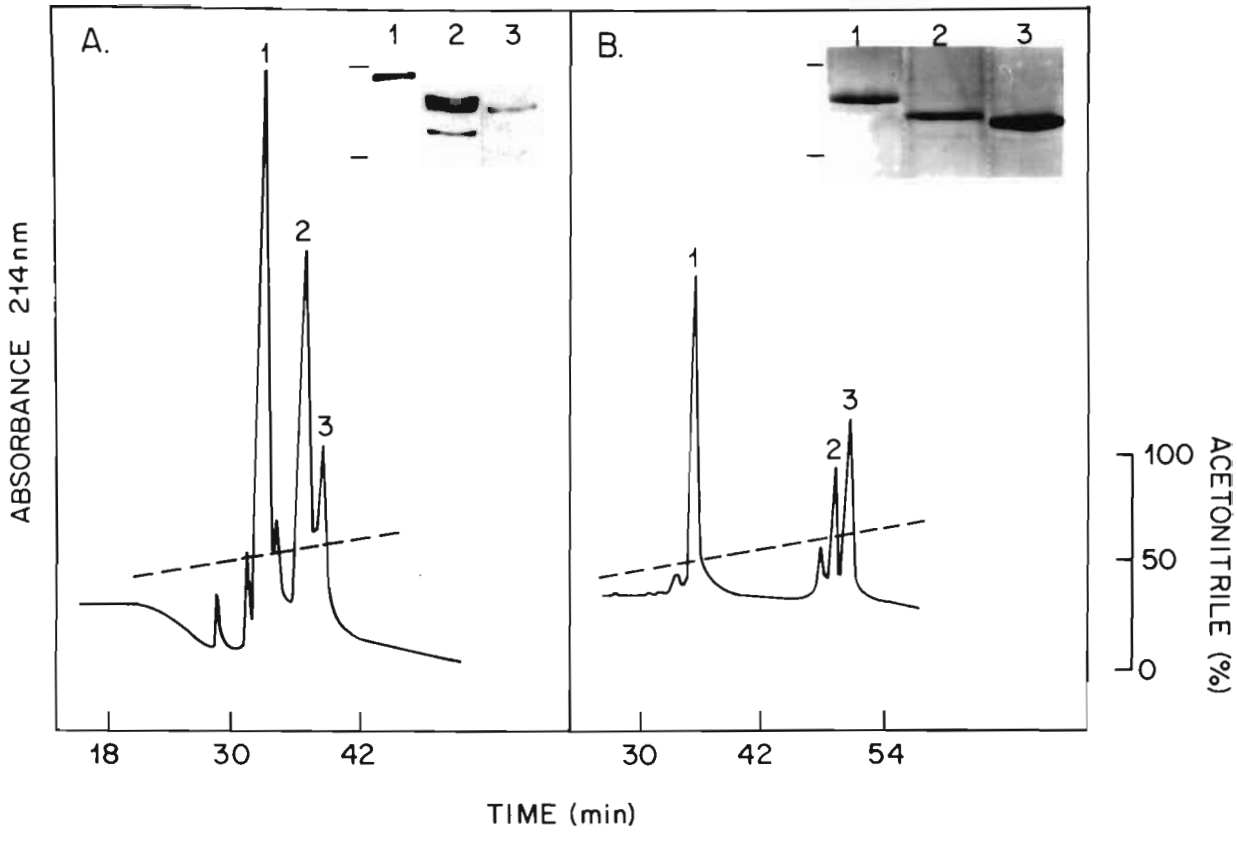
antisera raised against *T. pallidum* detergent-phase proteins was used, only the 47- and 17-kD antigens were identified. The 47-kD antigen reacted with monoclonal antibody 11E3. However, neither the 47- nor 17-kD contaminating proteins were visible on Coomassie brilliant blue stained gels indicating that contamination of the endoflagella with these proteins was minor. Silver-stained SDS-PAGE gels nevertheless demonstrated the 47-kD antigen contaminant, but the 17-kD antigen was not visualized.

- The degree of contamination with the 47-kD protein was estimated using 47-kD protein purified by immunoaffinity chromatography. Immunoblots of varying amounts of purified antigen using a constant amount of purified 11E3 antibody as probe indicated that approximately 25 ng of 47-kD antigen was present per mg of endoflagella.

On the basis of these analyses, the *T. pallidum* endoflagella preparations were judged to be at least 97% pure by weight.

3.1.2 Purification of component endoflagellar proteins. The elution profile of *T. pallidum* and *T. phagedenis* endoflagellar proteins separated by reverse-phase HPLC is shown in Figure 11; *T. pertenue* endoflagella had an identical elution profile to *T. pallidum*. Each of the component proteins eluted in a relatively hydrophobic region of the gradient. The major peaks were analyzed by SDS-PAGE (Figure 11, Insets) and Western blotting. The first major peak contained the sheath proteins: the 37-kD protein for

Figure 11. Purification of component endoflagellar proteins. Reverse-phase HPLC elution profiles of *T. phagedenis* (**Panel A**) and *T. pallidum* subsp. *pallidum* (**Panel B**) endoflagellar proteins. The acetonitrile gradient is indicated by the dashed lines. Insets show silver stained SDS-polyacrylamide gels of indicated peak fractions. The position of molecular weight markers of 43 and 30 kD respectively is indicated on the left of each inset panel.

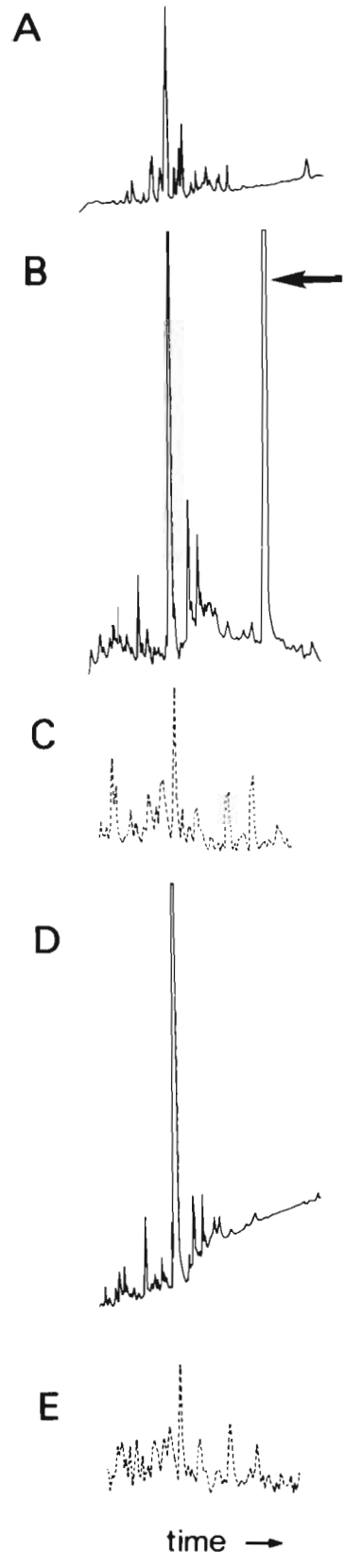

A.
B.
1
2
3
ABSORBANCE 214nm
18
30
42
54
TIME (min)
100
50
0
ACETONITRILE (%)

T. pallidum (Figure 11B, Peak 1) and the 39-kD protein for *T. phagedenis* (Figure 11A, Peak 1). Two distinct proteins of M_r 33.5 (Figure 11, Peak 2) and 33 kD (Figure 11, Peak 3) were identified repeatedly for all three endoflagella species, confirming that the 33-kD doublet seen on SDS-PAGE of intact endoflagella (Figure 10) consists of at least two proteins with distinct physicochemical properties.

3.1.3 Comparative tryptic peptide analysis of endoflagellar sheath proteins. To compare the primary structure of the sheath proteins from the three organisms, tryptic digestions of each purified protein were analysed by HPLC (Figure 12). The elution profile of *T. phagedenis* (Figure 12A) is different from that of either *T. pallidum* (Figure 12B) or *T. pertenue* (Figure 12D). The elution profiles of *T. pallidum* and *T. pertenue* are very similar; in order to correct for any variations in flow pressure during the HPLC between analyses, trace amount of radiolabelled *T. phagedenis* 39-kD sheath protein were included within the tryptic digestion to act as an internal marker (Figure 12C and E). These data indicate that the sheath proteins of *T. pallidum* and *T. phagedenis* have distinct primary structures; however, the sheath proteins of *T. pallidum* and *T. pertenue* cannot be readily distinguished, an observation consistent with the known close genetic relationship between these two organisms.

3.1.4 Amino terminal sequence analysis of component endoflagellar proteins. Purified endoflagellar sheath and core proteins from

Figure 12. Comparison of HPLC elution profiles of tryptic digests of the endoflagellar sheath proteins of *T. phagedenis*, *T. pallidum* subsp. *pallidum*, and *T. pallidum* subsp. *pertenue*. Purified endoflagellar sheath proteins from *T. phagedenis* (**Panel A**), *T. pallidum* (**Panel B**), and *T. pertenue* (**Panel D**) were digested overnight with trypsin and the resulting peptides separated by HPLC. In the cases of *T. pallidum* and *T. pertenue*, radio-labelled *T. phagedenis* 39-kD protein was included in the respective digestion mixtures and acted as an internal marker to correct for variations between HPLC runs (**Panel C** for *T. pallidum*, and **Panel E** for *T. pertenue*). The late peak (arrow) in the *T. pallidum* sheath protein profile (**Panel B**) is undigested protein. For Panels A, B, and D (solid line), the vertical axis is A_{214}. For Panels C and E (broken line), the vertical axis is counts per minute determined by scintillation counting. In all cases the horizontal axis is elution time.



each of the three species were analyzed by N-terminal sequence analysis in duplicate (Table 5). The 21 N-terminal amino acids of the purified *T. pallidum* 37-kD antigen were identical to those published by Norris *et al.*[100] and differed by only one amino acid from those reported by Blanco *et al.*[123] The N-terminal amino acid sequences of the core proteins were identical to those previously reported.[100] There was no difference between *T. pallidum* and *T. pertenue* in the amino acid sequences for any of the three proteins.

Ten peptides generated by tryptic digestion of the *T. pallidum* endoflagellar sheath protein were analyzed by automated N-terminal amino acid sequence analysis (Table 6). These peptide sequences were used subsequently to confirm the amino acid sequence of FlaA deduced from the nucleotide sequence analysis (see 3.2.8).

3.2 Determination of the primary structure of mature FlaA

3.2.1 Monoclonal antibody screening of a recombinant DNA library.

It was not known if *flaA*, the gene encoding the 37-kD sheath protein, contained a promoter which would enable expression of the recombinant antigen in *E. coli*. As other treponemal antigens have been cloned and expressed in *E. coli* under the control of their native treponemal promoter, it was decided to screen a *T. pallidum* genomic library using a monoclonal antibody specific for the endoflagellar sheath protein. The reactivity of murine monoclonal antibody 4H4-20 with the endoflagellar sheath protein

Table 5. N-terminal amino acid sequences for *T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue* and *T. phagedenis* endoflagellar proteins.

	Amino acid residue*
Organism	1 5 10 15 20 25
Sheath proteins of M_r 37-39 kD	
T. pallidum subsp. *pallidum*	DESVLIDFAKLNADIMADKSG
T. pallidum subsp. *pertenue*	.....
T. phagedenis	EQAT....G.....VP.KN.NMTQN
Core proteins of M_r 34.5 kD	
T. pallidum subsp. *pallidum*	MIINHNMSAMFAQRTLGH
T. pallidum subsp. *pertenue*	.....X.....
T. phagedenis	.....A.....VT.NAIGKD
Core proteins of M_r 33 kD	
T. pallidum subsp. *pallidum*	MIINHNMSAMFSQRTLGHKN
T. pallidum subsp. *pertenue*	.....X.
T. phagedenis	.....A.....NT.LSVQKN

* Amino acid residues are designated by the single-letter nomenclature. Residues identical to those of the corresponding *T. pallidum* protein are indicated by a dots.

Table 6. Amino acid sequence of peptides generated by tryptic digestion of purified *T. pallidum* subsp. *pallidum* endoflagellar sheath protein (see 3.1.4)*.

		Location in pre-Fla†	
Designation	Amino acid sequence	n	AA
FlaA.1	LNADIMADKSGG	187-222	30-41
FlaA.2	SSLAVAQWEVVLN	307-345	70-82
FlaA.3	VIEAPVSEG	385-411	96-104
FlaA.4	FEDGY	598-612	167-171
FlaA.5	SIAVNTYGMNY	643-675	182-192
FlaA.6	YFMGYLLFD	727-753	210-218
FlaA.7	ELVWNNPSYI	763-792	222-231
FlaA.8	LYPVYPAS	820-843	241-248
FlaA.9	QQQVL	1051-1065	318-322
FlaA.10	LATEVGF	1087-1104	330-335

* Amino acid residues are designated by the single-letter nomenclature.

† n = location of nucleotide sequence encoding peptide in Figure 19; AA = location of peptide within pre-FlaA with amino acid position 1 being the first amino acid of pre-FlaA.

of *T. pallidum* was confirmed initially by Western blot analysis using whole *T. pallidum* and subsequently using isolated *T. pallidum* endoflagella. Monoclonal antibody 4H4-20 was used to screen 40,000 clones from a pBR322 *T. pallidum* genomic library using the radio-immunocolony blot procedure. No antibody reactive clones were detected.

3.2.2 Characterization of murine monoclonal antibody H9-2. Murine monoclonal antibody, H9-2, generated by immunizing Balb/c mice with sonicated whole *T. pallidum*, was reported by Lukehart and co-workers.[105] The known reactivity of this antibody with a 37-kD antigen of *T. pallidum* suggested that it was directed against an epitope of the endoflagellar sheath protein; however, the specificity of H9-2 for this antigen was not previously demonstrated.[105] Prior to using this monoclonal antibody for further studies, experiments to unequivocally characterize the specificity of the antibody were performed.

Prior to the availability of a mono-specific rabbit anti-*T. pallidum* endoflagella antisera, immunological analyses were performed with the strongly cross-reactive anti-*T. phagedenis* endoflagella antisera.[100,193] In one experiment, 2D-PAGE-Western blots of whole *T. pallidum* were incubated first with rabbit antisera directed against *T. phagedenis* endoflagella and then bound antibody was detected with horseradish peroxidase-staphylococcal Protein A. The membrane was then incubated with H9-2 murine ascites and [^{125}I]-goat anti-mouse IgG followed by

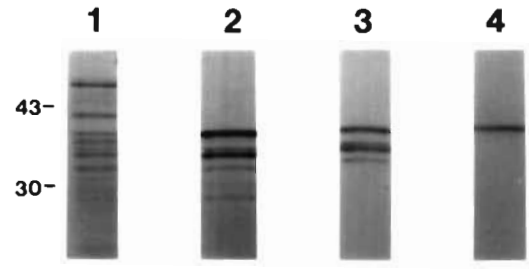
autoradiography. The antisera directed against the endoflagella identified a group of proteins (Figure 13, Panel B) with a 2D-PAGE profile very similar to that described by Norris *et al.* for isolated *T. pallidum* endoflagella.[100] In contrast, H9-2 reacted with a single polypeptide (Figure 13, Panel C) which coincided with the 37-kD endoflagellar antigen recognized by the polyclonal antisera (Figure 13, Panel B). Monoclonal antibody H9-2 also immunoprecipitated the same group of radioiodinated proteins as rabbit antisera raised against *T. phagedenis* endoflagella. Final confirmation of the specificity of H9-2 for the 37-kD endoflagellar protein was obtained for *T. pallidum* endoflagella. In contrast to the rabbit anti-*T. pallidum* endoflagella antisera (Figure 13, Panel A, Lane 3), H9-2 reacted only with the 37-kD protein (Figure 13, Panel A, lane 4).

The specificity of H9-2 for *T. pallidum* was determined by immunoblotting using a panel of nonpathogenic treponemes (Figure 14). As expected, H9-2 reacts with *T. pertenue*. It also reacts with *T. denticola* and *T. scoliodontum*, but not with *T. phagedenis* nor *T. vincentii*. H9-2 does not react by Western blot with the sheath protein of *S. aurantia*.

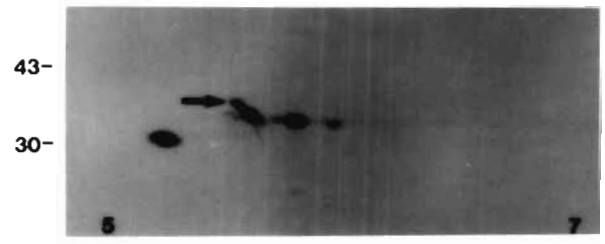
3.2.3 Purification of T. pallidum genomic DNA. For the planned investigations a number of genomic libraries needed to be constructed in *E. coli* using moderately large *T. pallidum* chromosomal DNA fragments. In order to maximize the quality of the extracted DNA, a number of extraction procedures were tried

Figure 13. Murine monoclonal antibody H9-2 reacts with the 37-kD endoflagellar sheath protein of *T. pallidum* subsp. *pallidum*. **Panel A.** SDS-PAGE and Western blot analysis. **Lanes 1 and 2:** *T. pallidum* whole cell lysate and isolated endoflagella, respectively, stained with Coomassie brilliant blue; **Lanes 3 and 4:** immunoblots of isolated *T. pallidum* endoflagella probed with rabbit antisera raised against *T. pallidum* endoflagella, and H9-2, respectively. Samples were separated on a 12.5% SDS-polyacrylamide gel. **Panels B and C.** 2D-PAGE immunoblots of whole *T. pallidum*. Nitrocellulose transfers were probed sequentially with antisera against endoflagella of *T. phagedenis* and horseradish peroxidase-staphylococcal Protein A (**Panel B**). This was followed by reaction with H9-2 and [^{125}I]-radiolabelled goat anti-mouse IgG, and autoradiography (**Panel C**). Arrows designate the 37-kD polypeptide recognized by both the polyclonal antisera and H9-2. pH gradient for isoelectric focusing is shown under Panels B and C. Molecular weight in kD for the second dimension is indicated on the left in each panel.

A.



B.



C.

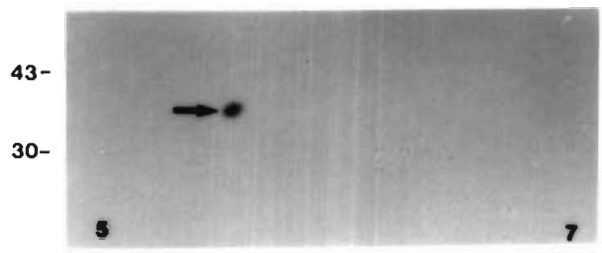
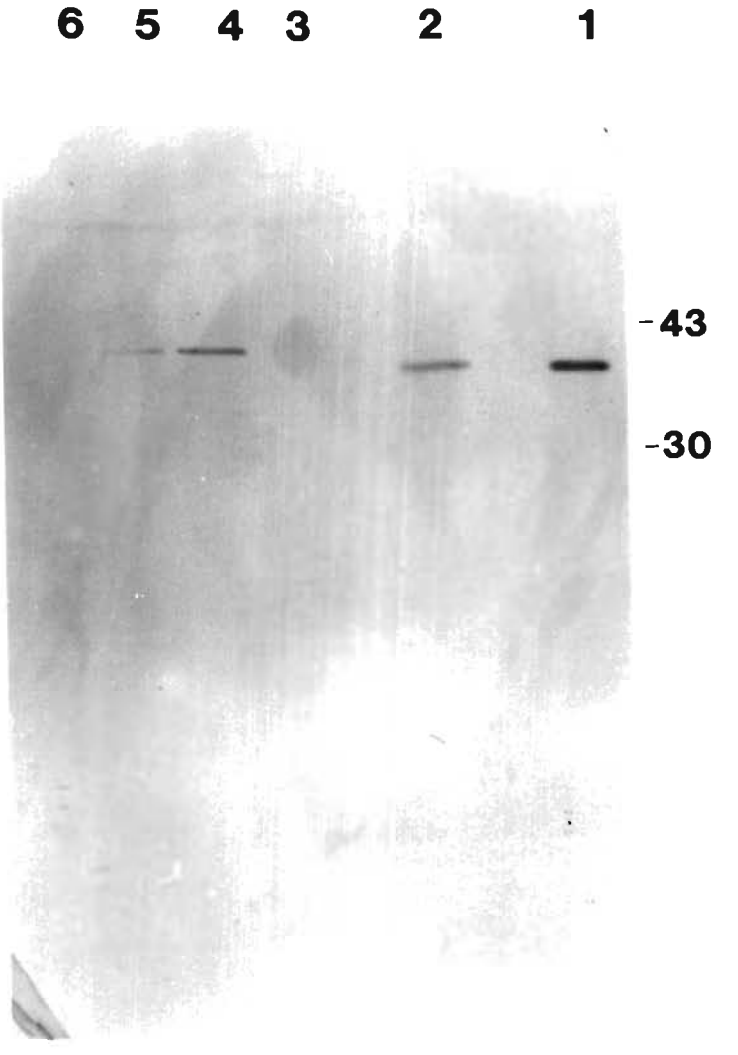


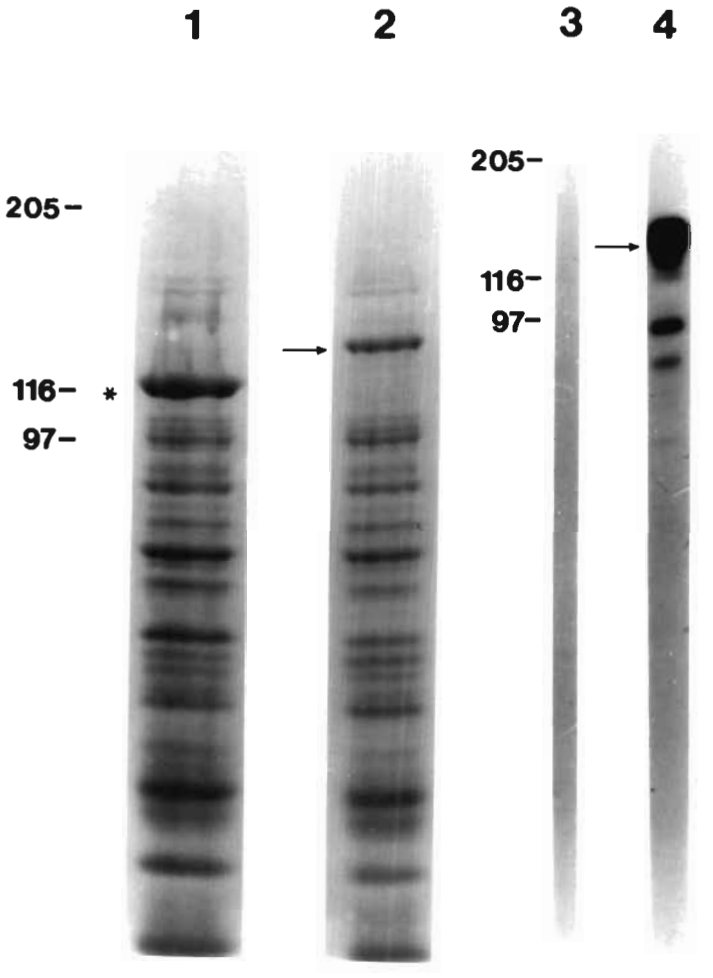
Figure 14. Reactivity of murine monoclonal antibody H9-2 with SDS-denatured proteins of several treponemes. Western blot probed with H9-2. **Lane 1:** *T. pallidum* subsp. *pallidum*; **Lane 2:** *T. pallidum* subsp. *pertenue*; **Lane 3:** *T. phagedenis*; **Lane 4:** *T. scoliodontum*; **Lane 5:** *T. denticola*; **Lane 6:** *T. vincentii*. Samples were separated on a 12.5% SDS-polyacrylamide gel. Molecular weight in kD is indicated on the right.


6
5
4
3
2
1
43
30

as indicated in the methods section (see 2.19). 'Smearing' of DNA on agarose gels and inability to construct satisfactory libraries was a common problem if frozen organisms were used as the source of the DNA. In addition, significant 'smearing' of the DNA seemed to occur with all procedures, but was least noticeable with procedure D. For this reason, procedure D was used to generate the chromosomal DNA used for library construction.

3.2.4 Characterization of clone λA34. Monoclonal antibody H9-2 was used to screen a *T. pallidum* genomic library constructed using λgt11; the recombinant phage from an antibody-reactive *E. coli* clone, designated A34, was used to lysogenize *E. coli* Y1089. Interestingly, the λA34 lysogen produced blue colonies when grown on LB agar plates containing IPTG and X-Gal. Crude cell lysates of λA34 and λgt11 lysogens, respectively, were prepared following induction with IPTG. Comparison of these lysates by SDS-PAGE revealed that the 116-kD band representing β-galactosidase in the λgt11 lysogen was replaced by a new protein of M_r 133 kD in the λA34 lysogen (Figure 15, Lanes 1 and 2). This polypeptide reacted with both H9-2 (Figure 15, Lane 4) and rabbit antisera raised to β-galactosidase, indicating that the 133-kD protein was a fusion of β-galactosidase and a portion of the 37-kD endoflagellar sheath protein. The H9-2-reactive lower molecular mass proteins in the λA34 lysogen (Figure 15, Lane 4) presumably represent proteolytic breakdown products as they were also recognized by the anti-β-galactosidase antiserum.

Figure 15. Characterization of the β-galactosidase fusion protein produced by λA34 *E. coli* lysogen. **Lanes 1 and 2:** Crude cell lysates of λgt11 and λA34 lysogens, respectively, separated on a 7% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. **Lanes 3 and 4:** Crude cell lysates of λgt11 and λA34, respectively, were separated on a 10% SDS-polyacrylamide gel and immunoblots were probed with monoclonal antibody H9-2 and [I^{125}]-radiolabelled goat anti-mouse IgG. Arrow and asterisk indicated A34 fusion protein and β-galactosidase, respectively. Molecular weight in kD is indicated on the left.


1
2
3
4
205-
116-
97-
*
205-
116-
97-

*Eco*RI endonuclease digestion of DNA purified from phage λA34 revealed a small insert, approximately 0.13 kb in size. An insert of this size would code for only 4 kD of the 17 kD predicted from SDS-PAGE analyses of the fusion protein. Restriction endonuclease analysis using *Pvu*I and *Kpn*I, restriction sites flanking the insert within λgt11[214], also demonstrated an insert of approximately 0.13 kb. In addition, a PCR amplification experiment further excluded the possibility that a multimeric insert or an internal *Eco*RI site had not been detected. Using primers flanking the *Eco*RI insertion site in λgt11, the PCR product obtained from the λA34 DNA was only 0.13 kb larger than that from λgt11 DNA (Figure 16). These data do not provide an explanation for the discrepancy between the observed and expected molecular weight of the fusion protein; it is probable that the A34 fusion protein migrates anomalously in SDS-polyacrylamide gels.

3.2.5 Southern analysis of T. pallidum genomic DNA with the λA34 probe. The λA34 insert, subcloned into pUC19 to create pRI1, provided a probe for subsequent library screening and Southern analyses. Specificity of this DNA probe for *T. pallidum* DNA was confirmed by Southern blot analysis. The probe hybridized to fragments of 11, 12, 11, and 2.5 kb in *T. pallidum* chromosomal DNA digested with *Pst*I, *Bam*HI, *Xho*I, and *Hin*dIII, respectively (Figure 17). No hybridization with rabbit mitochondrial DNA, *T. phagedenis* or *E. coli* chromosomal DNA was detected, but it did hybridize with *T. pertenue* chromosomal DNA.

Figure 16. The *T. pallidum* subsp. *pallidum* chromosomal DNA insert of λA34 is not multimeric. Amplification of the insert DNA of λA34 DNA (**A34**) and λgt11 (**gt11**) was by the polymerase chain reaction using oBG1 and oBG2 as primers. PCR products were analysed by ethidium bromide-agarose gel electrophoresis (2% [wt/vol]). Molecular weight standards (in base pairs) are indicated on the left.

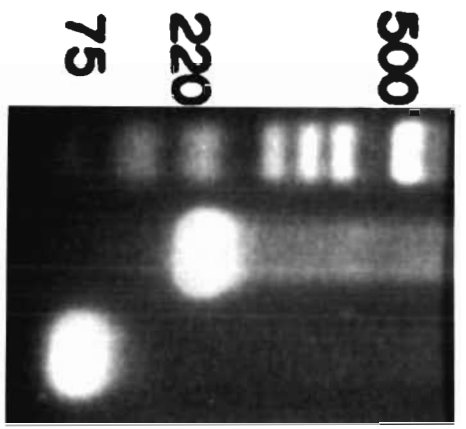
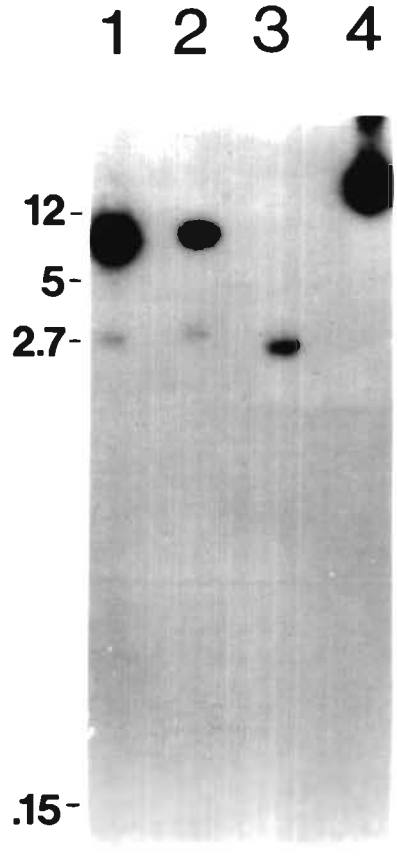

75
220
500
Standard
A34
gt11

Figure 17. Southern hybridization of *T. pallidum* subsp. *pallidum* chromosomal DNA using the *Eco*RI-*Eco*RI fragment from pRI1 as probe. Complete *Pst*I (**lane 1**), *Bam*HI (**lane 2**), *Hin*dIII (**lane 3**) digests and undigested chromosomal DNA (**lane 4**) separated on an 0.8% [wt/vol] agarose gel. Molecular weight standards (in kb) are indicated on the left.


1 2 3 4
12
5
2.7
.15

3.2.6 T. pallidum genomic library construction for "chromosome walking". The initial intention was to piece together the entire 37-kD gene by "chromosome walking" using the pRI1 probe. The following chromosomal DNA libraries were constructed and screened with the pRI1 probe:

- four libraries constructed in pUC19 using size-fractionated (1.5 to 5 kb) *T. pallidum* DNA digested to completion with *Hin*dIII.
- two libraries constructed in pUC19 using size-fractionated (8 to 15 kb) *T. pallidum* DNA digested to completion with *Pst*I.
- four libraries constructed in λ2001 using *T. pallidum* DNA digested to completion with *Bam*HI.
- one library constructed in λ2001 using *T. pallidum* DNA digested to completion with *Xho*I.

In total, in excess of 1 x 10^6 phage and 0.5 x 10^6 plasmid clones were screened by colony blot hybridization with the pRI1 probe. Unfortunately no pRI1-reactive clones were identified.

3.2.7 Screening and clone characterization from a randomly generated gene library. The inability to find pRI1-hybridizing clones in these directed libraries suggested that this region of the *T. pallidum* chromosome was in some way 'toxic' to *E. coli*. It was hypothesized that overlapping clones could be identified in a random genomic library. The random pBR322 library previously used for the antibody screening was therefore screened by colony blot hybridization with the pRI1 probe. Thirty reactive clones

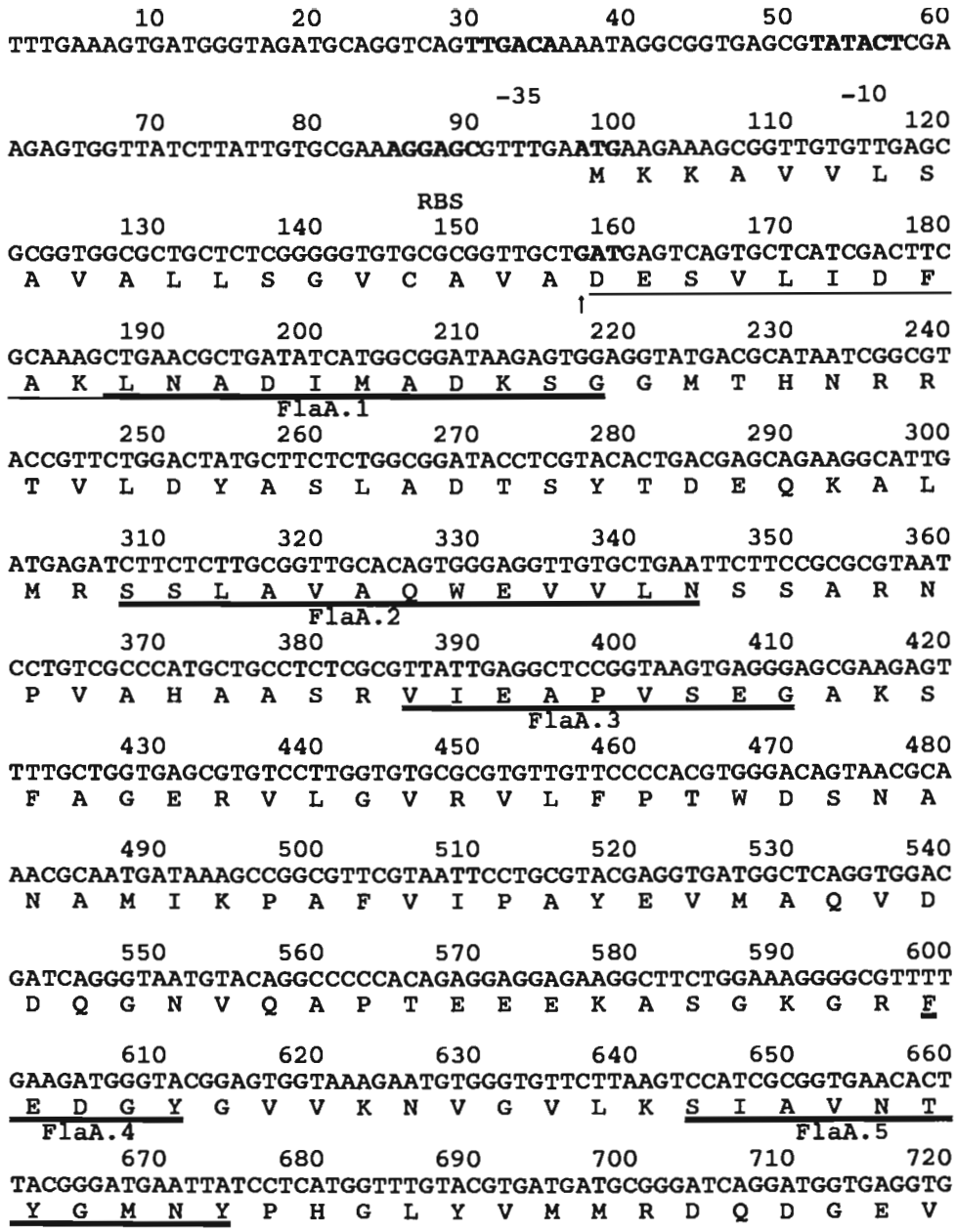
were isolated and their plasmids were purified and restriction endonuclease mapped. Six different chimeric plasmids, pRI2 through pRI7, were identified (Figure 18). The pRI1 probe hybridized with the 5′ *Pst*I-*Sal*I fragment of pRI3 by Southern blot analysis. This provided the starting point for subsequent nucleotide sequence analysis.

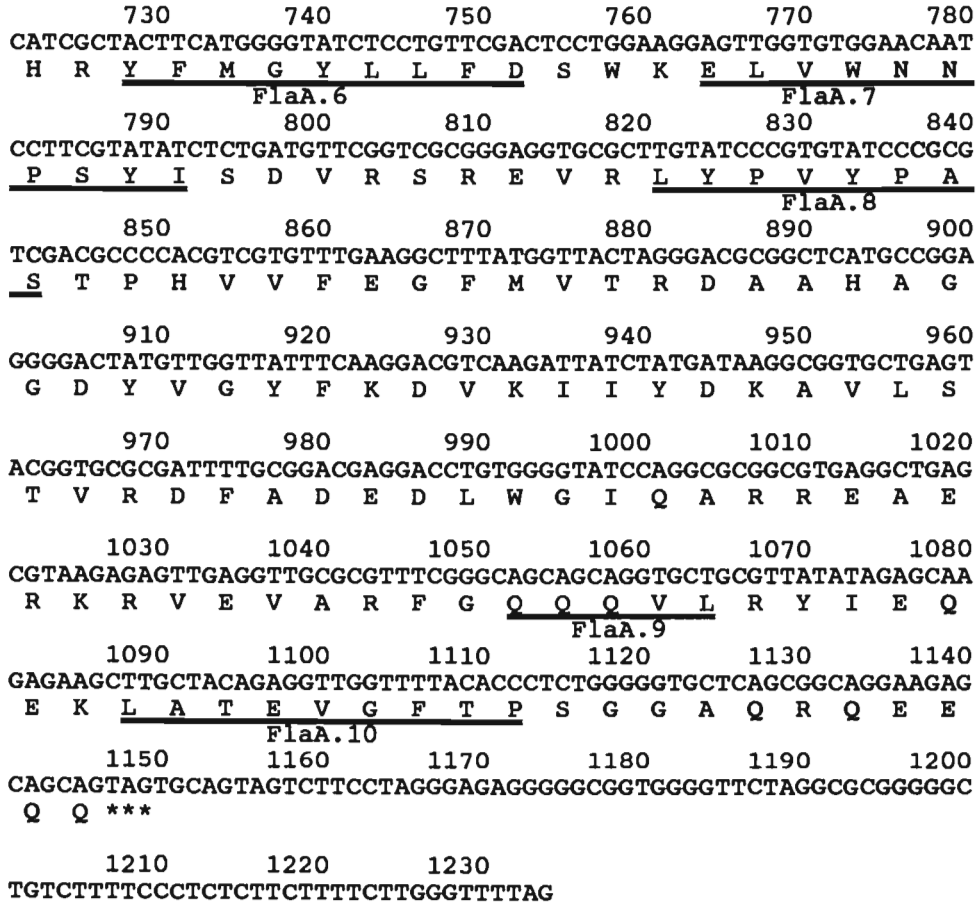
3.2.8 Nucleotide sequence analysis of pRI4. M13 and pUC subclones derived from plasmids pRI1, pRI3 and pRI4 were used to sequence both DNA strands (Figure 18). Based upon the nucleotide sequence analysis, it was determined that the insert in pRI4 encoded for all but the nine N-terminal amino acids of the native 37-kD protein. The amino acid sequence of the mature 37-kD protein was determined by combining the N-terminal and the DNA-derived sequences (Figure 19, base pairs 157-1149). Twenty eight per cent (91/330) of the DNA-derived amino acid sequence of FlaA was confirmed by N-terminal sequence analysis of the tryptic peptides (Table 6 and Figure 19); in all cases, there was complete correlation between the native amino acid sequence and that deduced from the nucleotide sequence. It is not clear how the peptide beginning with glutamine (Figure 19, amino acid 319, nucelotide 1052) was generated as a result of trypsin digestion. The insert of λA34 (pRI1) was localized to base pairs 508 through 626 (Figure 19, amino acids 138 through 177).

Figure 18. Partial restriction maps of the inserts from pRI1, from the chimeric plasmids identified by screening the pBR322 *T. pallidum* subsp. *pallidum* genomic library with the pRI1 probe, and from the subclones created from pRI3 and pRI4.

EcoRI
EcoRI
pRI1
pRI2
PstI
SalI
HindIII
KpnI
HindIII
EcoRI
ClaI
KpnI
PvuI
ClaI
PvuI
PstI
pRI3
PstI
ScaI
SmaI
pRI3.1
pRI3.3
pRI3.4
pRI3.5
pRI3.6
0.5 kb
PstI
EcoRI
pRI4
PstI
EcoRV
pRI4.1
pRI4.2
pRI4.3
pRI4.5
pRI4.6
pRI5
PstI
pRI6
PstI
pRI7
EcoRI
PstI

Figure 19. Nucleotide sequence of *fla*A and deduced amino acid sequence of pre-FlaA. Consensus *E. coli* DNA-dependent RNA polymerase promoter (-35, -10) and ribosomal binding (RBS) sites are indicated. Amino acid sequence determined by N-terminal sequence analysis of native FlaA is underlined (nucleotides 157-219, amino acids 21-41). Amino acid sequence determined by N-terminal sequence analysis of tryptic peptides is indicated by double underlining. The stop codon is indicated by '***'. A vertical arrow (↑) indicates the putative signal peptidase cleavage site. The codons encoding the first amino acids of pre-FlaA and FlaA, respectively, are indicated in bold. This nucleotide sequence data has been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the accession numbers M26525 and M27915.


10 20 30 40 50 60
TTTGAAAGTGATGGGTAGATGCAGGTCAGTTGACAAAATAGGCGGTGAGCGTATACTCGA
-35 -10
70 80 90 100 110 120
AGAGTGGTTATCTTATTGTGCGAAAGGAGCGTTTGAATGAAGAAAGCGGTTGTGTTGAGC
M K K A V V L S
RBS
130 140 150 160 170 180
GCGGTGGCGCTGCTCTCGGGGGTGTGCGCGGTTGCTGATGAGTCAGTGCTCATCGACTTC
A V A L L S G V C A V A D E S V L I D F
190 200 210 220 230 240
GCAAAGCTGAACGCTGATATCATGGCGGATAAGAGTGGAGGTATGACGCATAATCGGCGT
A K L N A D I M A D K S G G M T H N R R
FlaA.1
250 260 270 280 290 300
ACCGTTCTGGACTATGCTTCTCTGGCGGATACCTCGTACACTGACGAGCAGAAGGCATTG
T V L D Y A S L A D T S Y T D E Q K A L
310 320 330 340 350 360
ATGAGATCTTCTCTTGCGGTTGCACAGTGGGAGGTTGTGCTGAATTCTTCCGCGCGTAAT
M R S S L A V A Q W E V V L N S S A R N
FlaA.2
370 380 390 400 410 420
CCTGTCGCCCATGCTGCCTCTCGCGTTATTGAGGCTCCGGTAAGTGAGGGAGCGAAGAGT
P V A H A A S R V I E A P V S E G A K S
FlaA.3
430 440 450 460 470 480
TTTGCTGGTGAGCGTGTCCTTGGTGTGCGCGTGTTGTTCCCCACGTGGGACAGTAACGCA
F A G E R V L G V R V L F P T W D S N A
490 500 510 520 530 540
AACGCAATGATAAAGCCGGCGTTCGTAATTCCTGCGTACGAGGTGATGGCTCAGGTGGAC
N A M I K P A F V I P A Y E V M A Q V D
550 560 570 580 590 600
GATCAGGGTAATGTACAGGCCCCCACAGAGGAGGAGAAGGCTTCTGGAAAGGGGCGTTTT
D Q G N V Q A P T E E E K A S G K G R F
610 620 630 640 650 660
GAAGATGGGTACGGAGTGGTAAAGAATGTGGGTGTTCTTAAGTCCATCGCGGTGAACACT
E D G Y G V V K N V G V L K S I A V N T
FlaA.4
FlaA.5
670 680 690 700 710 720
TACGGGATGAATTATCCTCATGGTTTGTACGTGATGATGCGGGATCAGGATGGTGAGGTG
Y G M N Y P H G L Y V M M R D Q D G E V

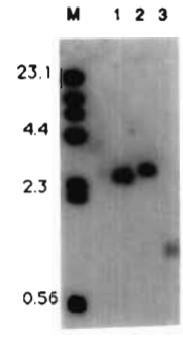

730 740 750 760 770 780
CATCGCTACTTCATGGGGTATCTCCTGTTCGACTCCTGGAAGGAGTTGGTGTGGAACAAT
H R Y F M G Y L L F D S W K E L V W N N
FlaA.6
FlaA.7
790 800 810 820 830 840
CCTTCGTATATCTCTGATGTTCGGTCGCGGGAGGTGCGCTTGTATCCCGTGTATCCCGCG
P S Y I S D V R S R E V R L Y P V Y P A
FlaA.8
850 860 870 880 890 900
TCGACGCCCCACGTCGTGTTTGAAGGCTTTATGGTTACTAGGGACGCGGCTCATGCCGGA
S T P H V V F E G F M V T R D A A H A G
910 920 930 940 950 960
GGGGACTATGTTGGTTATTTCAAGGACGTCAAGATTATCTATGATAAGGCGGTGCTGAGT
G D Y V G Y F K D V K I I Y D K A V L S
970 980 990 1000 1010 1020
ACGGTGCGCGATTTTGCGGACGAGGACCTGTGGGGTATCCAGGCGCGGCGTGAGGCTGAG
T V R D F A D E D L W G I Q A R R E A E
1030 1040 1050 1060 1070 1080
CGTAAGAGAGTTGAGGTTGCGCGTTTCGGGCAGCAGCAGGTGCTGCGTTATATAGAGCAA
R K R V E V A R F G Q Q Q V L R Y I E Q
FlaA.9
1090 1100 1110 1120 1130 1140
GAGAAGCTTGCTACAGAGGTTGGTTTTACACCCTCTGGGGGTGCTCAGCGGCAGGAAGAG
E K L A T E V G F T P S G G A Q R Q E E
FlaA.10
1150 1160 1170 1180 1190 1200
CAGCAGTAGTGCAGTAGTCTTCCTAGGGAGAGGGGGCGGTGGGGTTCTAGGCGCGGGGGC
Q Q ***
1210 1220 1230
TGTCTTTTCCCTCTCTTCTTTTCTTGGGTTTTAG

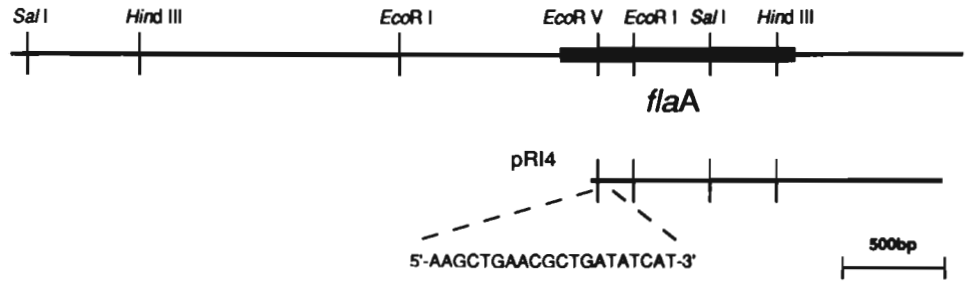
3.3 Completion of the nucleotide sequence of *flaA*

3.3.1 Southern analysis of T. pallidum genomic DNA with oligonucleotide probe oRI3. Further "chromosome walking" was planned using the data obtained from the sequence analysis. It was argued that a small DNA fragment immediately upstream of the 5′ end of the known *flaA* sequence might not be 'toxic' in *E. coli*. A detailed map of the *T. pallidum* genome with respect to *flaA* was generated. Oligonucleotide oRI3, derived from the 5′ end of the coding strand of the pRI4 insert, hybridized in Southern analyses with fragments of 2.5, 2.7, and 0.9 kb from *T. pallidum* chromosomal DNA digested to completion with *Hin*dIII, *Sal*I, and *Eco*RI (Figure 20), respectively. By combining these data with the restriction map of pRI4, a detailed genetic map was derived (Figure 20).

It is clear from Figure 20 that the N-terminal coding region of *flaA* should reside on a 0.9 kb *Eco*RI-*Eco*RI fragment. An additional genomic library was therefore generated in λgt11 from size-fractionated (0.5 to 2 kb) *T. pallidum* chromosomal DNA digested to completion with *Eco*RI. Oligonucleotide probe oRI3 was then used to screen 1 x 10^6 clones in this library and in the pBR322 and original λgt11 libraries. No reactive clones were identified. These results, in conjunction with the failure of previous attempts to clone the upstream regions of *flaA* (see 3.2.6), implied that conventional cloning strategies would not enable completion of the nucleotide sequence of *flaA*.

Figure 20. Construction of *T. pallidum* subsp. *pallidum* genetic map with respect to *fla*A. Southern hybridization of *T. pallidum* chromosomal DNA digested to completion with *Hin*dIII (**lane 1**), *Sal*I (**lane 2**), and *Eco*RI (**lane 3**), and probed with oRI3 (sequence derived from the 5′ end of pRI4 insert). Samples were separated on an 0.8% [wt/vol] agarose gel. Molecular weight standards (in kb) are indicated on the left.

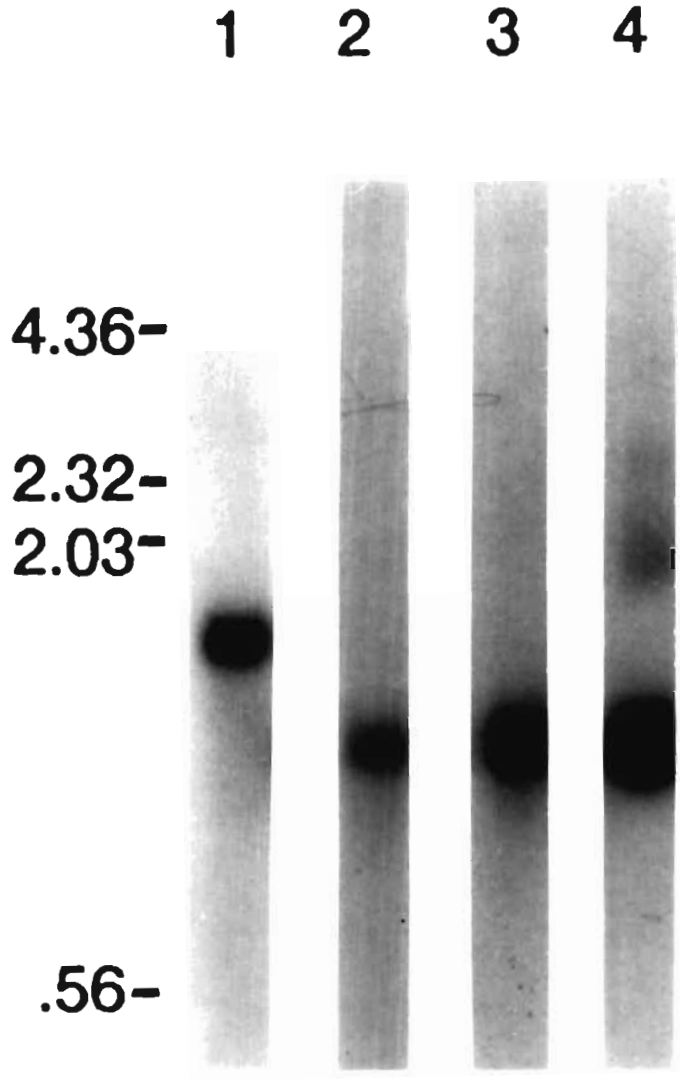

M
1
2
3
23.1
4.4
2.3
0.56


Sal I
Hind III
EcoR I
EcoR V
EcoR I
Sal I
Hind III
flaA
pRI4
5'-AAGCTGAACGCTGATATCAT-3'
500bp

3.3.2 Determining the remaining nucleotide sequence of flaA using the polymerase chain reaction. An alternative strategy based upon the use of inverse-PCR[206] was adopted in an attempt to determine the nucleotide sequence of the upstream portion of *fla*A. This technique enables selective amplification of a region of unknown nucleotide sequence adjacent to a region of known sequence (Figure 7).

The promoter/N-terminal coding region of *fla*A resides on a 2.5 kb *Hin*dIII genomic DNA fragment (Figure 20). This fragment contains *Sal*I and *Eco*RI sites in the region of known nucleotide sequence, and a second *Eco*RI site in the unsequenced region (Figure 20). Inverse-PCR was performed as shown in Figure 7. *T. pallidum* chromosomal DNA was digested to completion with *Hin*dIII, circularized in the presence of T4 DNA ligase, and then linearized with *Sal*I. Oligonucleotides oRI9 and oRI10, which flank the unsequenced area in the linearized chromosomal DNA, were used as the PCR primers. The resulting 1.8 kb product had an internal *Eco*RI site and hybridized with oRI3, confirming that the correct region had been amplified (Figure 21, Lane 1). For unclear reasons, these PCR experiments worked somewhat unpredictably and yielded rather small amounts of product; attempts to re-amplify the purified PCR product using both routine and asymmetric PCR also were unsuccessful. Furthermore, the inverse-PCR product were not able to be cloned into *E. coli* using either pUC or pTTQ vectors; pTTQ vectors contain a transcriptional stop downstream

Figure 21. Use of the inverse-polymerase chain reaction technique to amplify upstream regions of *fla*A. Southern hybridization of PCR products using oRI3 as the probe. **Lane 1:** inverse-PCR product using primers oRI9 and oRI10, **Lane 2:** PCR product using primers oRI11 and oRI1, **Lane 3:** PCR product using primers oRI18 and oRI7, **Lane 4:** pRI18 digested with *Bam*HI and *Pst*I. Samples were separated on a 1% [wt/vol] agarose gel. Molecular weight standards (in kb) are indicated on the left.


1
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4.36-
2.32-
2.03-
.56-

of the cloned fragment and are particularly useful for cloning strong promoters.[185]

An alternative strategy therefore was devised to obtain a limited amount of nucleotide sequence within the unsequenced region of the inverse-PCR product but upstream of the control regions of *fla*A. Using a primer derived from this new sequence, routine PCR could then be performed from *T. pallidum* chromosomal DNA with the expectation that this new product could be more easily manipulated than the original inverse-PCR product. Southern hybridization of the inverse-PCR product, digested with *Sau*3A I and probed with itself, indicated multiple *Sau*3A I sites were present within the inverse-PCR product. It was inferred that at least one of these *Sau*3A I DNA fragments should be upstream of the control regions of *fla*A. The inverse-PCR product was therefore digested to completion with *Sau*3A I and the resulting DNA fragments were ligated into M13mp19 digested with *Bam*HI. A clone was identified from this M13 library by DNA-DNA hybridization using the entire inverse-PCR product as a probe and the nucleotide sequence of the insert was determined. oRI11, a primer derived from this sequence, and oRI1, a primer derived from the *fla*A sequence downstream of both oRI3 and oRI19, were used to amplify a 1.2 kb *T. pallidum* chromosomal DNA fragment (Figure 21, Lane 2). Since approximately 0.3 kb would be sufficient to encode the N-terminus, including a leader peptide, and the promoter, this product was of sufficient size to contain all of the desired upstream portion of *fla*A.

Oligonucleotides oRI1 and oRI11, with oRI11 in excess, were then used in an asymmetric PCR to generate single stranded DNA using *T. pallidum* chromosomal DNA as the template. Nucleotide sequence obtained from the product using oRI9 as the primer was combined with that previously determined to provide the sequence of the entire *flaA* gene (Figure 19). Three other PCR-derived products containing the 5′ end of the open reading frame of *flaA* yielded nucleotide sequences identical to those obtained from the asymmetric PCR product.

3.3.3 Structural features of FlaA. The *flaA* gene contains a consensus *E. coli* promoter[215] and a ribosomal binding site (RBS)[216,217] (Figure 19). No transcriptional stop was identified in the nucleotide sequence downstream of the stop codon. A 20 amino acid signal sequence, including a typical signal peptidase I cleavage site[218] (Figure 19, vertical arrow), immediately precedes the N-terminus previously determined by N-terminal sequence analysis of the native antigen.[100,123] The precursor protein (pre-FlaA) contains 350 amino acids with a calculated molecular weight of 38,860; the mature protein (FlaA) contains 330 amino acids with a calculated molecular weight of 36,948. The deduced amino acid sequence of the first 21 amino acids of FlaA matches precisely that of the native FlaA.[100,123]

FlaA has 46 acidic (Asp, Glu) amino acids, 37 basic (Arg, Lys) amino acids, with the remaining amino acids being neutral; of the neutral amino acids, 111 are hydrophobic (Phe, Trp, Tyr, Ile,

Leu, Met, Val). The G+C content of the gene encoding the 350 amino acids is 54.1%, consistent with previously published G+C ratios of 52.4 to 53.7% for *T. pallidum* (Nichols).[51] The absence of cysteine residues in the derived sequence of FlaA is in agreement with the amino acid analysis of Blanco *et al.*[123] The presence within FlaA of 19 amino acid doublets and two amino acid triplets is a striking feature of the primary structure.

Hydrophilicity analyses by the algorithms of Kyte and Doolittle[219] (Figure 22) and Chou and Fasman[220] demonstrate the characteristic features of a signal peptide in the first 20 amino acids[221,222]; they do not demonstrate any large hydrophobic areas likely to be membrane spanning domains within the mature protein. A prominent hydrophilic domain is predicted from amino acids 150 through 170; this region contains the portion of the protein encoded by the λA34 phage clone which reacted with H9-2.

The nucleotide sequence for the *S. aurantia* flagellar filament surface antigen has been published[223]; the *T. pallidum* 37-kD endoflagellar sheath protein is analogous structurally to this protein. Although there were only minor homology overall between these two proteins, four areas within the N-terminal half of each protein showed homology (Table 7). The most striking of these lies partially within the major hydrophilic domain expressed by λA34; however H9-2 does not react with *S. aurantia*.

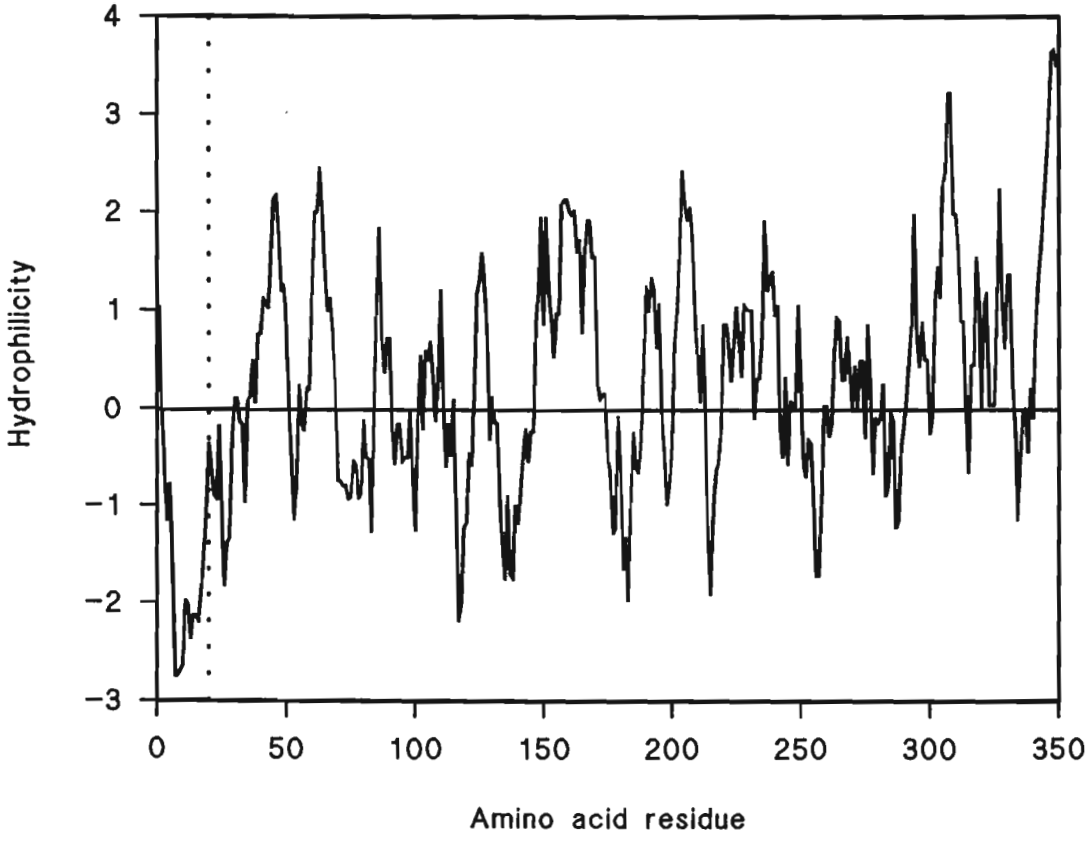


Figure 22. Hydrophilicity analysis of pre-FlaA according to the algorithm of Kyte and Doolittle.[219] The vertical dashed line indicates the site of signal peptide cleavage.

Table 7. Areas of homology between the *flaA* gene product of *T. pallidum* and *S. aurantia**.

T. pallidum	25	LIDFAKL 31
S. aurantia	26	....S.. 32
T. pallidum	111	GNRVLGVRVLFP 122
S. aurantia	106	..T.M...IH.. 117
T. pallidum	130	AMIKPAFVIPAY 141
S. aurantia	125	.V...P.T.... 136
T. pallidum	166	GRFEDGYGVVKNVGVLKSI 184
S. aurantia	151	.Q. ..F..L.....I... 168

* Amino acid residues are designated by the single-letter nomenclature. Residues identical to those of the *T. pallidum* protein are indicated by dots. The first amino acid of the precursor protein for each organism is designated to be position 1.

DNA and protein homology searches were performed using the NBRF protein database (Release 18.0, September, 1988) and the GenBank database (Release 58.0, December, 1988). No other obvious homologies were identified for FlaA.

3.4 Expression of recombinant FlaA

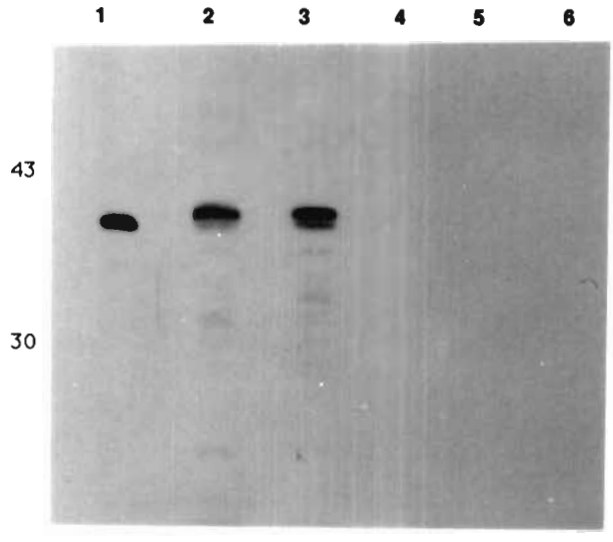
3.4.1 Construction of an E. coli clone expressing recombinant sheath protein. It was assumed that the native treponemal promoter would have to be substituted with a controllable *E. coli* promoter to express FlaA in *E. coli*. PCR was the most convenient method to obtain a clonable fragment containing *flaA* exclusive of its promoter sequences. Three oligonucleotides, oRI7, oRI17, and oRI18, were synthesized for these experiments. oRI7 is complementary to a region downstream of the *flaA* termination codon; it also contains a *Pst*I site near its 5′ end. oRI17 and oRI18 were derived from the sense strand of *flaA* beginning 3 and 30 base pairs upstream of the RBS, respectively. oRI18 also contains two mismatches near its 5′ end which overlaps the -10 promoter sequence; these were included to ensure inactivation of the native promoter. PCR experiments using *T. pallidum* chromosomal DNA with oRI7 and either oRI17 or oRI18 produced the predicted 1.2 kb products (Figure 21, Lane 3). Phosphorylated, purified PCR product digested with *Pst*I was ligated into pBluescript II KS- that had been digested with *Sma*I and *Pst*I. Plasmids pRI17 and pRI18, which contain *flaA* in the correct orientation for expression from the T7 promoter, were identified by restriction endonuclease mapping and Southern hybridization

with oRI3 (Figure 21, Lane 4).

Each of these plasmids was used to transform HB101(pGP1-2) so that expression would be temperature inducible.[186] Following induction, immunoblot analysis using monoclonal antibody H9-2 identified a doublet composed of 39- and 37-kD proteins in HB101(pRI18/pGP1-2) (Figure 23, Lane 2) but not in HB101(pRI17/pGP1-2). No H9-2 immunoreactive material was detected when the cultures were grown only at 30°C (Figure 23, Lanes 5 and 6). Corresponding bands were not readily seen on Coomassie brilliant blue stained gel.

Because *Taq* polymerase lacks proofreading function, misincorporation of nucleotides may occur during PCR.[224-226] For this reason, 2D-PAGE immunoblotting was used to confirm that the native and recombinant FlaA proteins were identical and that the 39-kD H9-2-reactive recombinant protein was the FlaA precursor. First, [^{35}S]-labelled HB101(pRI8/pGP1-2) was co-electrophoresed with isolated *T. pallidum* endoflagella. Immunoblot analysis with H9-2 demonstrated that the native 37-kD antigen (Figure 24, Panel 1) co-migrated with the 37-kD radiolabelled protein (Figure 24A, Panel 2). In a second experiment, radiolabelled HB101(pRI18/pGP1-2) expressing predominantly pre-FlaA (from processing experiments, see 3.4.2) was co-electrophoresed with unlabelled HB101(pRI18/pGP1-2) whole cell lysates (Figure 24). The radiolabelled FlaA precursor (Figure 24, Panel 2) co-migrated with the H9-2-reactive 39-kD antigen identified by immunoblotting

Figure 23. Analysis of *E. coli* clones expressing recombinant FlaA. The Western blot was incubated sequentially in monoclonal antibody H9-2 and horseradish peroxidase-conjugated rabbit anti-mouse IgG. A schematic of the recombinant DNA construct is shown below the blot. Cells were either induced by growing at 42°C for expression as described in Methods (**Lanes 2,3 and 4**), or grown at 30°C (**Lanes 5 and 6**). **Lane 1:** *T. pallidum*, **Lane 2 and 5:** HB101(pRI18/pGP1-2), **Lane 3:** HB101(pRI19/pGP1-2), **Lane 4 and 6:** HB101(pBluescript II KS-/pGP1-2). Samples were separated on a 10% SDS-polyacrylamide gel. Molecular weight in kD is indicated on the left.


1
2
3
4
5
6
43
30

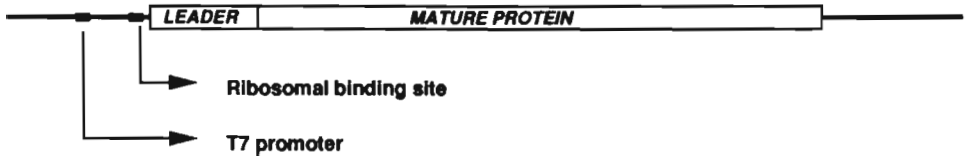
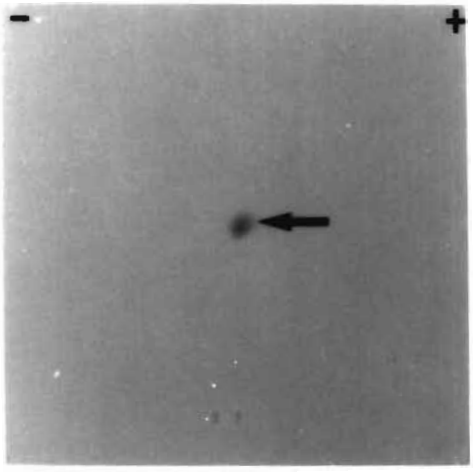
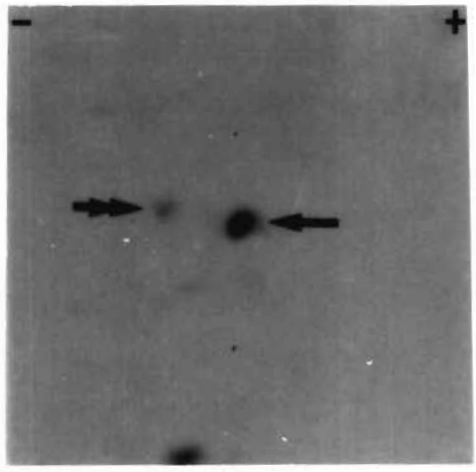

LEADER
MATURE PROTEIN
Ribosomal binding site
T7 promoter

Figure 24. Two dimensional electrophoretic analyses of recombinant FlaA. Samples were separated by IEF in the first dimension and by SDS-PAGE on a 10% SDS-polyacrylamide gel in the second dimension. After transfer to nitrocellulose, the blots were incubated sequentially with H9-2 and peroxidase-conjugated rabbit anti-mouse immunoglobulin (**Panel 1**), and then subjected to autoradiography (**Panel 2**). FlaA (**single arrow**) and pre-FlaA (**double arrow**) are indicated. The basic and acidic ends of the IEF gel are marked with "-" and "+", respectively. **Panel A.** [^{35}S]-labelled HB101(pRI18/pGP1-2) was co-electrophoresed with isolated *T. pallidum* subsp. *pallidum* endoflagella. **Panel B.** [^{35}S]-labelled HB101(pRI18/pGP1-2) predominantly expressing pre-FlaA was co-electrophoresed with HB101(pRI18/pGP1-2).

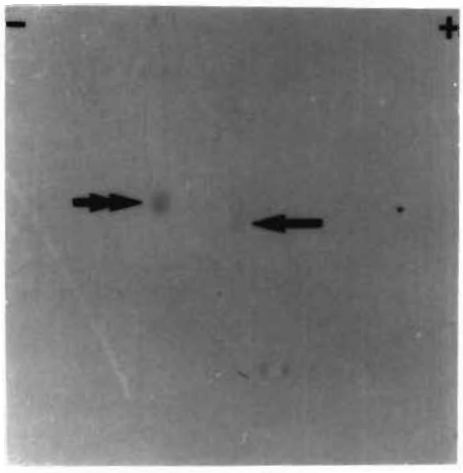
A. 1



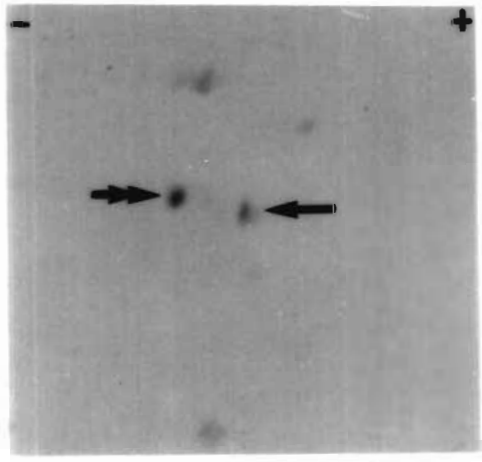
2



B. 1



2



(Figure 24, Panel 1). As predicted by the presence of two lysines in the signal sequence, the FlaA precursor was significantly more basic than the mature protein during isoelectric focusing (Figure 24A, Panel 2, double arrow).

Despite the above 2D-PAGE results, the possibility still existed that the recombinant antigen contained an amino acid substitution which did not affect its pI. Such substitution(s) could adversely impact future structure-function analyses of FlaA. As an alternative to sequencing the entire insert of pRI18 for comparison with the known sequence, an additional chimeric plasmid was constructed subsequently from fragments of known nucleotide sequence. Nucleotide sequence analysis of pRI18 showed that the 5′ sequence matched the expected sequence up to the *Eco*RI site. pRI19 was constructed in pBluescript II KS- from the 0.3 kb *Bam*HI-*Eco*RI fragment of pRI18, which encodes the RBS, the leader and amino acids 1 through 62 of the mature protein, and the 1.5 kb *Eco*RI-*Pst*I fragment of pRI4.3, which encodes the remainder of the mature protein. Immunoblots of SDS-PAGE (Figure 23, Lane 3) and 2D-PAGE gels showed that HB101(pRI19/pGP1-2) produced a H9-2-reactive 39- and 37-kD doublet identical to that of HB101(pRI18/pGP1-2).

Growth curves of HB101(pRI18/pGP1-2) and HB101(pRI19/pGP1-2) were compared to that of HB101(pBluescript II KS-/pGP1-2). At 42°C, a small difference in the growth rates for both HB101(pRI18/pGP1-2) and HB101(pRI19/pGP1-2) was consistently observed (Figure 25).

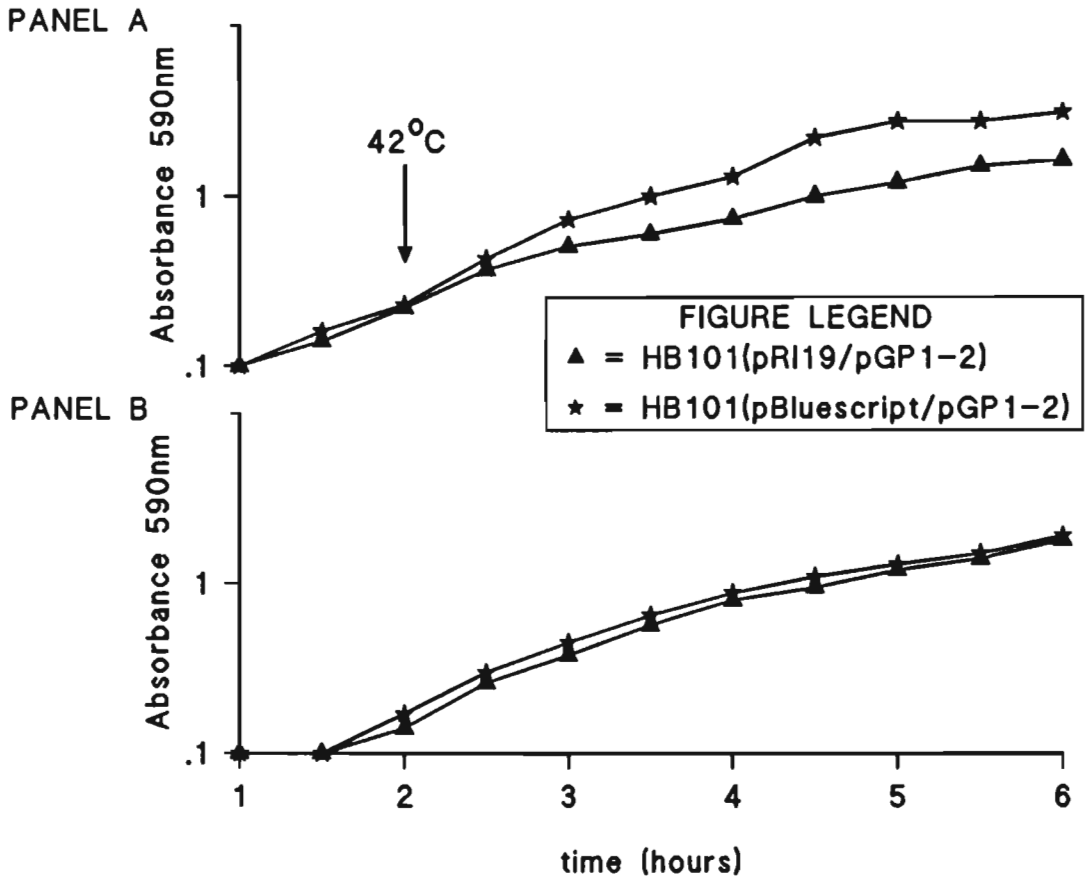


Figure 25. Expression of recombinant FlaA reduces the growth rate of *E. coli*. Representative growth curves for HB101(pRI19/pGP1-2) or HB101(pBluescript II KS-/pGP1-2) derived from one of three experiments. **Panel A.** Cells were grown at 30°C for the first two hours, then shifted to 42°C (arrow) for the remainder of the experiment. **Panel B.** Cells were grown only at 30°C.

Viable colonies were produced from samples taken at all time point.

3.4.2 Analysis of processing of FlaA. In pulse-chase experiments using HB101(pRI18/pGP1-2), FlaA was synthesized as a 39-kD precursor which was subsequently processed to the 37-kD mature protein (Figure 26A); even after a 60 min chase, a significant amount of precursor was still present (Figure 26A). Processing of pre-FlaA in HB101(pRI19/pGP1-2) showed similar results. In a parallel control experiment using HB101(pT7-3/pGP1-2) incubated under identical conditions, β-lactamase was processed completely with virtually no β-lactamase precursor detected at 60 min.

Ethanol, a non-specific inhibitor of membrane translocation by secretory proteins[227], was used in experiments with ORN103(pRI18/pGP1-2) minicells. Although substantial accumulation of precursor occurred even in the absence of ethanol, inhibition of processing was ethanol concentration dependent (Figure 26B).

3.4.3 Localization of recombinant antigen in E. coli. Spheroplasts derived from HB101(pRI18/pGP1-2) were osmotically shocked to release selectively soluble periplasmic proteins. All of the FlaA remained associated with the cell pellet (Figure 27A) while β-lactamase was easily detectable in the supernatant. In a separate experiment, HB101(pRI18/pGP1-2) were disrupted in a French press and membranes were collected by differential ultracentrifugation. The supernatant, comprising the periplasmic

Figure 26. Processing of FlaA in *E. coli*. The location of the 37-kD sheath protein and its precursor on the gel are indicated on the left. **Panel A.** HB101(pRI18/pGP1-2) were pulsed with Trans-[^{35}S]-label for 1 min and then chased for the times shown with 0.1% [wt/vol] methionine. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and subjected to autoradiography. **Panel B.** ORN103(pRI18/pGP1-2) minicells were labelled with Trans-[^{35}S]-label in the presence of different concentrations of ethanol, electrophoresed on a 10% SDS-polyacrylamide gel, and subjected to autoradiography. Ethanol concentrations [vol/vol] are shown above each lane.

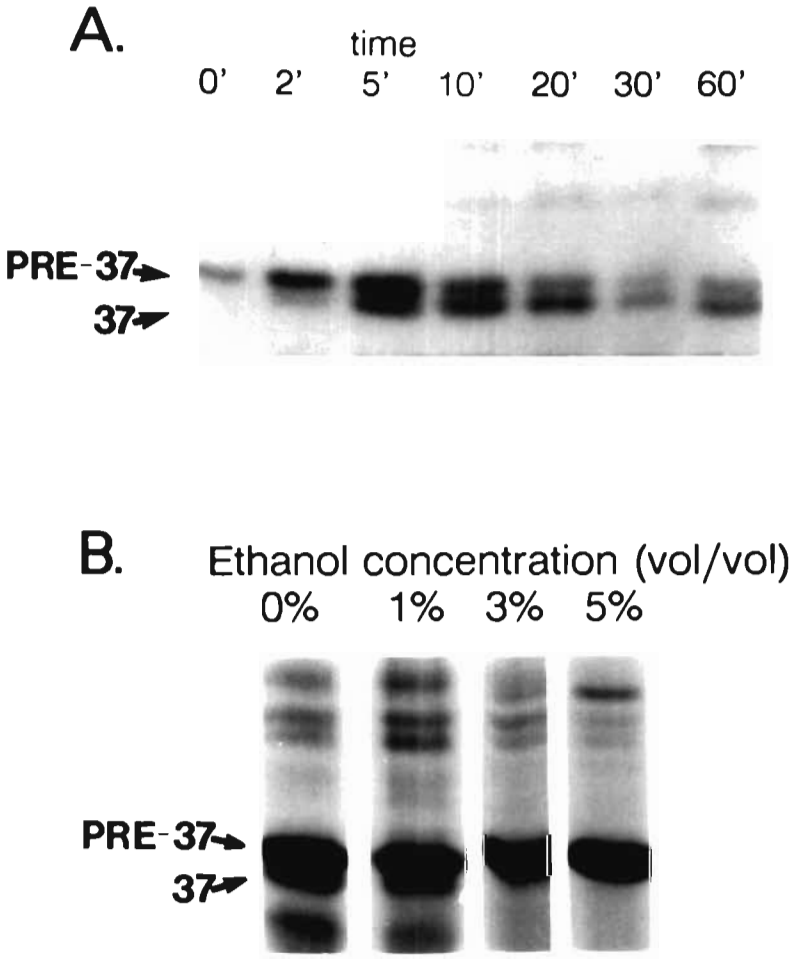
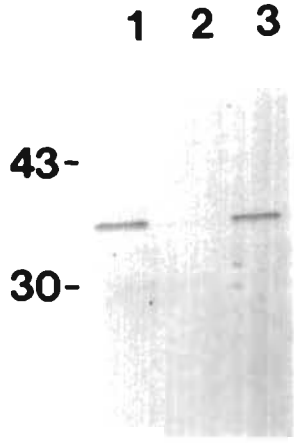
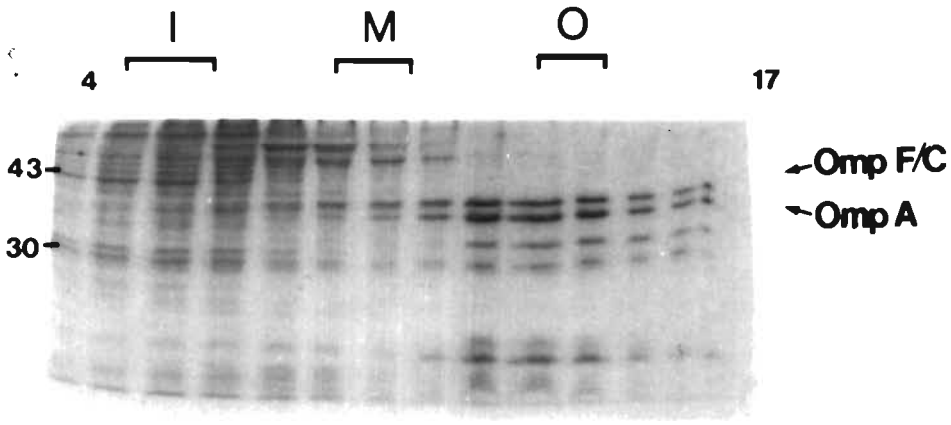

A.
time
0'
2'
5'
10'
20'
30'
60'
PRE-37
37
B.
Ethanol concentration (vol/vol)
0%
1%
3%
5%
PRE-37
37

Figure 27. Fractionation of *E. coli* clones expressing FlaA. **Panel A.** Immunoblot analysis with H9-2 of soluble periplasmic proteins released from HB101(pRI18/pGP1-2) spheroplasts. **Lane 1:** starting material, **Lane 2:** supernatant following osmotic shock of spheroplasts, **Lane 3:** insoluble pellet. Samples were separated on a 12.5% SDS-polyacrylamide gel. Molecular weight in kD is indicated on the left. **Panel B.** 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant blue (**Panel B1**) and corresponding immunoblot probed with monoclonal antibody H9-2 (**Panel B2**) of cell envelopes of HB101(pRI18/pGP1-2) separated by sucrose density gradient centrifugation into inner (I) and outer (O) membranes, and an intermediate membrane (M) fraction. Fractions #4 to #17 are shown. Molecular weight in kD is indicated on the left. The *E. coli* outer membrane proteins, OmpA and OmpF/C, are indicated.

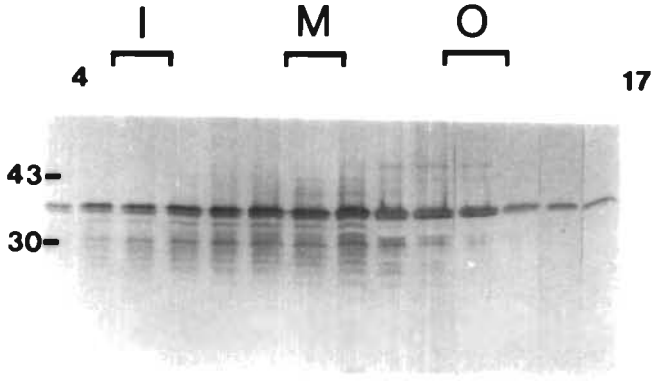
A.



B.1.



2.

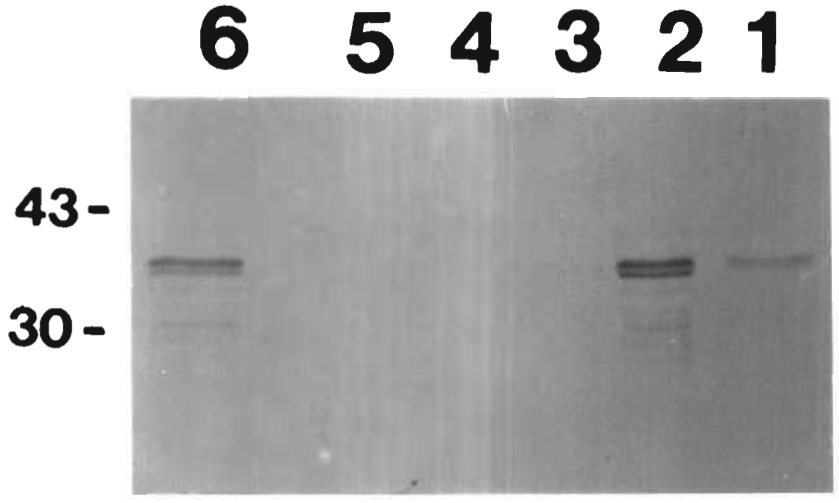


and cytoplasmic fractions, did not contain H9-2-reactive material. SDS-PAGE of the membranes fractionated on a sucrose gradient revealed protein profiles characteristic of *E. coli* inner and outer membranes (Figure 27B, Panel 1). The buoyant densities of the outer and inner membrane fractions were 1.230 g/cc and 1.167 g/cc, respectively. Immunoblot analysis of the gradient fractions (Figure 27B, Panel 2) revealed the surprising result that both the precursor and the mature protein were present in all fractions. Fractionation studies using HB101(pRI19/pGP1-2) showed identical results.

Attempts were made to selectively solubilize FlaA away from the *E. coli* membrane. When HB101(pRI19/pGP1-2) membranes were incubated in 1% [vol/vol] Triton X-114, the recombinant FlaA remained with the membrane fraction (Figure 28, Lane 2). Sarkosyl, 1% [wt/vol], completely solubilized the recombinant proteins (Figure 28, Lane 6).

3.4.4 Expression of a LacZ'-FlaA protein. It could be argued that removal of the signal peptide might increase production of FlaA. This was achieved by expression of FlaA as a fusion protein. An additional oligonucleotide, oRI23, was synthesized for these experiments. oRI23 was derived from the sense strand of *flaA* beginning 15 base pairs upstream of the first codon of the mature protein. It contained a series of mismatches near its 5' end (nucleotides 5 through 15 of the oligonucleotide) which encode for a bovine protease X_a site immediately upstream of the first

Figure 28. Detergent solubility of recombinant FlaA. *E. coli* membranes from HB101(pGP1-2/pRI19) were incubated in PBS containing either 1% [vol/vol] Triton X-114 or in 1% [wt/vol] sarkosyl. Western blot probed with monoclonal antibody H9-2. **Lane 1:** HB101(pGP1-2/pRI19) membranes; **Lane 2:** insoluble material after Triton X-114 extraction; **Lane 3:** Triton X-114 aqueous phase; **Lane 4:** Triton X-114 detergent phase; **Lane 5:** insoluble material after sarkosyl extraction; **Lane 6:** sarkosyl soluble material. Samples were separated on a 10% SDS-polyacrylamide gel. Molecular weight in kD is indicated on the left.


6
5
4
3
2
1
43-
30-

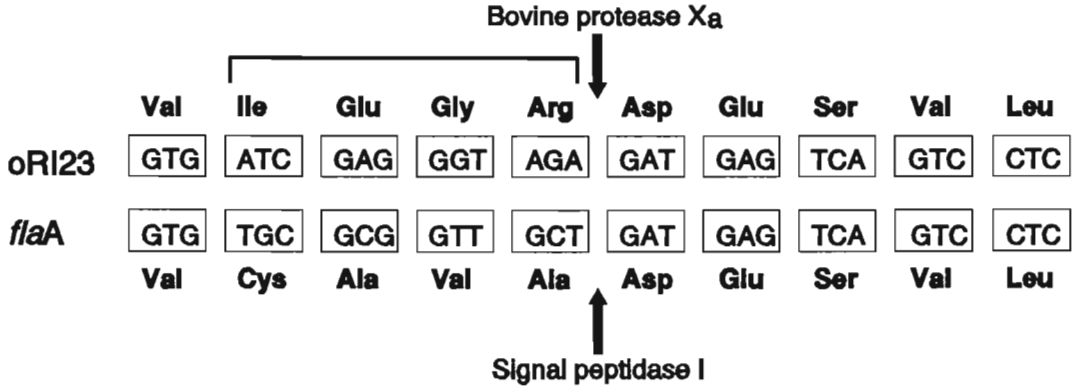
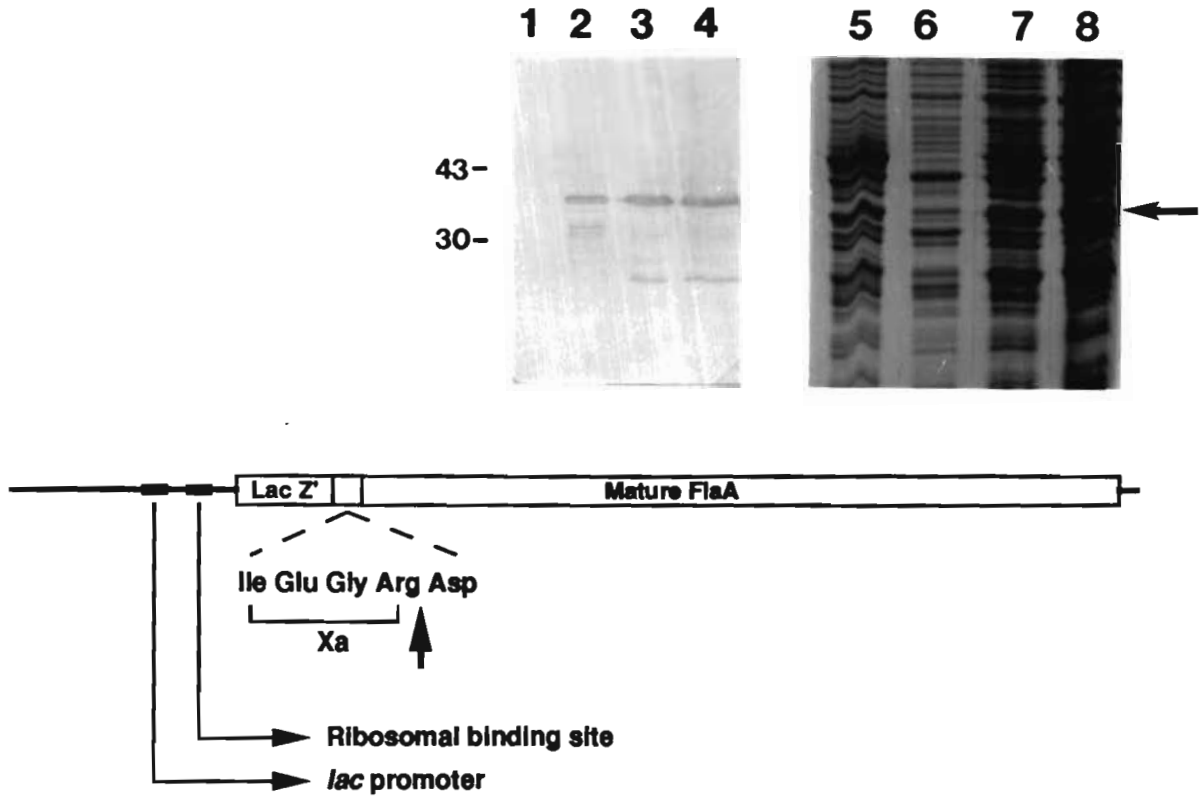


Figure 29. Introduction of a bovine protease X_a site by using mismatched PCR primers. A bovine protease X_a site[228] (Ile-Glu-Gly-Arg) was engineered into the hybrid protein immediately upstream of the first amino acid (Asp) of mature FlaA by using a mismatched PCR primer (oRI23). Following purification, the hybrid protein should be able to be proteolytically cleaved by bovine protease X_a to yield 'mature' FlaA with the same N-terminal sequence as is found in the native protein.

amino acid of the mature protein[228] (Figure 29). PCR amplification using purified pRI19 with oRI7 and oRI23 yielded the predicted 1 kb product. Phosphorylated, purified PCR product digested with *Pst*I was ligated into pUC18 digested with *Sma*I and *Pst*I. Plasmid, pRI22.2, which should produce a LacZ'-FlaA fusion protein (Figure 30, schematic diagram) was identified by restriction endonuclease mapping, and was used to transform DH5α. Immunoblot analysis using monoclonal antibody H9-2 demonstrated that DH5α(pRI22.2) produced a 39 kD protein, both with and without induction with IPTG. In order to remove the possibility of point mutations being introduced by *Taq* polymerase infidelity, a further chimeric plasmid, pRI23, was constructed. Nucleotide sequence analysis of pRI22.2 showed that the 5' sequence matched the expected sequence up to the *Eco*RV site. pRI22.2 was digested to completion with *Eco*RV and *Pst*I; the 2.8 kb fragment contains the origin of replication for the plasmid and the 5' end of the gene encoding the hybrid protein. pRI23 was produced by ligating this fragment with the 1.7 kb *Eco*RV-*Pst*I fragment from pRI4, encoding the remainder of *fla*A. pRI23 was identified by restriction endonuclease mapping and used to transform DH5α. DH5α(pRI23) produces a protein of 39 kD (Figure 30, Lanes 3 and 4). Corresponding bands were not readily seen on Coomassie brilliant blue stained gels as the hybrid protein co-migrated with a major *E. coli* protein. Degradation of the hybrid protein is indicated by the lower molecular weight species detected in the immunoblots (Figure 30, Lanes 3 and 4).

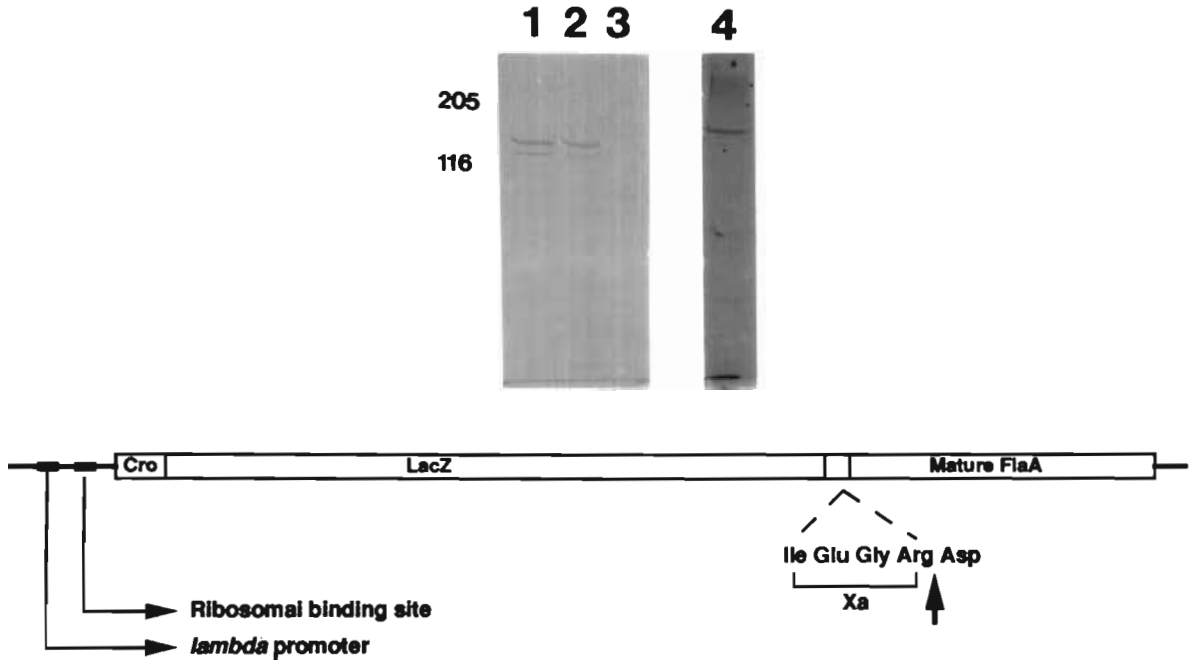
Figure 30. Expression of LacZ'-FlaA protein. A DNA fragment encoding FlaA with the 5' end encoding for a Protease X_a digestion site (indicated by arrow on schematic diagram) was introduced into plasmid pUC18; this yielded a *lacZ'-flaA* gene fusion. The fusion protein (**arrow at right**) co-migrates with *E. coli* proteins but is visible as a smear on Coomassie brilliant blue staining in Lanes 7 and 8. **Lanes 1,5:** DH5α(pUC18); **Lanes 2,6:** HB101(pGP1-2/pRI19) membranes; **Lanes 3,7:** DH5α(pRI23); **Lane 4,8:** DH5α(pRI23) induced with IPTG. Samples were separated on 12.5% SDS-polyacrylamide gels and either stained with Coomassie brilliant blue (**Lanes 5-6**) or transferred to nitrocellulose for Western blotting with monoclonal antibody H9-2 (**Lanes 1-4**). Molecular weight in kD is indicated on the left.


1 2 3 4
5 6 7 8
43-
30-
Lac Z'
Mature FlaA
Ile Glu Gly Arg Asp
Xa
Ribosomal binding site
lac promoter

3.4.5 Expression of a Cro-LacZ-FlaA protein. A second hybrid protein was expressed from a chimeric plasmid, pRI24, using the vector pEX1. In this vector, *fla*A is inserted near the 3′ end of the coding region of *cro-lac*Z to make a *cro-lac*Z-*fla*A gene fusion[187] (Figure 31, schematic diagram). Production of the hybrid protein is under the control of a strong P_L λ promoter (Figure 31, schematic diagram), and a temperature sensitive λ repressor gene is also present on the expression plasmid. To construct pRI24, pRI23 was digested with *Kpn*I and the 3′ overhang was filled using T4 DNA polymerase. Following digestion with *Pst*I, the 2.3 kb *Kpn*I-*Pst*I fragment was ligated into dephosphorylated pEX1 that had been digested with *Sma*I and *Pst*I. The ligation mixture was used to transform DH5α and a recombinant clone, DH5α(pRI24), was selected by immunoblot analysis with monoclonal antibody H9-2. DH5α(pRI24) produces a 150 kD protein (Figure 31, Lane 1). HB101(pRI24) or DH5α(pRI24) were both found to express low levels of the Cro-LacZ-FlaA protein when the cells were grown continuously at 37°C. However, no protein was detected when the cells were heat shocked at 42°C to induce hyper-expression from the λ promoter. The hybrid protein expressed at 37°C was partially purified from HB101(pRI24) by immunoaffinity chromatography (Figure 31, Lanes 2 and 4); the hybrid protein was detected in elution fractions 1 and 2. Less that 2 μg of protein was recovered from 1 l of cell culture.

3.4.6 Solubilization and partial purification of LacZ′-FlaA. As the Cro-LacZ-FlaA hybrid protein could not be produced in large

Figure 31. Expression and purification of the Cro-LacZ-FlaA protein. A DNA fragment encoding FlaA with the 5′ end encoding for a Protease X_a digestion site (indicated by arrow on schematic diagram) was introduced into plasmid pEX1; the resulting gene product was a Cro-LacZ-FlaA hybrid protein. **Lane 1:** DH5α(pRI24) grown at 37°C; **Lane 2,4:** Cro-LacZ-FlaA protein purified by adsorption to a anti-β-galactosidase immunoaffinity column; **Lane 3:** mock purification using DH5α(pEX1). Samples were separated on a 7% SDS-polyacrylamide gel and either stained with a silver stain (**Lane 4**) or transferred to nitrocellulose for Western blotting with monoclonal H9-2 (**Lanes 1-3**). Molecular weight in kD is indicated on the left.


1 2 3
4
205
116
Cro
LacZ
Mature FlaA
Ile Glu Gly Arg Asp
Xa
Ribosomal binding site
lambda promoter

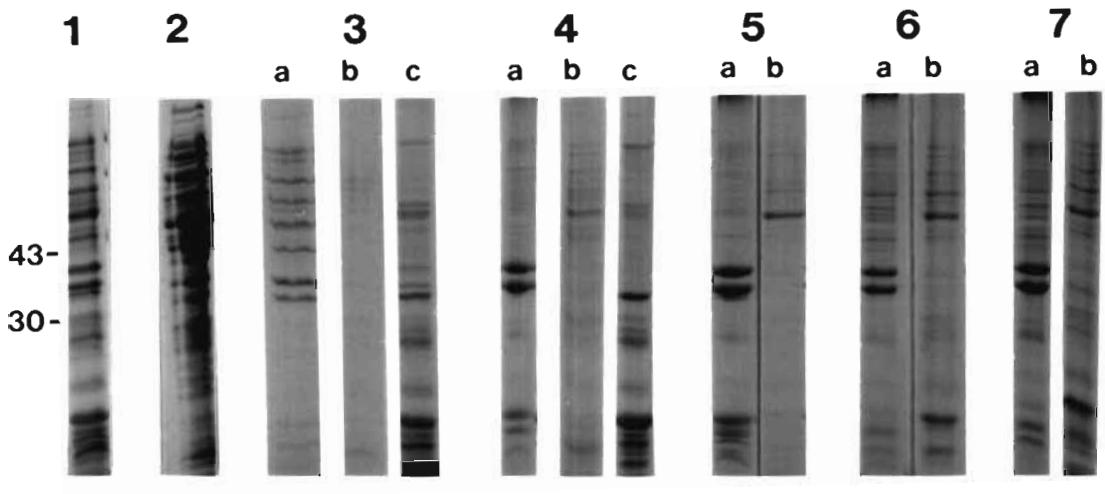
amounts, attempts to purify FlaA were concentrated on the LacZ'-FlaA protein. Experiments were performed to identify the cellular location of the LacZ'-FlaA protein within *E. coli*. DH5α(pRI23) were disrupted in a French press and the cell membranes recovered by ultracentrifugation. The majority of the H9-2 reactive material remained with the membrane fraction (Figure 32, Panel 2), suggesting a similar membrane association of the hybrid protein as found for recombinant FlaA (see 3.4.3).

In an attempt to selectively remove the hybrid protein from the membranes, membranes of DH5α(pRI23) were incubated in a 10 mM HEPES [pH 7.4] containing either 1% [wt/vol] sarkosyl, 1% [vol/vol] Triton X-114, 6 M guanidine hydrochloride, 6 M urea, 1% [wt/vol] sodium deoxycholate, 0.5 M or 1 M NaCl. Unsolubilized material was recovered by ultracentrifugation, and the two fractions were analysed both by Coomassie brilliant blue staining of SDS-PAGE gels and by Western blotting with H9-2 (Figure 32). The protein was fully solubilized by sarkosyl, guanidine hydrochloride and urea, and partially solubilized by sodium deoxycholate. Triton X-114 and NaCl did not solubilize the hybrid protein away from the *E. coli* membranes although subsequent studies with urea solubilized-LacZ'-FlaA indicated that it is soluble in the aqueous phase following phase partitioning with Triton X-114 (Figure 33).

As sarkosyl is difficult to remove by dialysis and is likely to yield an irreversibly denatured protein, subsequent studies

Figure 32. The solubility of the LacZ'-FlaA protein following treatment with guanidine hydrochloride, urea, sodium deoxycholate and sodium chloride. *E. coli* membranes from DH5α(pRI23) (**Panel 2**) were separated from soluble fractions (**Panel 1**) by ultracentrifugation and incubated in either 6 M guanidine hydrochloride-10 mM HEPES [pH 7.4] (**Panel 3**), 6 M urea-10 mM HEPES [pH 7.4] (**Panel 4**), 1% [wt/vol] sodium deoxycholate in 10 mM HEPES [pH 7.4] (**Panel 5**), 0.5 M NaCl-10 mM HEPES [pH 7.4] (**Panel 6**), or 1 M NaCl-10 mM HEPES [pH 7.4] (**Panel 7**). Following incubation at room temperature for 60 min, insoluble material (**Lane a**) was separated from the supernatant (**Lane b**) by centrifugation. If the hybrid protein was present in the supernatant, the salt was removed by dialysis against 10 mM HEPES [pH 7.4] and aggregates removed by further centrifugation; the supernatant was analyzed to detect soluble hybrid protein (**lane c**). Samples were separated on a 12.5% SDS-polyacrylamide gel and either stained with Coomassie brilliant blue (**Large panel A**) or transferred to nitrocellulose for Western blotting with monoclonal H9-2 (**Large panel B**). Molecular weight in kD is indicated on the left.

A.



B.

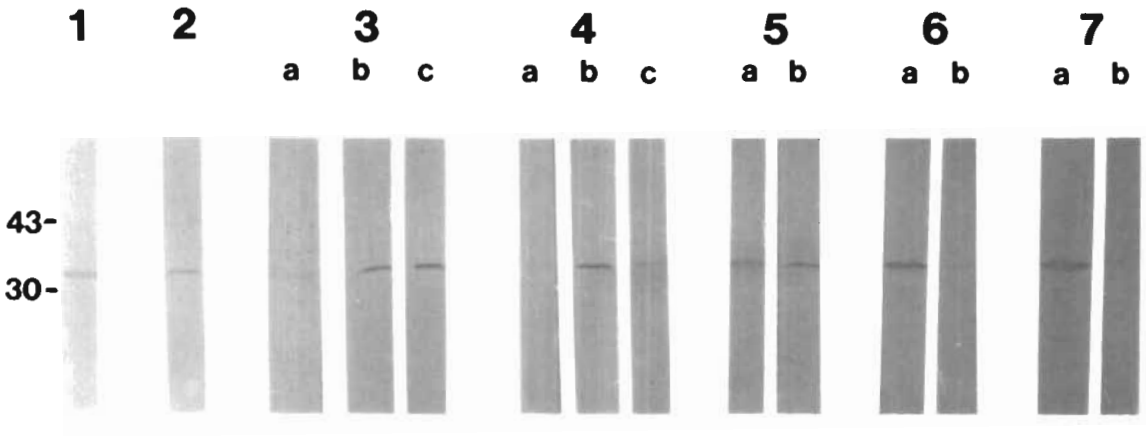
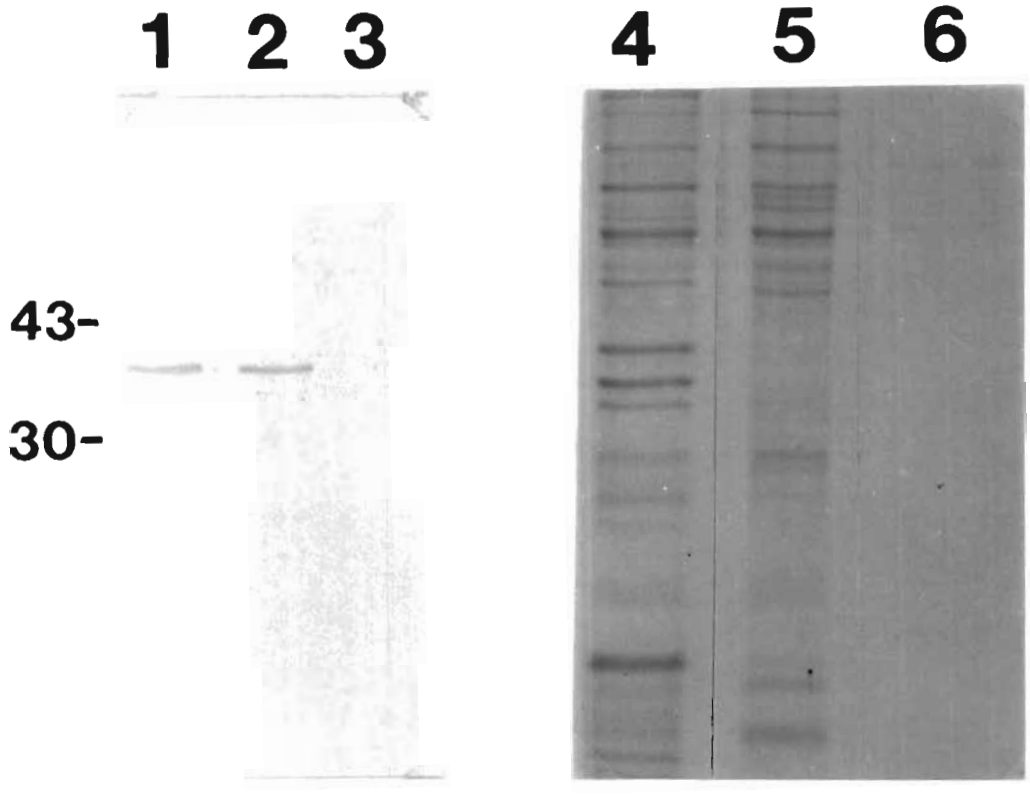


Figure 33. Triton X-114 solubility of urea-solubilized LacZ'-FlaA protein. *E. coli* membranes from DH5α(pRI23) were incubated in 6 M urea-10 mM HEPES [pH 7.4] and the supernatant, following ultracentrifugation, was dialysed against 10 mM HEPES [pH 7.4] and then extracted in 2% [vol/vol] Triton X-114. **Lanes 1,4:** *E. coli* DH5α(pRI23) membranes; **Lanes 2,6:** Triton X-114 aqueous phase; **Lanes 3,5:** Triton X-114 detergent phase. Samples were separated a 12.5% SDS-polyacrylamide gel and either stained with Coomassie brilliant blue (**Lanes 4-6**) or transferred to nitrocellulose for Western blotting with H9-2 (**Lanes 1-3**). Molecular weight in kD is indicated on the left.



concentrated on guanidine hydrochloride- and urea-solubilized LacZ'-FlaA. Dialysis of the solutions containing the solubilized protein to remove the chaotrope resulted in the visual appearance of aggregates, more marked in the guanidine treated solution than in that treated with urea. These aggregates were removed by centrifugation at 40,000 x g; most of the immunoreactivity was lost from the supernatant for the guanidine-solubilized material, whereas virtually no immuno-reactivity was lost in the urea-solubilized material (Figure 32, Panels 3 and 4, Lanes b and c).

Urea solubilized LacZ'-FlaA was passed over an H9-2 immuno-affinity column. H9-2-reactive material was present in elution fractions 3 to 6. Analysis of these fractions by SDS-PAGE followed by silver staining demonstrated that they contained small amounts of the LacZ'-FlaA protein heavily contaminated with *E. coli* proteins. When the aggregates following dialysis were removed by ultracentrifugation at 240,000 x g, no H9-2-reactive material was recovered following immunoaffinity chromatography.

4. Discussion

Venereal syphilis is a sexually transmitted disease caused by the spirochaete *T. pallidum*. A significant increase in reported cases of syphilis in the United States has occurred in the last few years[2], adding impetus to research aimed at understanding the pathogenesis of this disease. Motility is a characteristic feature of all spirochaetes[43] and is thought to contribute to the ability of *T. pallidum* to invade and disseminate within mammalian hosts.[71] The putative organelles of motility, the endoflagella[61,76-78], are complex polymeric structures within the periplasmic space of the organism[126] and consist of a core surrounded by a protein sheath or outer layer.[122,126,127] They are composed primarily of polypeptides of M_r 37, 33.5 and 33 kD,[100,122,123] with immunological studies demonstrating that the 37-kD protein comprises the sheath.[127]

The endoflagellar proteins are amongst the better characterized of the *T. pallidum* antigens[100,104,122,123,126,193] and they are a relatively abundant component of the organism. Intact endoflagella have been purified in sufficient quantities to allow for initial antigenic studies. In addition to their role as putative virulence factors, the endoflagellar antigens stimulate B and T cell responses in humans[82,85] and in experimental syphilis[87,89,169] suggesting that they also may play a role in the immunopathogenesis of the disease.

The sheath protein was selected for the detailed investigation reported in this thesis for several compelling reasons. In the

first instance, it is the most surface-exposed of the endoflagellar components.[78,123,126,127] and both N-terminal amino acid sequence analysis,[100,123] and antigenic analyses[100,123,193] indicate that the core proteins are relatively conserved among the treponemes, whereas the sheath proteins are more divergent. Furthermore, the host develops both humoral[82,85,87,89,93,96,168] and cell-mediated[169] immune responses to the 37-kD antigen.

The antigenic analysis of *T. pallidum* proteins traditionally has been severely limited by the inability to cultivate *T. pallidum* continuously *in vitro*. For nearly a decade, recombinant DNA methodologies have been used to clone *T. pallidum* proteins in *E. coli* in an attempt to overcome this limitation.[61,107,109,112,115,128,136-159] Studies carried out in this thesis were aimed at expressing recombinant *T. pallidum* endoflagellar sheath protein in *E. coli*. Treponemal endoflagella were purified in sufficient quantity to allow for purification of component proteins for N-terminal and internal amino acid sequence analysis. Murine monoclonal antibody H9-2 was found to be specific for the *T. pallidum* endoflagellar sheath protein and was used to detect λA34 in a λgt11 *T. pallidum* genomic library. The primary structure of the mature sheath protein was determined by combining amino acid sequence of the native protein with nucleotide sequence derived from plasmid pRI4, which was identified in a pBR322 *T. pallidum* genomic library using the DNA insert of λA34 as probe. As the N-terminal coding and regulatory regions of *fla*A could not be cloned in *E. coli*, a novel approach using inverse- and asymmetric-PCR was used

to complete the nucleotide sequence of *flaA*. FlaA, expressed in *E. coli* in a temperature-inducible T7 polymerase system, was poorly produced and inefficiently processed. A LacZ'-FlaA protein therefore was overproduced in *E. coli* to circumvent these problems, but complete purification of the recombinant antigen was not achieved as the hybrid protein associated with *E. coli* membranes.

4.1 Purification of treponemal endoflagella

At the time these studies were initiated, the only published procedure for isolation of *T. pallidum* endoflagella was that of Penn *et al.*[122] Attempts at isolating *T. pallidum* endoflagella using this procedure[122] yielded small quantities of impure material. Blanco *et al.* demonstrated that intact endoflagella purified from the cultivatable treponeme *T. phagedenis* can be dissociated into component proteins by incubation in SDS, 8 M urea, 8 M guanidine hydrochloride, and acid (pH 2.0) solutions, but are stable in neutral or mild alkaline solutions (pH 8.0), 0.1% [wt/vol] sarkosyl, and 0.1% [vol/vol] NP-40.[193] In this work, whole *T. pallidum* were extracted in sarkosyl; electron microscopy and SDS-PAGE analysis demonstrated that the insoluble residue following this extraction consisted predominantly of the endoflagella and cytoskeletal matrix. This observation formed the basis for the isolation procedure used in these studies. Milligram quantities of endoflagella, which were at least 97% pure by weight, were isolated for a number of studies including immunological analyses, purification of component endoflagellar

proteins by reverse-phase HPLC for N-terminal sequence analysis, and tryptic peptide analysis. A similar procedure for the isolation of *B. burgdorferi* endoflagella has been described independently[124]; this procedure involves repeated extractions in sarkosyl with the purified endoflagella being recovered by density gradient centrifugation.

In these studies, endoflagella were isolated from three different treponemes, *T. pallidum*, *T. pertenue*, and *T. phagedenis*. *T. pallidum* and *T. pertenue* endoflagella protein composition appear to be identical on the basis of SDS-PAGE and HPLC elution profiles. N-terminal sequence analysis of the sheath proteins and of two of the core proteins of *T. pallidum* and *T. pertenue* were identical. Further, HPLC tryptic peptide elution profiles of the endoflagellar sheath proteins of *T. pallidum* and *T. pertenue* were indistinguishable. These results, which suggest that the primary structure of the core and sheath proteins of these two organisms are identical, are consistent with the close antigenic and genetic relationship known to exist between *T. pallidum* and *T. pertenue*.[40]

4.2 The nucleotide sequence of *flaA*

Nearly all of the *T. pallidum* antigens cloned and expressed in *E. coli*, to date, were identified in genomic libraries using monoclonal or polyclonal antibodies.[107,109,112,115,128,137-142,145-159] It is clear, however, that only a limited number of recombinant *T. pallidum* proteins can be identified using techniques dependent

upon expression from native treponemal promoters. The difficulties experienced by a number of investigators in cloning the endoflagellar proteins[115,144,229] have emphasized the need to employ alternative strategies. Pallesen and Hindersson[144] used degenerate oligonucleotide probes derived from the N-terminal amino acid sequence of the endoflagellar core proteins to identify a gene, designated *fla*B2. DNA sequence analysis revealed that *fla*B2 lacked a consensus promoter, a finding which most likely explained the lack of its expression in *E. coli*. Champion *et al.* used a similar strategy to identify the operon encoding two other core proteins, *fla*B1 and *fla*B3.[143]

Cloning of the 37-kD endoflagellar sheath protein has proved to be particularly difficult. Previous attempts to identify recombinant clones expressing the 37-kD endoflagellar sheath protein in non-expression *T. pallidum* genomic libraries have been unsuccessful.[115,229] The λgt11 expression system has provided an alternative means for cloning proteins which are not expressed from their native promoters.[170,230-234] For example, the major outer membrane protein of *Chlamydia trachomatis* serovar L_2 could not be identified in a λ1059 genomic library by antibody screening[230]; however, a recombinant clone expressing an epitope of this protein was detected by antibody screening of a λgt11 genomic library, and the chlamydial DNA insert of this clone provided the probe which subsequently allowed the complete nucleotide sequence of the gene to be determined[231]. The λgt11 expression system has also proved useful in cloning mycobacterial genes[232-234] and the

yeast RNA polymerase II genes.[170]

In the work reported in this thesis, monoclonal antibody H9-2 reacted specifically with the 37-kD endoflagellar sheath protein and was used to identify a clone, A34, in the λgt11 genomic library. The A34 hybrid protein retains β-galactosidase activity as the λA34 lysogen colonies are blue when grown on LB agar containing IPTG and X-Gal, indicating that the treponemal DNA insert does not introduce a premature translational stop in the *lacZ* mRNA transcript. While SDS-PAGE analysis revealed the A34 hybrid protein to be 17 kD larger than native β-galactosidase, the DNA insert encoded for only approximately 4 kD of additional peptide. The DNA insert is not multimeric and nucleotide sequence analysis does not indicate any potential glycosylation sites nor that the protein is very basic. Decreased SDS binding of the hybrid protein leading to anomalous gel migration is a possible explanation for the observed discrepancy in electrophoretic mobility and the difference in the estimated molecular weight.

Exhaustive attempts to clone the entire gene or any portion that contained the putative promoter and N-terminus by generating overlapping clones using a directed cloning approach were unsuccessful. As an alternative approach, a random gene library constructed in pBR322 was screened. Nucleotide sequence analysis of pRI4, which was identified in this library, indicated that this plasmid encoded for all but the nine N-terminal amino acids of the 37-kD sheath protein. Because the N-terminal amino acid

sequence obtained from the purified native antigen overlapped that derived from the nucleotide sequence, it was possible to deduce the primary structure of the mature 37-kD protein.

In order to complete the nucleotide sequence of *fla*A, a strategy based on inverse-PCR was employed. Initially, it was planned to use the inverse-PCR product as a template for nucleotide sequence analysis. However, a product was detected in only a few experiments and could not be used as a template for subsequent PCR reactions. Complementarity between oRI9 and oRI10 (Table 3), with resultant "primer-dimer" formation during PCR[226], and/or variability in circularization of the chromosomal DNA may have contributed to this problem. The inability to clone the relevant portion of the inverse-PCR product was not surprising in view of the previous failures to clone the upstream regions of *fla*A. Using sequence derived from a *Sau*3A I fragment cloned from the inverse-PCR product, the complete nucleotide sequence of *fla*A was finally obtained by asymmetric PCR.

The failure to clone the *fla*A gene may have been due to control factors similar to those known to occur in other bacterial systems. For example, production of the components of *Bacillus subtilis* flagella is co-ordinated at the level of transcription by a specific sigma factor, σ^{28}.[235-237] *B. subtilis* mutants in which σ^{28} synthesis is blocked contain no flagellin[235] and no detectable *hag* mRNA transcripts (*hag* is the gene encoding flagellin).[236] Both *E. coli* and *S. typhimurium* flagella-associated genes contain

consensus σ^{28}-promoter sites, suggesting that this is a common mechanism of controlling flagella assembly across species.[237] A consensus promoter sequence for σ^{28} associated-DNA-dependent RNA polymerase is present in *T. pallidum flaB2* and in the operon encoding the *T. pallidum* FlaB1 and FlaB3 endoflagellar core proteins.[143,144,237] In contrast, conventional *E. coli* promoter and RBS sequences were identified in *flaA*. Only 30 base pairs of nucleotide sequence upstream of the promoter sites were obtained; as σ^{28}-promoter sequences may be over 100 base pairs upstream of the initiation codon[237], their presence in *flaA* cannot be excluded entirely.

Hydrophilicity analyses demonstrated a major hydrophilic peak in the region of the protein encoded by the DNA insert in λA34 which encodes the epitope recognized by H9-2. Localization of the H9-2 epitope to this region of the protein is consistent with the previously determined correlation between B cell epitopes and large hydrophilic peaks.[238] Furthermore, the λA3 hybrid protein induces a proliferative response in splenic lymphocytes from rabbits with experimental syphilis.[61] Analysis of the primary structure of FlaA using the algorithm of Margalit *et al.*[239] predicted three T-cell epitopes either partially or completely encoded by the λA34 DNA insert: amino acids 141 through 147 (Block length = 7, Amphipathic score = 13.8), amino acids 142 through 147 (Block length = 11, Amphipathic score = 11.2), and amino acids 171 through 185 (Block length = 11, Amphipathic score = 32.5). In addition, this third putative epitope contains a

lysine residue near the carboxyl-terminus, increasing the probability that this is a true epitope.[239]

Although the FlaA protein from *S. aurantia*[223] shares little overall sequence homology with that of *T. pallidum*, four areas of homology were identified in the N-terminal half of the protein. The area of greatest homology lies partially within the major hydrophilic domain described above, although H9-2 does not react with *S. aurantia* by Western blot. It is possible that these areas of homology represent either functionally conserved domains or domains necessary for the assembly of the endoflagella. A striking feature of the primary structure of *T. pallidum* FlaA is the presence of 19 amino acid doublets and two amino acid triplets. Interestingly, *S. aurantia* FlaA protein shows a similar pattern with 13 doublets and four triplets, suggesting that this sequence pattern has either functional and/or structural significance.

4.3 Cloning toxic gene products in *E. coli*

The inability to clone putative bacterial virulence factors in *E. coli* is not unique to FlaA. Other examples include the major outer membrane proteins of *Haemophilus influenzae*[240,241] and *N. gonorrhoeae*[242,243], both of which are presumed to be 'toxic' when expressed in *E. coli* from their native promoters. Nucleotide sequence analysis of these putatively 'toxic' proteins often has been accomplished by generating overlapping clones, one of which encoded the putative promoter and N-terminus.[241-243] In the case of

the *N. gonorrhoeae* major outer membrane protein P1, overlapping clones were identified by either screening a λgt11 genomic DNA library with monoclonal antibodies[243] or by screening plasmid genomic DNA libraries constructed from small DNA fragments with oligonucleotides derived from N-terminal amino acid sequences.[242] Munson *et al.*[241] determined the nucleotide sequence of the *H. influenzae* type b major outer membrane protein P2 by first screening a λgt11 genomic DNA library with an oligonucleotide probe derived from N-terminal sequence of the native protein. The DNA insert from this clone could not be subcloned into either a high or a low copy number plasmid, but eventually was able to be subcloned into M13. Nucleotide sequence derived from this clone provided the probes for identifying overlapping clones. In contrast, Hansen *et al.*[240] used a shuttle vector to initially clone the same *H. influenzae* protein in *H. influenzae* and to subsequently perform sequence analysis in *E. coli*.

The nucleotide sequence of *fla*A could not be completed by generating overlapping clones. DNA fragments which encoded for as little as the first 85 amino acids of pre-FlaA in association with the upstream regulatory regions could not be cloned in either plasmid or phage vectors. Similar difficulties were encountered in cloning the regulatory region of the *H. influenzae* type b major outer membrane protein in *E. coli*[244]; in this case the entire gene was cloned in a shuttle vector and nucleotide sequence analysis could be performed without subcloning. Brahamsha and Greenberg[223] identified a clone expressing the

analogous protein of *S. aurantia* along with its putative leader peptide by immunological screening of a genomic library utilizing the hybrid *tac* promoter[245]; however, no native promoter sequence was identified. Assuming that the genetic organization of these two organisms is similar with respect to the endoflagellar proteins, their data suggest that the leader peptide alone is not the cause of toxicity. Rather the promoter, either alone or in association with the N-terminal peptide, or products encoded by upstream DNA may be responsible for putative toxicity. In this regard, it has been known for a number of years that cloning 'strong' promoters can be difficult.[246] Bacteriophage T5 promoters exhibiting high signal strength have been successfully cloned by placing strong transcriptional stops immediately downstream of the putative promoter sequence.[246] The regulatory regions of *fla*A could not be cloned using pTTQ vectors which contain such a strong transcriptional stop.[185]

The reason for the inability to clone the regulatory regions of *fla*A remain unclear. In any event, these problems no doubt account for the difficulties encountered by several groups attempting to clone this antigen. The strategy ultimately employed in these studies should be useful for obtaining sequences from other unclonable portions of genes. In fact, this already has been demonstrated independently by Berish *et al.*[247] who used a similar strategy to determine the nucleotide sequence of the iron binding protein of *N. gonorrhoeae*. These workers used oligonucleotide probes based on N-terminal and internal amino

acid sequence to identify overlapping clones in genomic DNA libraries; the combined nucleotide sequence contained an open reading frame encoding the precursor protein but no regulatory sequences. In contrast to *flaA*, their inverse-PCR DNA fragment encoding the regulatory regions was able to be cloned into M13 for direct sequence analysis.

4.4 Purification of *T. pallidum* chromosomal DNA

Numerous *T. pallidum* genomic DNA libraries were constructed during the course of this research. The initial libraries contained few recombinant clones. The chromosomal DNA used to construct these libraries, although of satisfactory molecular weight, ran as a 'smear' when assessed by ethidium bromide-agarose gel electrophoresis. Genomic DNA extracted from *E. coli* did not produce this 'smear' and libraries constructed from this DNA contained acceptable numbers of clones. It was assumed that the smearing indicated degradation of the chromosomal DNA prior to extraction and it was hypothesized that rabbit cellular exonucleases were the cause of the degradation. Further, the smearing was worse when the chromosomal DNA was extracted from organisms which had been frozen at -70°C prior to use. Several different procedures for chromosomal DNA extraction were used before a satisfactory procedure was developed (see 2.19, Procedure D).

This procedure has a number of specific features. First, chromosomal DNA was isolated only from *T. pallidum* freshly

extracted from rabbit testicles. Second, rabbit cellular exonucleases were inhibited by high concentrations of EDTA prior to disruption of the *T. pallidum* membranes and the organisms were incubated in proteinase K for five minutes prior to the addition of detergent to digest any exonucleases. Third, lysozyme was not used in the extraction procedure as its addition did not increase the yield of DNA. By not using lysozyme, prolonged incubation prior to the addition of proteinase K was avoided. Lysozyme appeared to have little or no activity against *T. pallidum* peptidoglycan, but this was not assessed more formally. Mechanical shearing of the chromosomal DNA was minimized as no ethanol precipitation step was required to concentrate the extracted DNA.

4.5 Expression of FlaA in *E. coli*

Expression of FlaA in *E. coli* was achieved by using PCR-derived constructs in a temperature-inducible T7 expression system.[186] T7 RNA polymerase is a rifampin-resistant, single polypeptide DNA-dependent RNA polymerase which recognizes a conserved specific 27 base pair promoter sequence; *E. coli* DNA-dependent RNA polymerase is inhibited by rifampin. In the expression system used, T7 RNA polymerase is transcribed from a plasmid, pGP1-2, under the control of a strong λ P_L promoter; a temperature-sensitive λ repressor, encoded on the same plasmid, is active at 30°C but is inactive at 42°C. Transcription of the *flaA* under the control of the T7 promoter is minimal at 30°C since the λ repressor inhibits transcription of the T7 RNA polymerase gene. However, when the temperature is shifted to 42°C, T7 RNA

polymerase is manufactured resulting in expression of pre-FlaA. Following the addition of rifampin, protein synthesis derived from *E. coli* promoters is inhibited and pre-FlaA is almost exclusively produced. The insert of the first construct, pRI17, encoded for only 3 base pairs upstream of the RBS and did not express FlaA. Analysis of the mRNA transcript suggested that a stem-loop could form between the *Sma*I site of the vector polylinker and the RBS of *flaA* leading to inactivation of the RBS. Expression was achieved with an alternative construct, pRI18, whose insert encoded for 30 base pairs upstream of the RBS.

Nucleotide sequence analysis indicated that mature FlaA arises from cleavage of a signal peptide.[218,221] Immunoblot analysis with monoclonal antibody H9-2 revealed a 37- and 39-kD doublet in the *E. coli* transformants. The 37-kD protein co-migrated with the native FlaA by both SDS- and 2D-PAGE analyses; similar analyses using [^{35}S]-labelled pre-FlaA confirmed that the 39-kD antigen was the accumulated precursor. While the reason(s) for inefficient processing is unclear, several possibilities were excluded. First, it is unlikely that the temperature shock required for FlaA expression contributed to the abnormal processing. In the pulse-chase experiments, bacteria were induced at 42°C for 25 min, equilibrated at 30°C prior to the addition of the radiolabel, and then chased at the same temperature. Further, β-lactamase in controls was efficiently processed under the same conditions. Second, although the amino acid sequence of the

signal peptide was derived entirely from PCR products, PCR conditions associated with the lowest misincorporation rates were chosen to minimize the possibility of errors introduced by the infidelity of *Taq* polymerase.[224-226] In addition, the nucleotide sequences of the signal peptides of several PCR-derived constructs were identical. Third, pre-FlaA also accumulated when expressed from pRI19, indicating that unidentified mutations downstream of the cleavage site in pRI18 were not responsible for the inefficient processing. Fourth, the observations that the cleavage site so closely matches the consensus site for *E. coli* signal peptidase I[218] and that the native and the recombinant FlaA had identical electrophoretic mobilities strongly argue against the possibility that a difference in cleavage site specificity exists between the *E. coli* and *T. pallidum* signal peptidases.

Overloading of the *E. coli* export machinery is the most likely explanation for inefficient processing of FlaA. This phenomenon is well recognized in *E. coli* clones overproducing native *E. coli* secretory proteins.[248-252] For example, *E. coli* overproducing *E. coli* phosphate binding protein, PhoS, accumulate its precursor form, pre-PhoS, in the cytoplasm[251]. Cell fractionation studies indicate that pre-PhoS is present in the soluble cytoplasmic and inner membrane fractions[252]. No degradation of pre-PhoS protein occurs when spheroplasts of overproducing cells are exposed to trypsin, indicating that none of the accumulated precursor is translocated across the inner membrane[249]. Further the inner membrane associated pre-PhoS is more resistant to trypsin

treatment than the cytoplasmic protein, suggesting that the proteins in each subcellular compartment are in different configurations[249]; as mature PhoS was trypsin resistant, the authors speculated that the cytoplasmic pre-PhoS had a configuration which was closer to that of PhoS. Further, the retarded maturation of PhoS is not due to limiting amounts of signal peptidase I. Overproduction of PhoS and signal peptidase in the same cell has no effect on the retarded maturation of PhoS.[248] In addition, overproduction of PhoS retards the maturation of β-lactamase and PhoA expressed in the same cell.[248] These data indicate that the retarded maturation of PhoS is due to a general failure of the cytoplasmic export apparatus to maintain the precursor in a translational-competent form; once the overproduced precursor has attained a stable configuration within the cytoplasm there appear to be no mechanism(s) to unfold the protein into a translational-competent form (e.g. an unfoldase). As a consequence, pre-PhoS accumulates within the cytoplasm. This hypothesis has been confirmed, in part, by the observation that in *E. coli* expressing LacZ hybrid proteins, this defect in export can be overcome by overproducing one of the following heat shock proteins-- GroEL, GroELS, or DnaK.[253] These heat shock proteins are involved in the cytoplasmic export apparatus.[254]

Other cloned treponemal secretory proteins are also inefficiently processed in *E. coli*. The treponemal basic membrane protein is slowly processed in maxicells and the authors comment that they have noted similar kinetics of processing for a number of cloned

T. pallidum antigens.[138] In pulse-chase experiments expressing TmpA and TmpB in minicells, precursor proteins are easily identifiable after 90 min of chase.[156] In similar experiments, precursor to the 34-kD protein is still present following a 120 min chase.[154] Further, precursors for both TmpA and TmpB accumulate when they are overproduced in *E. coli*.[156,157] In combination with the FlaA processing data, these findings suggest that *T. pallidum* secretory proteins are, in general, inefficiently processed in *E. coli*. One might speculate that toxicity due to progressive accumulation of pre-FlaA during unregulated expression explains the inability to clone *flaA* with its native promoter. The diminished growth of *E. coli* clones expressing FlaA at the relatively low levels achieved in this study supports this contention, although there are alternative explanations for this finding (e.g. the increased metabolic burden of transcription).

4.6 Subcellular localization of FlaA in *E. coli*

The processing experiments indicated that recombinant FlaA should be detectable within the periplasmic space. However, FlaA was not detected in the supernatants from either osmotically shocked spheroplasts or disrupted whole cells. Instead, both precursor and mature protein were found in association with the inner and outer membrane fractions. FlaA in *T. pallidum* is likely to be present as a polymer, but not so in *E. coli*. Therefore, recombinant FlaA may be relatively insoluble and have aggregated onto both the inner and outer membranes following either translocation and processing or cell disruption; proteins are not released

during osmotic shock and/or spheroplasting unless they are soluble within the periplasmic space.[255] Unexported pre-FlaA accumulated within the cytoplasm most likely aggregated onto the membranes following cell disruption.[256]

A number of cloned *T. pallidum* proteins have been localized putatively to the outer membrane in *E. coli* using a variety of methodologies.[137,139,154,156,157] A 38-kD antigen was localized to the *E. coli* outer membrane using sucrose density gradient centrifugation.[139] Sucrose density gradient centrifugation, lack of Triton X-100 solubility, and immunoelectron microscopy all localize TmpA and TmpB to the outer membrane in *E. coli* overproducing these antigens.[157] The 34-kD[154] and 47-kD[137] antigens have been localized in *E. coli* by sarkosyl solubility studies. The 34-kD antigen was partially solubilized suggesting at least some of the antigen is associated with the inner membrane. Interestingly, the 47-kD antigen was localized to the inner membrane at low levels of expression, but when overproduced in an expression system was present equally in the inner and outer membrane fractions. These data have often been used to support an outer membrane location for the native antigens in *T. pallidum*. There are now significant reasons to question the validity of this approach. Given the ultrastructural differences between the outer membranes of *T. pallidum* and *E. coli*[46,47], it is unclear whether location in the outer membrane of *E. coli* is relevant to the native antigen in *T. pallidum*. Further, fractionation results in *E. coli* may not even be representative

of the true subcellular location of the cloned antigen.[256] Aberrant outer membrane localization is well recognized in *E. coli* clones expressing genetically manipulated native or hybrid secretory proteins analyzed using the same techniques.[256] Indeed, the *T. pallidum* 47-kD fractionation data[137] suggests that the level of protein expression alters the subcellular location as determined by standard methods. The FlaA fractionation data emphasize the need for caution in extrapolating *E. coli* fractionation data to *T. pallidum*.

4.7 Expression of FlaA as a fusion protein

Although expression of recombinant FlaA in *E. coli* was achieved, only small amounts of mature FlaA were produced. As outlined above, it was argued that the low level of expression was a consequence of inefficient processing of the overproduced protein. Three possible solutions to this problem were considered:

- expression of FlaA as a hybrid protein with elimination of the signal peptide, an approach previously described by Dallas *et al.*[138] for the expression of the treponemal basic membrane protein.

- expression of pre-FlaA using either a single copy or a low copy number plasmid.[257-260] This has the potential to decrease the production of the protein to a point where it did not overload the export machinery. In addition, expression of FlaA in *E. coli* under these circumstances may not be 'toxic', enabling a simplified expression system.

- expression of pre-FlaA in a Gram positive organism. Expression of pre-FlaA under the control of vegetative promoter in a *spo*$^-$ mutant of *B. subtilis*[261,262] has two particularly attractive features. First, mature FlaA would be excreted into the surrounding liquid media. The use of a *spo*$^-$ mutant would minimize the presence of secreted proteolytic enzymes. Second, even if pre-FlaA accumulated cytoplasmically due to hyper-expression, only mature FlaA would be recovered from the supernatant. Using a fermenter, large quantities of organisms could then be cultured for protein production.

The first approach was used here as it seemed the most expeditious. Single copy plasmids can be difficult to work with and there was no guarantee that FlaA could be expressed in *E. coli* in this manner; for example, the toxic *H. influenzae* type b major outer membrane protein could not be cloned using a low copy number plasmid.[241] Furthermore, recombinant DNA methodologies using Gram positive organisms are much less developed than for Gram negative organisms and the necessary cloning vectors and strains were not available.

Two gene fusions were constructed. For subsequent purification of FlaA from the hybrid protein, a bovine protease X_a cleavage site was engineered in the hybrid protein immediately upstream of the first amino acid of FlaA. Bovine protease X_a, one of the proteins involved in the coagulation cascade, was chosen as the amino acid sequence recognized by this protease (Ile-Glu-Gly-Arg)

is not present in FlaA[228,263], and this protease recognizes its cleavage site even in denatured proteins.[263]

Theoretically, the Cro-LacZ-FlaA hybrid protein had two advantages over LacZ'-FlaA. The Cro-LacZ-FlaA hybrid protein can be purified on a anti-β-galactosidase immunoaffinity column and it was probable that the β-galactosidase component of the hybrid protein would maintain it as a soluble, non-membrane associated protein. The main disadvantage of this system is that insertion of peptides larger than 40 kD may result in extensive degradation of the hybrid protein.[187] Unfortunately this proved to be the case with the Cro-LacZ-FlaA protein. In particular, subjecting the bacteria to heat shock at 42°C to hyperexpress the hybrid protein resulted in total destruction of the protein.

In contrast, the LacZ'-FlaA protein, which is of significantly lower molecular weight, could be expressed in *E. coli* in larger amounts. However, subsequent purification of this protein was not achieved. The LacZ'-FlaA protein is isolated along with the *E. coli* membranes following cellular disruption suggesting it associates with the membranes in the same manner as pre-FlaA and FlaA. The hybrid protein can be dissociated away from the membranes and solubilized by the ionic detergent sarkosyl, and the chaotropes guanidine hydrochloride and urea. As sarkosyl is difficult to remove prior to subsequent isolation procedures, further experiments were performed with guanidine- and urea-solubilized LacZ'-FlaA. Removal of the chaotrope by dialysis

resulted in the formation of visible aggregates, possibly containing hydrophobic membrane proteins.

After ultracentrifugation at 40,000 x g to remove these aggregates, reduced amounts of LacZ'-FlaA could be detected in the supernatant of the guanidine-solubilized material. In contrast, the supernatant from the urea-solubilized material still contained H9-2 immunoreactive material. All subsequent studies were performed with the urea-solubilized material. Small quantities of LacZ'-FlaA, heavily contaminated with *E. coli* proteins, were recovered following immunoaffinity purification of this material. It was hypothesized that the *E. coli* contaminating proteins were from small aggregates which were not removed by ultracentrifugation at 40,000 x g. In a second experiment in which any aggregates were removed by ultracentrifugation at 240,000 x g, no immunoreactive LacZ'-FlaA remained in the supernatant. These data indicate that the LacZ'-FlaA protein does not remain soluble in the absence of urea.

4.8 Future experimentation

The presence of the LacZ'-FlaA protein in the aqueous phase following phase-partitioning of the urea-solubilized protein in Triton X-114 suggests that the hybrid protein is hydrophilic and will not require the presence of a detergent to remain soluble following purification. In order to maintain soluble LacZ'-FlaA, aggregates to which the protein can associate cannot be allowed to form. Two possible experimental approaches to further purify

the urea-solubilized LacZ'-FlaA are:

- the addition of a nonionic detergent such as Triton X-100 or N-octyl glucoside to the urea-solubilized material prior to dialysis to remove the urea. Aggregate formation would be prevented by maintaining the membrane proteins in solution. Immunoaffinity chromatography would be carried out in the presence of detergent. The use of N-octyl glucoside would be advantageous as it can be removed by dialysis following purification.
- continued purification retaining the urea. Although immunoaffinity chromatography cannot be performed in the presence of urea, other protein separation methods including ion-exchange chromatography, gel-filtration chromatography, isoelectric focusing and chromatofocusing can. Urea is easily removable by dialysis at any stage of the purification process.

Once purified hybrid protein is obtained, then the protein will be cleaved proteolytically with bovine protease X_a. FlaA will be purified from the modified LacZ' peptide by immunoaffinity chromatography. The purified LacZ'-FlaA protein may or may not be denatured. The association of the urea-solubilized LacZ'-FlaA protein with the membrane aggregates following removal of the urea suggest that the membrane binding capabilities of the protein are not denatured irreversibly by urea. Provided relatively mild, non-denaturing purification methods were used following the urea-solubilization, it is probable that the purified hybrid protein will not be denatured completely. This

hypothesis is supported by observations of Roepe and Kaback[264] who purified *lac* permease from *E. coli*; the purified protein retained a native conformation despite the use of 5 M urea in the extraction procedure. The purification of FlaA in its native configuration is desirable for detailed B-cell epitope mapping studies, in which the native configuration is required to detect conformational epitopes, and for *in vitro* experiments to see if the endoflagellar sheath self-assembles.

The studies reported here will provide the basis for further molecular investigations of the endoflagellar apparatus and treponemal motility, including further studies of the organization of the gene family encoding the endoflagellar proteins. In other bacteria, analysis of the genetics of flagellar expression has proved useful in understanding the genetic organization and control of complex operons.[265] In concert with attempts to achieve continuous *in vitro* cultivation of *T. pallidum*, the ultimate goal of these studies will be to use the derived genetic information along with mutational analyses to elucidate the relationships between motility and virulence as has been accomplished with other bacterial pathogens.[266]

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APPENDICES

Appendix 1. Amino acid composition of pre-FlaA compared with the endoflagellar core proteins (FlaB1, FlaB2, and FlaB3)*.

Amino Acid	pre-FlaA		Core proteins	
	n	%	n	%
Alanine	39	11.1	105	12.3
Arginine	22	6.3	58	6.8
Asparagine	12	3.4	62	7.2
Aspartic Acid	22	6.3	41	4.8
Cysteine	1	0.3	0	0.0
Glutamine	15	4.3	70	8.2
Glutamic Acid	24	6.9	48	5.6
Glycine	25	7.2	64	7.4
Histidine	6	1.7	11	1.3
Isoleucine	11	3.1	72	8.4
Leucine	22	6.3	60	7.0
Lysine	17	4.9	24	2.8
Methionine	11	3.1	42	4.9
Phenylalanine	13	3.7	19	2.2
Proline	12	3.4	3	0.4
Serine	23	6.6	65	7.5
Threonine	12	3.4	46	5.4
Tryptophan	5	1.4	2	0.2
Tyrosine	16	4.6	18	2.1

Valine	42	12.0	47	5.5
TOTAL	350	100.0	857	100.0

*Abbreviations: n, number of amino acids

Appendix 2. Codon usage table for the gene encoding pre-FlaA and for all four genes (*fla*A, *fla*B1, *fla*B2, *fla*B3) encoding the major *T. pallidum* endoflagellar proteins*.

Amino Acid	Codon	Pre-FlaA		All endoflagellar proteins	
		n	%	n	%
Alanine	GCT	12	30.8	35	24.3
	GCC	4	10.3	18	12.5
	GCA	5	12.7	39	27.1
	GCG	18	46.2	52	36.1
		39	100.0	144	100.0
Arginine	CGT	8	36.4	21	26.3
	CGC	5	22.7	35	43.7
	CGA	0	0.0	4	5.0
	CGG	6	27.3	10	12.5
	AGA	2	9.1	6	7.5
	AGG	1	4.5	4	5.0
		22	100.0	80	100.0
Asparagine	AAT	7	58.3	24	32.4
	AAC	5	41.7	50	67.6
		12	100.0	74	100.0

Aspartic acid	GAT	11	50.0	29	46.0
	GAC	11	50.0	34	54.0
		22	100.0	63	100.0
Cysteine	TGT	0	0.0	0	0.0
	TGC	1	100.0	1	100.0
		1	100.0	1	100.0
Glutamine	CAA	1	6.7	14	16.5
	CAG	14	93.3	71	83.5
		15	100.0	85	100.0
Glutamic acid	GAA	3	12.5	22	30.6
	GAG	21	87.5	50	69.4
		24	100.0	72	100.0
Glycine	GGT	11	44.0	30	33.7
	GGC	1	4.0	26	29.2
	GGA	5	20.0	15	16.9
	GGG	8	32.0	18	20.2
		25	100.0	89	100.0
Histidine	CAT	5	83.3	5	29.4
	CAC	1	16.7	12	70.6
		6	100.0	17	100.0

Isoleucine	ATT	3	27.3	26	31.3
	ATC	6	54.5	52	62.7
	ATA	2	18.2	5	6.0
		11	100.0	83	100.0
Leucine	TTA	0	0.0	1	1.2
	TTG	6	27.3	20	24.4
	CTT	4	18.2	20	24.4
	CTC	3	13.6	21	25.6
	CTA	0	0.0	1	1.2
	CTG	9	40.9	19	23.2
		22	100.0	82	100.0
Lysine	AAA	1	5.9	5	12.2
	AAG	16	94.1	36	87.8
		17	100.0	41	100.0
Methionine	ATG	11	100.0	53	100.0
		11	100.0	53	100.0
Phenylalanine	TTT	6	46.2	11	34.4
	TTC	7	53.8	21	65.6
		13	100.0	32	100.0

Proline	CCT	4	33.3	5	33.3
	CCC	6	50.0	7	46.7
	CCA	0	0.0	1	6.7
	CCG	2	16.7	2	13.3
		12	100.0	15	100.0
Serine	TCT	8	34.8	25	28.4
	TCC	3	13.0	17	19.3
	TCA	1	4.4	10	11.4
	TCG	5	21.7	12	13.6
	AGT	5	21.7	11	12.5
	AGC	1	4.4	13	14.8
		23	100.0	88	100.0
Threonine	ACT	3	25.0	17	29.3
	ACC	2	16.7	18	31.0
	ACA	3	25.0	9	15.5
	ACG	4	33.3	14	24.2
		12	100.0	58	100.0
Tryptophan	TGG	5	100.0	7	100.0
		5	100.0	7	100.0

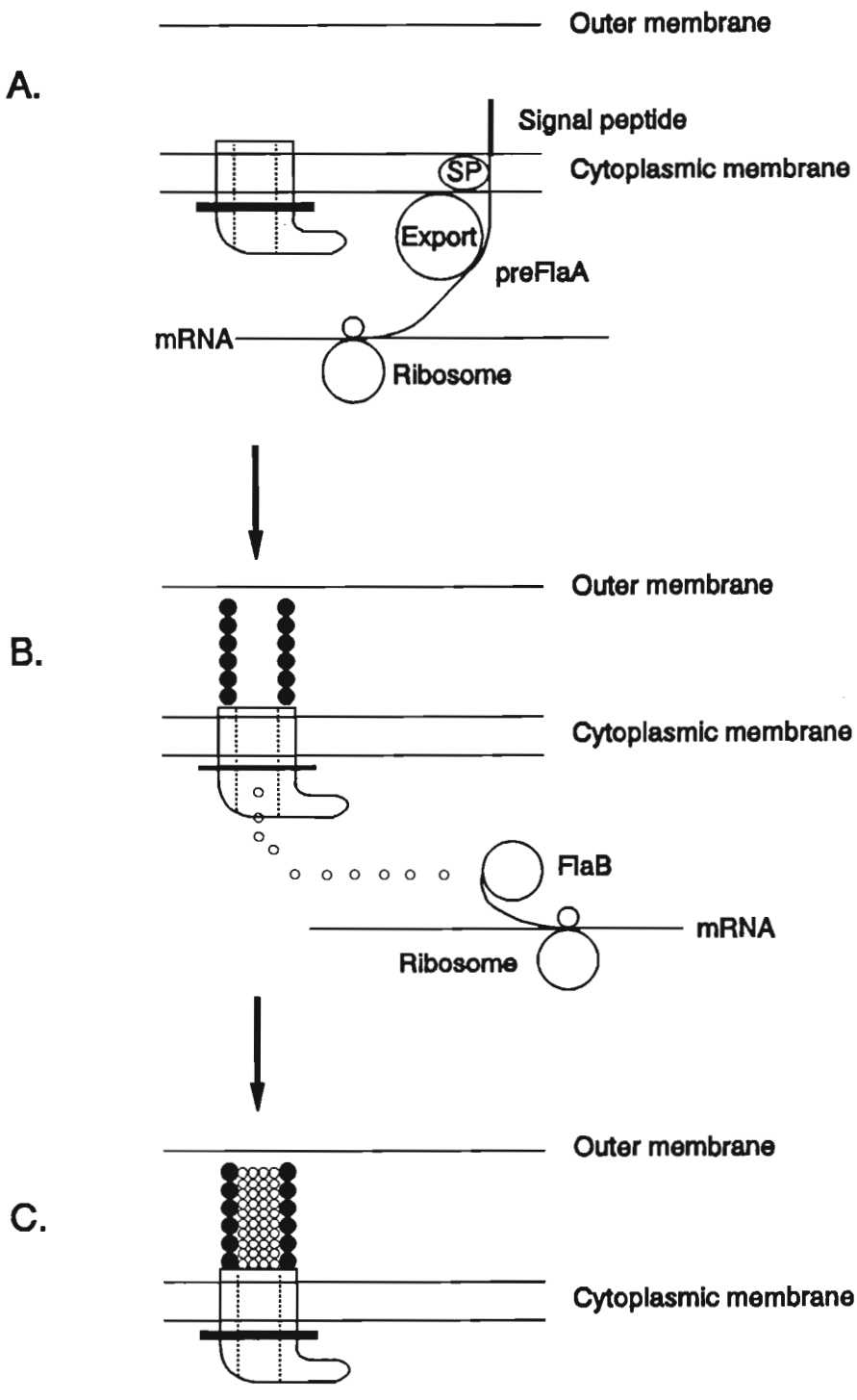
Tyrosine	TAT	10	62.5	13	38.2
	TAC	6	37.5	21	61.8
		16	100.0	34	100.0
Valine	GTT	13	31.0	25	28.1
	GTC	4	9.5	19	21.3
	GTA	4	9.5	11	12.4
	GTG	21	50.0	34	38.2
		42	100.0	89	100.0
TOTAL		350		1207	

*Abbreviations: n, number of each amino acid coded for by a particular codon; %, per cent of each amino acid coded for by a particular codon.

Appendix 3. Hypothetical model of treponemal endoflagella assembly.

During the course of the studies reported in this thesis, the following hypothesis was developed to explain how *T. pallidum* endoflagella is assembled (Figure 34). In *E. coli*, flagellin monomers are exported across the cell membrane by a poorly characterized, flagellum-specific mechanism which does not involve cleavage of an N-terminal signal.[267] It has been proposed that monomers are transported via a channel within the growing flagellin to its distal end. The *T. pallidum* core proteins (FlaB1, FlaB2, and FlaB3) do not have a signal sequence and share significant sequence homology with other bacterial flagellins, including *E. coli* flagellin[100,144]; presumably the endoflagella core assembles in a manner similar to that of *E. coli* flagella. Secretion by a signal peptide-dependent pathway is consistent with the hypothesis that FlaA forms the sheath by polymerizing around the growing core.

Figure 34. Hypothetical model for endoflagella assembly. **Panel A.** The base-plate/hook assembly has been formed with a channel in the centre (dashed lines). Pre-FlaA is translated from the mRNA by ribosomes and the elongating peptide is maintained in the proper configuration for export across the cytoplasmic membrane by the "export" protein complex (**EXPORT**). The signal peptide is cleaved by signal peptidase I (**SP**). **Panel B.** Once FlaA is free in the periplasmic space, it assembles into its proper configuration (black dot) and the sheath of the endoflagella self-assembles. Core proteins (**FlaB**) are translated and attain their tertiary structure in the cytoplasm. **Panel C.** They are transported through the channel in the base-plate/hook structure by a signal peptidase-independent mechanism, self-assembling as a core within the sheath.


A.
Outer membrane
Signal peptide
SP
Cytoplasmic membrane
Export
preFlaA
mRNA
Ribosome
B.
Outer membrane
Cytoplasmic membrane
FlaB
mRNA
Ribosome
C.
Outer membrane
Cytoplasmic membrane

SELECTED PUBLICATIONS RELATED TO THESE STUDIES

Isaacs RD, Hanke JH, Guzman-Verduzco L-M, Newport G, Agabian N, Norgard MV, Lukehart SA, Radolf JD. Molecular cloning and DNA sequence analysis of the 37-kilodalton endoflagellar sheath protein of *Treponema pallidum*. Infect Immun 1989;57:3403-3411.

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Molecular Cloning and DNA Sequence Analysis of the 37-Kilodalton Endoflagellar Sheath Protein Gene of *Treponema pallidum*

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We have used a combination of nucleotide and N-terminal-amino-acid-sequence analyses to determine the primary structure of the 37-kilodalton (kDa) endoflagellar outer layer, or sheath, protein. Initially, a λgt11 clone (designated λA34) expressing a portion of the 37-kDa protein was selected from a *Treponema pallidum* genomic library with a murine monoclonal antibody (H9-2) directed against an epitope of the 37-kDa protein. The insert from λA34 provided a probe with which a chimeric plasmid (pRI4) encoding all but the nine N-terminal amino acids of the entire protein was selected from a *T. pallidum*(pBR322) genomic library. The nine N-terminal amino acids determined by amino acid sequencing were combined with the DNA sequence encoded by pRI4 to determine the primary structure of the entire 37-kDa protein; the combined sequence made up a polypeptide with a calculated molecular mass of 36,948 Da. Approximately one-third of the deduced sequence was confirmed by N-terminal amino acid analysis of tryptic peptides from the purified 37-kDa protein. Repeated attempts to clone upstream portions of the gene (*flaA*) by using a variety of strategies were unsuccessful, suggesting that unregulated expression of the intact sheath protein or of its most amino-terminal portions is toxic in *Escherichia coli*. These studies should provide the basis for further molecular investigations of the endoflagellar apparatus and of treponemal motility.

The motility of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the causative bacterium of venereal syphilis, is thought to play a role in events critical to the pathogenesis of syphilis (55). Analyses of mutants of cultivatable spirochetes, including *Treponema phagedenis* (29), *Spirochaeta aurantia* (44), *Spirochaeta halophila* (44), and *Leptospira interrogans* (8), indicated that the periplasmic endoflagella are the organelles of motility. Although the inability to cultivate *T. pallidum* continuously in vitro has hindered similar genetic analyses of *T. pallidum*, it is presumed that the endoflagella serve an identical role in the pathogenic treponemes. Investigators from several laboratories have demonstrated that *T. pallidum* endoflagella are complex structures consisting of a core surrounded by a protein sheath or outer layer (11, 24, 45) and that they are composed primarily of polypeptides with relative molecular masses of 37, 33.5, and 33 kilodaltons (kDa) (5, 41, 45). Immunoelectron microscopy (11) shows that the 37-kDa protein makes up the sheath. Strong antigenic cross-reactivity exists between the sheath and the core endoflagellar proteins of *T. pallidum* and strains of nonpathogenic *T. phagedenis* (41, 46).

Recent ultrastructural analyses have demonstrated that *T. pallidum* has a unique outer membrane which contains few transmembrane proteins (47, 48). The precise membrane topography and locations of protein immunogens within the outer and cytoplasmic membrane compartments are currently the subjects of intensive investigation. Because the endoflagella represent components of the organism which have been well characterized immunologically (2, 5, 40, 41, 46) and by cellular location (24), they provide essential markers for further studies of the membrane topography of other protein antigens. In addition to playing roles as putative virulence factors and potential structural markers, the endoflagellar proteins, particularly the 37-kDa antigen, elicit immune responses early in experimental syphilis (3, 19, 30). Furthermore, isolated endoflagella are potent stimulators of splenic lymphocytes from rabbits with experimental syphilis (unpublished observations). It is possible that these responses contribute to the development of protective immunity.

We report here the molecular cloning and DNA sequence analysis of the 37-kDa endoflagellar sheath protein gene of *T. pallidum*. The 37-kDa sheath protein was selected for detailed investigation for two reasons. First, it is the most surface-exposed endoflagellar component (11). Second, N-terminal-amino-acid-sequence analysis (5, 41), antigenic analyses (5, 41, 46), and high-pressure liquid chromatography (HPLC) tryptic maps (unpublished observations) indicate that the core proteins are relatively conserved among the treponemes, whereas the sheath proteins are more divergent. In concordance with the nomenclature established by Brahamsha and Greenberg for *S. aurantia* (7), we have designated the gene encoding this protein *flaA*.

MATERIALS AND METHODS

Bacterial strains. *T. pallidum* Nichols was passaged and extracted as previously described (37, 47) and further purified, if required, by Percoll density gradient centrifugation (20). *T. phagedenis* biotype Reiter was cultivated in 2.9% (wt/vol) Spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% (vol/vol) heat-inactivated normal rabbit serum (Pel-Freez Biologicals, Rogers, Ark.) at 33°C.

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Escherichia coli Y1088, Y1089, and Y1090 were used for λgt11 library construction (62). *E. coli* JM83 (59), JM109 (59), and DH5α (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) were used as recipients for plasmid transformations. *E. coli* DH5αF′ (Bethesda Research Laboratories Life Technologies) was used in M13 sequencing experiments.

Plasmids and DNAs. Bacteriophage λgt11 (62) and plasmid pBR322 (6) were used for chromosomal-DNA library construction. Plasmids pUC18 and pUC19 were used for subcloning experiments (59). Plasmid DNA for double-stranded sequencing was extracted by using an alkaline lysis procedure (32) followed by treatment with RNase. Phage DNA was isolated by using a modification of the procedure of vande Woude et al. (32, 56).

T. pallidum chromosomal DNA for Southern analysis was prepared as follows. Freshly extracted treponemes were harvested in phosphate-buffered saline (PBS) (pH 7.4) by centrifugation at 39,000 × *g* for 20 min at 4°C and suspended gently in 5 ml of 50 mM Tris hydrochloride (pH 8.0)–50 mM EDTA. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 100 μg/ml, and the mixture was incubated at 50°C for 5 min. Sodium dodecyl sulfate (SDS) was then added (final concentration, 0.5% [wt/vol]), and the mixture was incubated for 2 to 4 h at 50°C. Sequential gentle extractions were performed with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and then chloroform-isoamyl alcohol (24:1). After the addition of 1.25 g of CsCl per ml to the aqueous phase, isopycnic density gradient centrifugation was performed in a V65 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 240,000 × *g* for 10 h at 20°C. The gradient was collected in 500-μl aliquots and DNA-bearing fractions were identified by ethidium bromide agarose gel electrophoresis before being dialyzed against 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

Isolation of treponemal endoflagella. *T. pallidum* was collected by centrifugation at 13,000 × *g* for 15 min at 4°C and washed twice by repeated centrifugation in PBS. The pellet was suspended in PBS containing 0.5% (wt/vol) *N*-lauroyl sarcosinate (Sigma Chemical Co., St. Louis, Mo.) and 5 mM EDTA and was incubated overnight with mixing at 4°C; 10 ml of detergent solution was used per 10^{10} organisms. The insoluble material was collected by centrifugation at 30,000 × *g* for 20 min at 4°C and washed twice with ice-cold PBS by repeated centrifugation. The washed pellet then was suspended in 10 ml of PBS and sheared in a blender (model 23; The VirTis Co., Inc., Gardiner, N.Y.) at medium speed for 10 min. The cytoplasmic bodies and sheared endoflagella were separated by centrifugation at 20,000 × *g* for 20 min at 4°C. The supernatant, containing sheared endoflagella, was decanted and retained. The pellet was suspended in 10 ml of PBS, sheared as described above for 15 min, and then combined with the retained supernatant. The combined supernatants were centrifuged at 100,000 × *g* for 60 min at 4°C in an SW40 Ti rotor (Beckman). The insoluble material was suspended in 26% (wt/wt) CsCl in PBS containing 0.02% (wt/vol) *N*-lauroyl sarcosinate and 5 mM EDTA and was then subjected to isopycnic density gradient centrifugation at 160,000 × *g* for 48 h at 4°C. The endoflagellar band was collected and dialyzed against distilled water, and the endoflagella were recovered by centrifugation at 100,000 × *g* for 60 min at 4°C. Protein concentrations were determined by the BCA protein assay micromethod (Pierce Chemical Co., Rockford, Ill.) by using bovine serum albumin as a standard.

T. phagedenis biotype Reiter endoflagella were isolated as described above, except that the *N*-lauroyl sarcosinate concentration during isopycnic centrifugation was 0.05% (wt/vol).

Purification and N-terminal-amino-acid-sequence analysis of the 37-kDa endoflagellar antigen and tryptic peptides. Isolated endoflagella were suspended in guanidine hydrochloride (final concentration, 3 M) and heated for 30 min at 60°C before being loaded onto an RP-300 reverse-phase column (2.1 by 30 mm [diameter]; Brownlee Laboratory, Santa Clara, Calif.) in 0.1% (vol/vol) trifluoroacetic acid (Pierce) in water for separation by HPLC by using the Applied Biosystems (Foster City, Calif.) model 130A separation system. The endoflagellar antigens were eluted sequentially in a gradient of increasing concentrations of 72% (vol/vol) acetonitrile (Mallinckrodt, Inc., Paris, Ky.) and 0.085% (vol/vol) trifluoroacetic acid in water. Elution profiles were monitored by A_{214}. The fraction containing purified 37-kDa antigen (5 to 10 μg) was dried under nitrogen and suspended in 50 μl of 0.1 M ammonium bicarbonate for overnight digestion with 50 to 100 ng of trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (Worthington Biochemical Corp., Freehold, N.J.) at 37°C. The tryptic peptides were separated as described above by using an RP-300 reverse-phase column (2.1 by 100 mm). Fractions containing either the purified 37-kDa antigen or eluted peptides were applied directly onto an Applied Biosystems model 477A protein sequencer for N-terminal-amino-acid-sequence determination.

Immunological reagents. Murine monoclonal antibody (MAb) H9-2 (immunoglobulin G1 [IgG1]) directed against a 37-kDa antigen of *T. pallidum* has been described previously (31). Rabbit antisera directed against isolated *T. pallidum* and *T. phagedenis* endoflagella were raised in female New Zealand White rabbits by priming with 100 μg of isolated endoflagella in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) intramuscularly followed by two intramuscular boosts of 50 μg each in incomplete Freund adjuvant (Difco) given 4 and 6 weeks later. Rabbit antisera directed against β-galactosidase were generated by using the same protocol.

Radiolabeling of treponemes and radioimmunoprecipitation. Percoll-purified *T. pallidum* was radiolabeled with $Na^{125}I$ by the lactoperoxidase method (1, 33). Whole radiolabeled treponemes solubilized with Zwittergent 3-12 (Calbiochem-Behring, La Jolla, Calif.) were immunoprecipitated with either H9-2 murine ascites or rabbit antisera raised against *T. phagedenis* endoflagella as described previously (33).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) procedures were performed as described previously (28, 47). Two-dimensional PAGE was performed by the procedure of O'Farrell (42) with modifications as described by Norris et al. (40). For Western blotting, H9-2 murine ascites was used in a dilution of 1:100; rabbit antisera raised against isolated endoflagella were used in a dilution of 1:250; and horseradish peroxidase conjugates of goat anti-mouse IgG, rabbit anti-goat IgG (Organon Teknika, Malvern, Pa.), and staphylococcal protein A (Boehringer Mannheim) were used in dilutions of 1:1,000. Goat anti-mouse IgG radiolabeled with ^{125}I was kindly provided by E. Vitetta (Dallas, Tex.).

Construction and screening of *T. pallidum* chromosomal-DNA libraries. Restriction and other DNA-modifying enzymes were obtained from commercial sources. *T. pallidum* chromosomal DNA was partially digested with DNase, and

fragments ranging in size from 250 to 1,100 base pairs (bp) were used to construct a λgt11 phage library (52, 61, 62). Briefly, digested DNA was fractionated by agarose gel electrophoresis, methylated with *Eco*RI methylase, ligated to *Eco*RI linkers, digested with *Eco*RI, ligated to λgt11 arms, and subsequently packaged as phage (52, 61, 62). Immunoscreening was performed following induction at 42°C by using nitrocellulose filters soaked in isopropyl-β-D-thiogalactopyranoside (52). The filters were incubated sequentially with antibody and horseradish peroxidase-conjugated goat anti-mouse IgG. Phage purified from plaque-purified, MAb-reactive *E. coli* clones were used to lysogenize *E. coli* Y1089 (61, 62).

A *T. pallidum* genomic library constructed by partially digesting chromosomal DNA with *Hae*III and *Alu*I and cloning into the *Pst*I site of pBR322 after G-C tailing has been described previously (39). For screening with DNA probes, duplicate nitrocellulose filters (Millipore Corp., Bedford, Mass.) were treated sequentially with 0.5 M NaOH, 1 M Tris hydrochloride (pH 7.6) and 0.5 M Tris hydrochloride (pH 7.6)–1 M NaCl before being baked at 80°C under vacuum for 2 h. DNA-DNA hybridization was performed as described below.

DNA-DNA hybridizations. DNA probes for Southern blot analysis were purified by electroelution of the individual DNA fragments from SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gels and were labeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Lisle, Ill.) by using random-primer labeling (Boehringer Mannheim) (16). DNA transfers to nylon membranes (Zetaprobe from Bio-Rad Laboratories, Richmond, Calif., or Hybond-N from Amersham Corp., Arlington Heights, Ill.) were performed by passive transfer (51). Membranes were prehybridized in 1 M NaCl, 10% (wt/vol) dextran sulfate (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.), and 1% (wt/vol) SDS at 65°C. Radiolabeled probe (final concentration, 2×10^5 to 5×10^5 cpm/ml) and heat-denatured salmon sperm DNA (final concentration, 100 μg/ml; Sigma) were added, and hybridization was performed at 65°C for 14 h. After two brief washes in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) at room temperature, the following stringency washes were performed: two washes in 2× SSC–0.1% SDS (wt/vol) at 65°C for 30 min followed by two washes in 0.1× SSC–0.1% SDS (wt/vol) at 65°C for 10 min. Autoradiography was performed with enhancing screens at −70°C with XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

DNA sequence analysis. DNA sequence analysis was performed by using the modification by Tabor and Richardson (54) of the dideoxy termination method of Sanger et al. (50) with modified T7 DNA polymerase (Sequenase 2.0; United States Biochemicals Corp., Cleveland, Ohio). Both M13 phage vectors mp18 and mp19 and plasmids pUC18 and pUC19 were used for sequencing reactions (59). In addition to the standard M13 primers, the following synthetic oligonucleotides were used in DNA sequence analysis: 5′-CACAGAGGAGGAGAAGG-3′ (nucleotides 381 to 397), 5′-AAATAACCAACATAGTC-3′ (complementary to nucleotides 721 to 737), and 5′-AACGCCAAAACACCGC-3′ (complementary to nucleotides 1051 to 1066). (The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the accession number M26525.)

PCR DNA amplification. Polymerase chain reaction (PCR) DNA amplification was performed by using 2.5 U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.) for 50 cycles with a thermocycler (Perkin Elmer Cetus) (49). Each PCR cycle consisted of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The PCR was carried out in a volume of 100 μl in the presence of 1× PCR buffer (10 mM Tris hydrochloride [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 200 μM (each) deoxynucleoside triphosphate, 100 ng of each primer, and approximately 1 ng of template DNA. The two primers used, 5′-GGTGGCGACGACTCCTGGAGCCCG-3′ and 5′-TTGACACCAGACCAACTGGTAATG-3′, flank the *Eco*RI insertion site of the β-galactosidase gene of the λgt11 template.

Computer analysis. The University of Wisconsin Genetics Computer Group sequence analysis software package (14) was used in conjunction with Microgenie software (Beckman) for DNA sequence analyses.

Electron microscopy of isolated endoflagella. Specimens for electron microscopy were prepared as described previously (47).

RESULTS

Isolation of treponemal endoflagella. Our procedure for isolation of intact endoflagella was based on the observation that extraction of treponemes in *N*-lauroyl sarcosinate produces an insoluble residue consisting of endoflagella attached to the cytoskeletal matrix (unpublished data). The endoflagella formed a distinct band in the CsCl gradient at a refractive index of 1.3628. Electron microscopy confirmed that the fractions collected from this band contained isolated endoflagella (data not shown). The electron microscopic appearance (45) and the SDS-PAGE profile of the isolated *T. pallidum* endoflagella were identical to those previously reported (5, 41, 45) (Fig. 1C, lane 2). On average, 1 mg of endoflagella was isolated from 2×10^{11} organisms.

Murine MAb H9-2 reacts with an epitope on the 37-kDa endoflagellar sheath protein. The known reactivity of MAb H9-2 with a 37-kDa antigen of *T. pallidum* suggested that it was directed against an epitope of the endoflagellar sheath protein; however, the specificity of H9-2 for this antigen was not previously demonstrated (31). It was essential to establish unequivocally the reactivity of MAb H9-2 with this antigen before the genomic libraries were screened.

Before a monospecific rabbit anti-*T. pallidum* endoflagellar antiserum was available, immunological analyses were performed with the strongly cross-reactive anti-*T. phagedenis* endoflagellar antisera (41, 46). In one experiment, two-dimensional PAGE–Western blots of whole *T. pallidum* were incubated initially with rabbit antisera directed against *T. phagedenis* endoflagella and horseradish peroxidase-staphylococcal protein A. This was followed by incubation with H9-2 murine ascites and ^{125}I-labeled goat anti-mouse IgG and autoradiography. The antisera directed against the endoflagella identified a group of proteins (Fig. 1A) with a two-dimensional PAGE profile very similar to that described by Norris et al. for isolated *T. pallidum* endoflagella (41). In contrast, H9-2 reacted with a single polypeptide (Fig. 1B) which coincided with the 37-kDa endoflagellar antigen recognized by the polyclonal antisera (Fig. 1A). MAb H9-2 also immunoprecipitated the same group of radioiodinated proteins as rabbit antisera raised against *T. phagedenis* endoflagella (data not shown). This observation is consistent with the demonstration by Limberger and Charon (29) that a MAb directed against an epitope of the sheath can immunoprecipitate all of the major endoflagellar polypeptides. Final confirmation of the specificities of H9-2 for the 37-kDa endoflagellar protein was obtained with *T. pallidum*

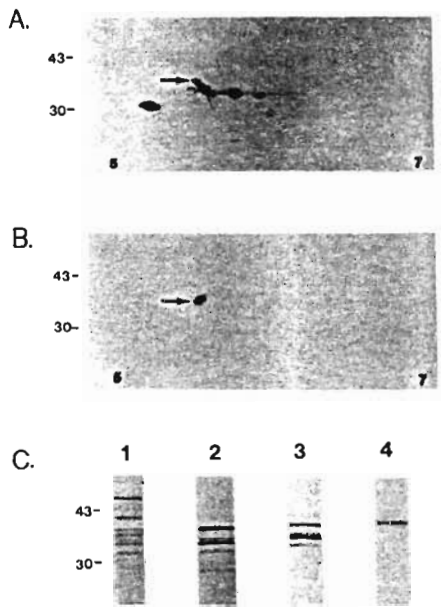

FIG. 1. Specificity of murine MAb H9-2 for the 37-kDa endoflagellar sheath protein of *T. pallidum*. (A and B) Two-dimensional PAGE immunoblots of whole *T. pallidum*. Nitrocellulose transfers were probed sequentially with antisera against endoflagella of *T. phagedenis* and horseradish peroxidase-staphylococcal protein A (A). This was followed by reaction with H9-2 and ^{125}I-labeled goat anti-mouse IgG and autoradiography (B). Arrows indicate the 37-kDa polypeptide recognized by the polyclonal antisera and MAb H9-2. The pH gradient for isoelectric focusing is shown at the bottom of each panel. (C) SDS-PAGE and Western blot analysis. Lanes 1 and 2, *T. pallidum* whole-cell lysate and isolated endoflagella, respectively, stained with Coomassie brilliant blue; lanes 3 and 4, immunoblots of isolated *T. pallidum* endoflagella probed with rabbit antisera raised against *T. pallidum* endoflagella and H9-2, respectively. Specimens were separated on a 12.5% polyacrylamide gel. Molecular masses (in kilodaltons) are shown on the left of each panel.

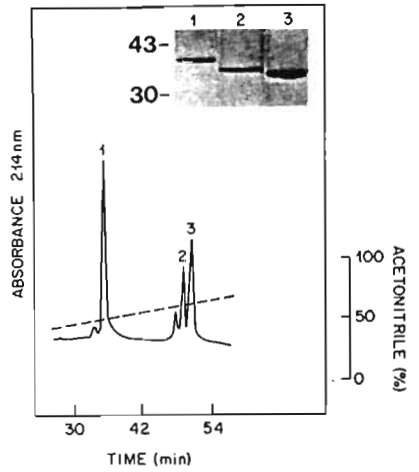

FIG. 2. Reverse-phase HPLC elution profile of *T. pallidum* endoflagellar proteins. Inset, Silver-stained polyacrylamide gel of indicated peaks; dashed line, acetonitrile gradient. Molecular masses (in kilodaltons) are shown on the left of the inset.

endoflagella, isolated as described above. Unlike rabbit anti-*T. pallidum* endoflagellar antisera (Fig. 1C, lane 3), H9-2 reacted only with the 37-kDa protein (Fig. 1C, lane 4).

Purification of the 37-kDa endoflagellar antigen. The elution profile of *T. pallidum* endoflagellar proteins separated by reverse-phase HPLC is shown in Fig. 2. Each of the component proteins eluted in a relatively hydrophobic region of the acetonitrile gradient. The major peaks were analyzed by SDS-PAGE (Fig. 2, inset) and Western blotting (data not shown). The first major peak contained the 37-kDa protein (Fig. 2, peak 1). Two distinct proteins with molecular masses of 33.5 kDa (Fig. 2, peak 2) and 33 kDa (Fig. 2, peak 3) were identified repeatedly, confirming that the 33-kDa doublet seen on SDS-PAGE of intact endoflagella (Fig. 1, lane 2) consists of at least two proteins with distinct physicochemical properties.

Expression of the epitope recognized by MAb H9-2 in *E. coli*. When the specificity of H9-2 for the endoflagellar sheath protein was established, a *T. pallidum* genomic library in λgt11 was screened with this MAb; the recombinant phage from an antibody-reactive *E. coli* clone, designated A34, was used to lysogenize *E. coli* Y1089. Crude cell lysates of λA34 and λgt11 lysogens were prepared following induction with isopropyl-β-D-thiogalactopyranoside. A comparison of these lysates by SDS-PAGE revealed that the 116-kDa band representing β-galactosidase in the λgt11 lysogen was replaced by a protein with a molecular mass of 133 kDa in the λA34 lysogen (Fig. 3, lanes 1 and 2). This polypeptide reacted with both MAb H9-2 (Fig. 3, lane 4) and rabbit antisera raised to β-galactosidase (data not shown), indicating that it was a fusion of β-galactosidase and a portion of the 37-kDa endoflagellar sheath protein. The H9-2-reactive lower-molecular-mass proteins in the λA34 lysogen (Fig. 3, lane 4) presumably represent proteolytic breakdown products, as they were also recognized by the anti-β-galactosidase antiserum (data not shown).

*Eco*RI endonuclease digestion of DNA purified from phage λA34 revealed a small insert, approximately 130 bp in size. An insert of this size would code for only 4 of the 17

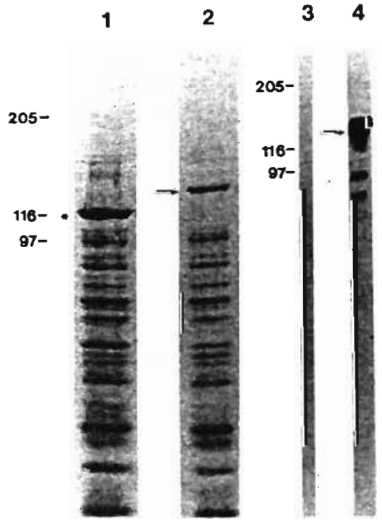

FIG. 3. Characterization of the β-galactosidase fusion protein produced by λA34 *E. coli* lysogen. Lanes 1 and 2, Crude cell lysates of λgt11 and λA34 lysogens, respectively, separated on a 7% polyacrylamide gel and stained with Coomassie brilliant blue; lanes 3 and 4, immunoblots with H9-2 and radioiodinated goat anti-mouse IgG of λgt11 and λA34 crude cell lysates, respectively, separated on a 10% polyacrylamide gel. Molecular masses (in kilodaltons) are shown on the left. Arrows and asterisk indicate A34 fusion protein and β-galactosidase, respectively.

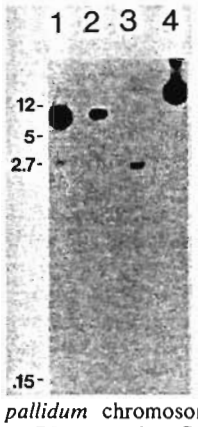

FIG. 4. Southern hybridization of *T. pallidum* chromosomal DNA with the *Eco*RI-*Eco*RI fragment from pRI1 as probe. Complete *Pst*I (lane 1), *Bam*HI (lane 2), and *Hin*dIII (lane 3) digests and undigested chromosomal DNA (lane 4) separated on a 0.8% agarose gel are shown. Kilobase-pair standards are shown on the left.

kDa predicted from SDS-PAGE analyses of the fusion protein. Restriction endonuclease analysis using *Pvu*I and *Kpn*I, restriction sites flanking the insert within λgt11 (61), also demonstrated an insert of approximately 130 bp (data not shown). In addition, a PCR amplification experiment further excluded the possibility that a multimeric insert or an internal *Eco*RI site had not been detected. When primers flanking the *Eco*RI insertion site in λgt11 were used, the PCR product obtained from the λA34 DNA was 130 bp larger than that obtained from λgt11 DNA (data not shown).

The λA34 insert, subcloned into pUC19 to create pRI1, provided the probe for subsequent library screening and Southern analyses. Specificity of this DNA probe for *T. pallidum* DNA was confirmed by Southern blot analysis. The probe hybridized to fragments of 11, 12, and 2.4 kbp in *T. pallidum* chromosomal DNA digested with *Pst*I, *Bam*HI, and *Hin*dIII, respectively (Fig. 4). No hybridization with rabbit mitochondrial DNA or *E. coli* chromosomal DNA was detected (data not shown).

Identification of the *flaA* gene in the pBR322 library. A pBR322 library (39) was screened by colony blot hybridization with the pRI1 probe. Thirty reactive clones were isolated, and their plasmids were purified and restriction enzyme mapped. Six different chimeric plasmids, pRI2 through pRI7, were identified (Fig. 5). The pRI1 probe hybridized with the 5′ *Pst*I-*Sal*I fragment of pRI3 by Southern blot analysis (data not shown). This provided the reference frame for subsequent nucleotide sequence analysis.

Amino acid and nucleotide sequence analysis of the 37-kDa antigen. The purified 37-kDa antigen and 10 peptides generated by tryptic digestion were analyzed by automated N-terminal-amino-acid-sequence analysis to provide both N-terminal and internal amino acid sequences (Fig. 6). The 21 N-terminal amino acids of the purified 37-kDa antigen were identical to those described by Norris et al. (41) and differed by only one amino acid from those reported by Blanco et al. (5).

M13 and pUC subclones derived from plasmids pRI1, pRI3, and pRI4 were used to sequence both DNA strands. On the basis of nucleotide sequence analysis, it was determined that the insert in pRI4 encoded for all but the nine N-terminal amino acids of the 37-kDa protein. The amino acid sequence of the entire 37-kDa protein has been determined by combining the N-terminal and the DNA-derived sequences (Fig. 6). Twenty-eight percent (91 of 330 amino acids) of the DNA-derived amino acid sequence was confirmed by N-terminal-sequence analysis of the tryptic peptides (Fig. 6); in all cases, there was complete correlation between the native amino acid sequence and that deduced from the nucleotide sequence. It is not clear how the peptide beginning with glutamine (Fig. 6, amino acid 298, nucleotide 868) was generated as a result of trypsin digestion. The insert of λA34 (pRI1) was localized to bp 328 through 444 (Fig. 6, amino acids 119 through 157).

The 37-kDa endoflagellar sheath protein contains 330 amino acids with a calculated molecular weight of 36,948. There are 46 acidic (Asp and Glu) and 37 basic (Arg and Lys) amino acids, and the remaining amino acids are neutral; of the neutral amino acids, 111 are hydrophobic (Phe, Trp, Tyr,

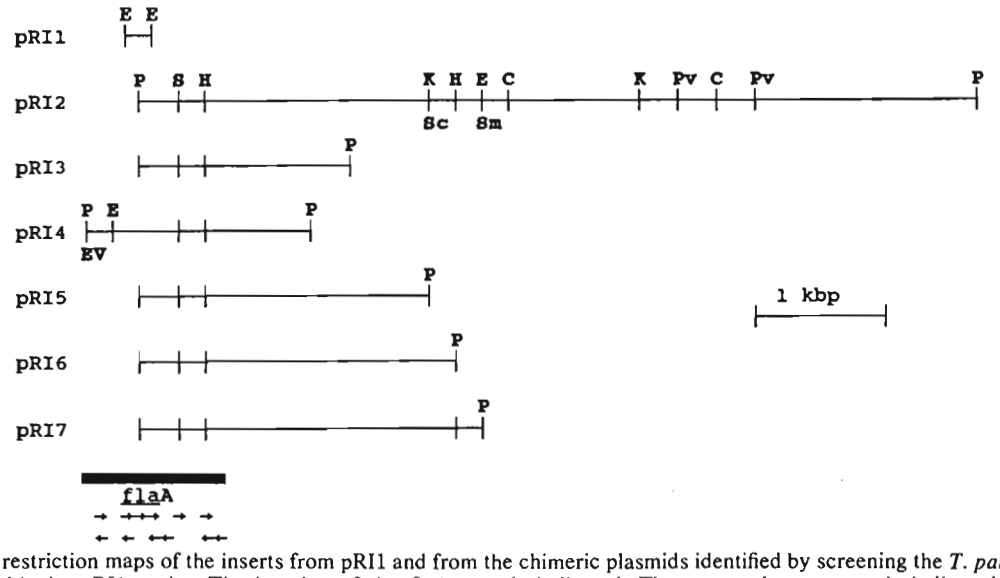

FIG. 5. Partial restriction maps of the inserts from pRI1 and from the chimeric plasmids identified by screening the *T. pallidum* (pBR322) genomic library with the pRI1 probe. The location of the *flaA* gene is indicated. The sequencing strategy is indicated by the arrows. Abbreviations: C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I.

```
                                                  10          20          30
                                        AAGCTGAACGCTGATATCATGGCGGATAAG
   AspGluSerValLeuIleAspPheAlaLysLeuAsnAlaAspIleMetAlaAspLys
        40          50          60          70          80          90
AGTGGAGGTATGACGCATAATCGGCGTACCGTTCTGGACTATGCTTCTCTGGCGGATACC
SerGlyGlyMetThrHisAsnArgArgThrValLeuAspTyrAlaSerLeuAlaAspThr
       100         110         120         130         140         150
TCGTACACTGACGAGCAGAAGGCATTGATGAGATCTTCTCTTGCGGTTGCACAGTGGGAG
SerTyrThrAspGluGlnLysAlaLeuMetArgSerSerLeuAlaValAlaGlnTrpGlu
       160         170         180         190         200         210
GTTGTGCTGAATTCTTCCGCGCGTAATCCTGTCGCCCATGCTGCCTCTCGCGTTATTGAG
ValValLeuAsnSerSerAlaArgAsnProValAlaHisAlaAlaSerArgValIleGlu
       220         230         240         250         260         270
GCTCCGGTAAGTGAGGGAGCGAAGAGTTTTGCTGGTGAGCGTGTCCTTGGTGTGCGCGTG
AlaProValSerGluGlyAlaLysSerPheAlaGlyGluArgValLeuGlyValArgVal
       280         290         300         310         320         330
TTGTTCCCCACGTGGGACAGTAACGCAAACGCAATGATAAAGCCGGCGTTCGTAATTCCT
LeuPheProThrTrpAspSerAsnAleAsnAlaMetIleLysProAlaPheValIlePro
       340         350         360         370         380         390
GCGTACGAGGTGATGGCTCAGGTGGACGATCAGGGTAATGTACAGGCCCCCACAGAGGAG
AlaTyrGluValMetAlaGlnValAspAspGlnGlyAsnValGlnAlaProThrGluGlu
       400         410         420         430         440         450
GAGAAGGCTTCTGGAAAGGGGCGTTTTGAAGATGGGTACGGAGTGGTAAAGAATGTGGGT
GluLysAlaSerGlyLysGlyArgPheGluAspGlyTyrGlyValValLysAsnValGly
       460         470         480         490         500         510
GTTCTTAAGTCCATCGCGGTGAACACTTACGGGATGAATTATCCTCATGGTTTGTACGTG
ValLeuLysSerIleAlaValAsnThrTyrGlyMetAsnTyrProHisGlyLeuTyrVal
       520         530         540         550         560         570
ATGATGCGGGATCAGGATGGTGAGGTGCATCGCTACTTCATGGGGTATCTCCTGTTCGAC
MetMetArgAspGlnAspGlyGluValHisArgTyrPheMetGlyTyrLeuLeuPheAsp
       580         590         600         610         620         630
TCCTGGAAGGAGTTGGTGTGGAACAATCCTTCGTATATCTCTGATGTTCGGTCGCGGGAG
SerTrpLysGluLeuValTrpAsnAsnProSerTyrIleSerAspValArgSerArgGlu
       640         650         660         670         680         690
GTGCGCTTGTATCCCGTGTATCCCGCGTCGACGCCCCACGTCGTGTTTGAAGGCTTTATG
ValArgLeuTyrProValTyrProAlaSerThrProHisValValPheGluGlyPheMet
       700         710         720         730         740         750
GTTACTAGGGACGCGGCTCATGCCGGAGGGGACTATGTTGGTTATTTCAAGGACGTCAAG
ValThrArgAspAlaAlaHisAlaGlyGlyAspTyrValGlyTyrPheLysAspValLys
       760         770         780         790         800         810
ATTATCTATGATAAGGCGGTGCTGAGTACGGTGCGCGATTTTGCGGACGAGGACCTGTGG
IleIleTyrAspLysAlaValLeuSerThrValArgAspPheAlaAspGluAspLeuTrp
       820         830         840         850         860         870
GGTATCCAGGCGCGGCGTGAGGCTGAGCGTAAGAGAGTTGAGGTTGCGCGTTTCGGGCAG
GlyIleGlnAlaArgArgGluAlaGluArgLysArgValGluValAlaArgPheGlyGln
       880         890         900         910         920         930
CAGCAGGTGCTGCGTTATATAGAGCAAGAGAAGCTTGCTACAGAGGTTGGTTTTACACCC
GlnGlnValLeuArgTyrIleGluGlnGluLysLeuAlaThrGluValGlyPheThrPro
       940         950         960
TCTGGGGGTGCTCAGCGGCAGGAAGAGCAGCAGTAG
SerGlyGlyAlaGlnArgGlnGluGluGlnGlnEnd
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FIG. 6. DNA and amino acid sequence of the entire 37-kDa endoflagellar sheath protein. Underlined areas represent amino acid sequences obtained by N-terminal-amino-acid-sequence analysis of either the purified native protein or tryptic peptides. The gene is numbered from the first nucleotide sequenced.

Ile, Leu, Met, and Val). The G+C content of the gene encoding the 321 amino acids derived from the nucleotide sequence is 54.1%, consistent with previously published G+C ratios of 52.4 to 53.7% for *T. pallidum* Nichols (36). The absence of cysteine residues in the derived sequence is in agreement with the amino acid analysis of Blanco et al. (5). The presence of 19 amino acid doublets and 2 amino acid triplets is a striking feature of the primary structure.

Hydrophilicity analyses by the algorithms of Kyte and Doolittle (27) (Fig. 7) and Chou and Fasman (10) (data not shown) do not demonstrate any large hydrophobic areas likely to be membrane-spanning domains. A prominent hydrophilic domain is predicted from amino acids 130 through 150; this region contains the portion of the protein encoded by the λA34 phage clone which reacted with MAb H9-2. Several T-cell epitopes are predicted by the algorithm of Margalit and co-workers (34). One of these (amino acids 146 to 170) is also partially encoded by the insert of λA34.

Homology analysis of *T. pallidum* FlaA. DNA and protein homology searches were performed by using the NBRF protein data base (release 18.0, September 1988) and the GenBank data base (release 58.0, December 1988); no significant homologies were identified. The sequence was also compared with that recently published for the homologous endoflagellar protein of *S. aurantia* (7). Four areas in the N-terminal half of each protein showed significant homology (data not shown). The greatest homology was between

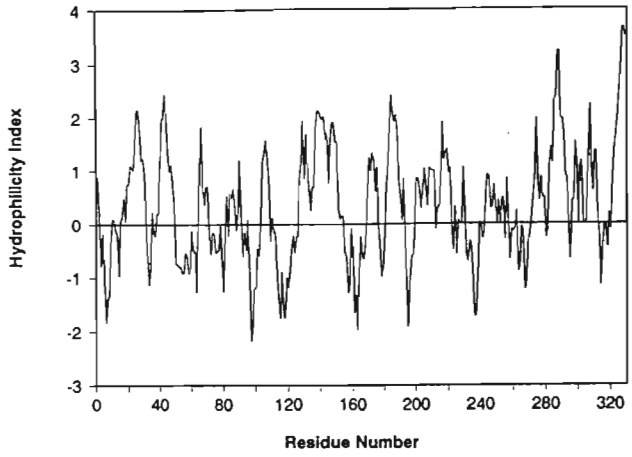

FIG. 7. Hydrophilicity analysis of the 37-kDa endoflagellar sheath protein according to the algorithm of Kyte and Doolittle (27).

amino acids 146 and 164 of *T. pallidum* and 130 and 147 of *S. aurantia* (7).

Attempts to clone the upstream region of the *flaA* gene. Initially, we attempted to clone the entire gene as one fragment by using a directed cloning approach based on Southern blot analysis (Fig. 4). Genomic libraries were generated in both phage and plasmid vectors from size-fractionated *T. pallidum* chromosomal DNA digested to completion with either *Hin*dIII (fragments 1.5 to 5 kbp in length) or *Bam*HI (fragments 8 to 15 kbp in length). No reactive clones were found after approximately 500,000 recombinant clones were screened with the pRI1 insert.

After the nucleotide sequence of pRI4 had been determined, the pBR322 and λgt11 libraries were screened with a synthetic oligonucleotide, 5′-AAGCTGAACGCTGATATC AT-3′ (nucleotides 1 to 20) in order to identify an overlapping clone which contained the upstream portion; no reactive clones were identified. Southern analysis of *T. pallidum* chromosomal DNA had shown that the upstream portion of the *flaA* gene through nucleotide 160 was contained on a 900-bp *Eco*RI-*Eco*RI fragment (data not shown). Therefore, a λgt11 genomic library generated from chromosomal DNA digested to completion with *Eco*RI (fragments 500 to 2,000 bp in length) was screened with the same synthetic oligonucleotide but contained no reactive clones (data not shown).

DISCUSSION

The endoflagellar proteins are among the best characterized of the *T. pallidum* antigens (2, 5, 24, 41, 45, 46). Norris et al. (41) have proposed that these proteins can be divided into two families, the class A and class B proteins, on the basis of N-terminal-amino-acid-sequence analysis. The sheath protein is the only member of the class A family, while the class B family includes all of the core proteins. Homology studies which use the N-terminal sequence data suggest that the core proteins are relatively conserved among the treponemes and also share significant homology with the other flagellins, including those of gram-positive organisms (13, 26, 35, 41, 58); the recently published sequence of one of the *T. pallidum* endoflagellar core proteins (FlaB2) supports this hypothesis (43). Although they share conserved epitopes, the sheath proteins are more antigenically divergent. Sequence analysis of the endoflagellar proteins, as reported here for the 37-kDa sheath protein, will provide further insights into these relationships.

In our hands, attempts to isolate *T. pallidum* endoflagella by using published procedures (45) yielded small quantities of impure material. Previous studies have demonstrated that isolated treponemal endoflagella are resistant to dissociation with a number of detergents, including *N*-lauroyl sarcosinate (5, 46). We observed that *N*-lauroyl sarcosinate extraction of whole treponemes selectively solublized the majority of treponemal antigens, leaving the endoflagella and cytoskeletal matrix intact. By using this observation as the basis for our isolation procedure, milligram quantities of endoflagella could be purified for immunological analyses and purification of component endoflagellar proteins by reverse-phase HPLC for N-terminal-sequence and tryptic peptide analyses. A similar procedure for the isolation of *Borrelia burgdorferi* endoflagella has been described independently (4).

Previous attempts to identify recombinant clones expressing the 37-kDa endoflagellar sheath protein in nonexpression *T. pallidum* genomic libraries have been unsuccessful (57; unpublished observations). The λgt11 expression system has provided an alternative means for cloning proteins which are not expressed from their native promoters (52, 53, 60–63). MAb H9-2 reacted specifically with the 37-kDa endoflagellar sheath protein and was subsequently used to identify a clone, A34, in the λgt11 genomic library. Interestingly, while SDS-PAGE analysis revealed the A34 fusion protein to be 17 kDa larger than native β-galactosidase, the DNA insert encoded only approximately 4 kDa of additional peptide. The reason for this discrepancy is unclear.

Nucleotide sequence analysis of pRI4 indicated that this plasmid encoded all but the nine N-terminal amino acids of the 37-kDa sheath protein. Because the N-terminal amino acid sequence obtained from the purified native antigen overlapped that derived from the nucleotide sequence, it was possible to deduce the primary structure of the entire 37-kDa protein. Given our subsequent inability to clone the N-terminal coding region of *flaA*, it was fortunate that an *Alu*I site was present in the N-terminal coding region of the gene. Hydrophilicity analyses demonstrated a major hydrophilic peak in the region of the protein encoded by the DNA insert in λA34 which encodes the epitope recognized by H9-2. Localization of the H9-2 epitope to this region of the protein is consistent with the previously determined correlation between B-cell epitopes and large hydrophilic peaks (23). Computer analyses predicted that the λA34 DNA insert would also encode a T-cell epitope (34); this has been confirmed by the finding that the β-galactosidase fusion protein induces a proliferative response in splenic lymphocytes from rabbits with experimental syphilis (unpublished data). These observations will provide the basis for comprehensive B- and T-cell epitope mapping of this important antigen.

Extensive attempts to clone the upstream portions of the *flaA* gene were made. The inability to clone certain genes because of the toxicity of the gene product has been described previously (9, 18, 22, 38), but in general these difficulties have been overcome by cloning the gene in portions (9, 18). However, we failed to clone fragments which code for as few as the first 65 amino acids of the protein in association with the upstream regions. Brahamsha and Greenberg (7) identified a clone expressing the analogous protein of *S. aurantia* along with its putative leader peptide by immunological screening of a genomic library utilizing the hybrid *tac* promoter; however, no promoter sequence was identified. Assuming that the genetic organizations of these two organisms are similar with respect to the endoflagellar proteins, the data suggest that the leader peptide alone is not the cause of toxicity. Rather, the promoter, either alone or in association with the N-terminal peptide or products encoded by upstream DNA, may be responsible for putative toxicity. Similar difficulties were encountered in cloning the promoter-leader region of the *Haemophilus influenzae* type b major outer membrane protein in *E. coli* (38; E. Hansen, personal communication). In this regard, it has been known for a number of years that cloning strong promoters can be difficult (17). It is also possible that the unavailability of a nondegenerate probe to the extreme N terminus of *flaA* contributed to our inability to identify clones containing this region. In any case, these problems probably account for the difficulties encountered by several groups attempting to clone this antigen.

The studies reported here will provide the basis for further molecular investigations of the endoflagellar apparatus and treponemal motility, including studies of the organization of the gene family encoding the endoflagellar proteins. Analyses of the genetics of flagellar expression in other bacteria have proved useful in understanding the genetic organization and control of complex operons (25). Furthermore, analysis of the upstream DNA may provide additional insights into our emerging understanding of the processing of treponemal antigens expressed in *E. coli* (12, 21). In concert with attempts to achieve continuous in vitro cultivation of *T. pallidum*, the ultimate goal of these studies will be to use the derived genetic information along with mutational analyses to elucidate the relationships between motility and virulence, as has been accomplished with other bacterial pathogens (15).

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ADDENDUM IN PROOF

Nucleotide sequence analysis of the upstream portion of the *flaA* gene has been completed using PCR amplification of *T. pallidum* chromosomal DNA. The *flaA* gene contains consensus *E. coli* −35 and −10 promoter sequences, a ribosome-binding site, and a typical leader with a signal peptidase I cleavage site, consistent with transport of this protein to the periplasmic space.

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Expression in *Escherichia coli* of the 37-Kilodalton Endoflagellar Sheath Protein of *Treponema pallidum* by Use of the Polymerase Chain Reaction and a T7 Expression System

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We previously reported the complete primary structure of the 37-kilodalton endoflagellar sheath protein (FlaA) of *Treponema pallidum*. However, we were unable to determine the nucleotide sequence of *flaA* upstream of amino acid 10. The desired nucleotide sequence was obtained by use of a strategy based upon the polymerase chain reaction and was found to contain a consensus *Escherichia coli* promoter, a ribosomal binding site, and a 20-amino-acid signal peptide. Expression of FlaA in *E. coli* was achieved by cloning polymerase chain reaction-derived constructs lacking the native *T. pallidum* promoter into a temperature-inducible T7 expression system. Pulse-chase and ethanol inhibition analyses of protein processing in *E. coli* cells and minicells, respectively, indicated that processing of the FlaA precursor was incomplete. Native and recombinant FlaA were identical as assessed by antibody reactivity and sodium dodecyl sulfate- and two-dimensional polyacrylamide gel electrophoretic mobilities. Soluble FlaA was not detected in either the cytoplasmic or the periplasmic fractions of *E. coli* transformants. Fractionation of *E. coli* cell envelopes unexpectedly revealed that FlaA precursor and FlaA were associated with both the cytoplasmic and outer membranes. This is the first report of expression in *E. coli* of a *T. pallidum* protein which could not be cloned or expressed with its native promoter. Our data also indicate that information obtained in *E. coli* regarding the subcellular location of cloned treponemal proteins must be cautiously extrapolated to *T. pallidum*.

Venereal syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* (*T. pallidum*). A significant increase in reported cases of syphilis in the United States has occurred in the last two years (13), adding impetus to research aimed at understanding the pathogenesis of this disease. Motility, a characteristic feature of all spirochetes (31), is thought to contribute to the ability of *T. pallidum* to invade and disseminate within mammalian hosts (71). The organelles of motility, the endoflagella (11, 33, 38, 55), are polymeric structures within the periplasmic space of the organism (32) consisting of 33- and 33.5-kilodalton (kDa) core and 37-kDa sheath subunits (8, 15, 48, 56). The endoflagellar antigens stimulate B- and T-cell responses in humans (5, 26; unpublished data) and in experimental syphilis (4, 25, 39; unpublished data), suggesting that they also may play a role in the immunopathogenesis of the disease.

Treponemal research has been hampered by the inability to cultivate *T. pallidum* continuously in vitro. Many investigators have circumvented this problem, in part, by expressing treponemal antigens in *Escherichia coli* (for a review, see reference 68). In the course of these studies, data obtained in *E. coli* regarding the cellular location and membrane topography of cloned treponemal antigens often have been extrapolated to the native *T. pallidum* proteins (14, 18, 28, 64, 68). However, to what extent *E. coli* can be used as a model for molecular analysis of *T. pallidum* proteins remains unclear. It is now recognized that these two organisms differ greatly with respect to both the protein and lipid compositions of their outer membranes and the relative antigenicities of their respective surfaces (59, 75). The periplasmic endoflagellar proteins are excellent candidates for addressing these issues as they are the only antigens of *T. pallidum* that have been localized unequivocally within the organism.

We previously reported the primary structure of the 37-kDa sheath protein of *T. pallidum* (FlaA) along with a partial nucleotide sequence of *flaA* (33). However, we were unable to clone the portion of *flaA* upstream of amino acid 10, presumably because of toxicity in *E. coli*. To determine the complete nucleotide sequence of *flaA* and to express FlaA in *E. coli*, we employed an alternative cloning strategy based upon the polymerase chain reaction (PCR) (61). This is the first report of expression in *E. coli* of a *T. pallidum* protein which could not be cloned or expressed with its native promoter. Our data also indicate that information obtained in *E. coli* regarding the subceliular location of cloned treponemal proteins must be cautiously extrapolated to *T. pallidum*.

(A preliminary report of this work has been presented (R. D. Isaacs and J. D. Radolf, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, D-160, p. 107].)

MATERIALS AND METHODS

Bacterial strains. *T. pallidum* (Nichols) was passaged by intratesticular inoculation into New Zealand White rabbits and extracted as previously described (27, 42, 58). *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and HB101 (10) were used as host strains for plasmid transformations. *E. coli* DH5αF′ (Bethesda Research Laboratories) and XL1-Blue (Stratagene, La Jolla, Calif.) were used as host strains for M13 phage (76). *E. coli* ORN103 was used for minicell analyses (50).

Plasmids and DNAs. Plasmid DNAs and oligonucleotides used in this study are listed in Table 1 and Fig. 1, respectively. *T. pallidum* chromosomal DNA was extracted as previously described (33).

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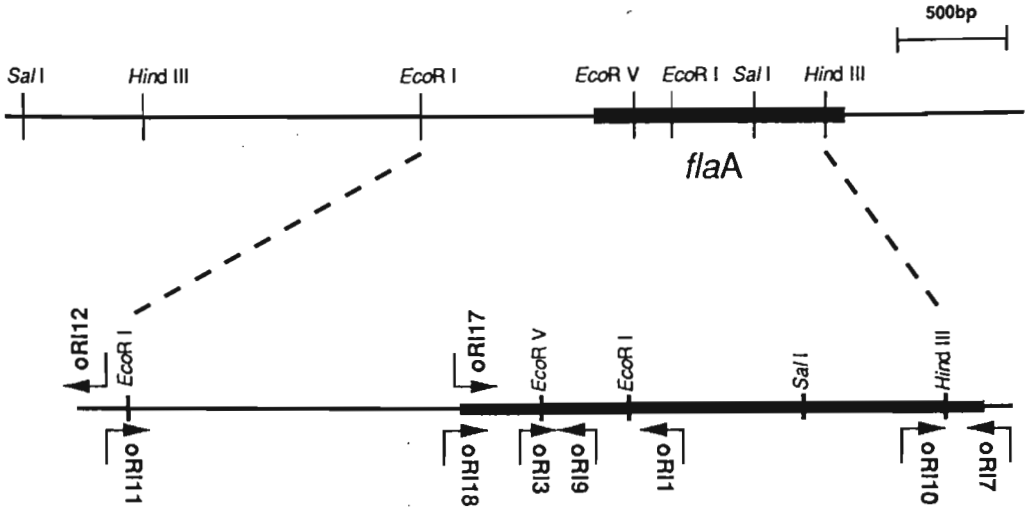

Designation	Sequence	Coding Strand	Location
oRI1	CCCATCTTCAAAACGCCCCT	-	590 - 609
oRI3	AAGCTGAACGCTGATATCAT	+	184 - 203
oRI7	CCCTGCAGAACGCCAAAACACCGC	-	1234 - 1257
oRI9	TCATACCTCCACTCTTA	-	210 - 226
oRI10	TAAGAGAGTTGAGGTTG	+	1023 - 1039
oRI11	TCATTATGACGCGGAAA	+	NK
oRI12	TTTCCGCGTCATAATGA	-	NK
oRI17	GAAAGGAGCGTTTGAATGAA	+	82 - 101
oRI18	TAGTCGACGAGTGGTTATCTTATT	+	54 - 77

FIG. 1. *T. pallidum* genetic map in the vicinity of *flaA*. The region of the chromosome between *flaA* (thick line) and the upstream *Eco*RI site has been expanded to show the locations of oligonucleotides used in these studies. The oligonucleotide sequences are 5′ to 3′, and this is indicated by the arrows. Oligonucleotides which are derived from the coding strand (+) and those that are complementary to the coding strand (−) are indicated. Nucleotide position 1 is the first nucleotide of the sequence shown in Fig. 3. The exact location of oRI11 and oRI12 is not known (NK), as their sequences were derived from a randomly selected fragment of the inverse-PCR product (see text).

SDS-PAGE and immunological reagents. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37, 58), two-dimensional PAGE (2D-PAGE) (33, 47, 49), and immunoblot analyses (33, 58) were performed as previously described. H9-2, a murine monoclonal antibody of subclass immunoglobulin G_1 specific for the 37-kDa endoflagellar sheath protein of *T. pallidum* (33, 40), was purified from hybridoma supernatants by using recombinant Protein G (Genex Corp., Gaithersburg, Md.) according to the instructions of the manufacturer. Horseradish peroxidase-conju-

TABLE 1. Plasmid DNAs used in this study

Designation	Description[a]	Source or reference
pBR322	Tc^r Ap^r, replicon pMB1	9
pUC19	Ap^r, replicon pMB1	76
pTTQ19	Ap^r, replicon pMB1	67
pBluescript II KS-	Ap^r, replicon pMB1, polylinker contains T7 promoter transcribing in opposite orientation to *lac* promoter	Stratagene, La Jolla, Calif.
pT7-3	Ap^r, replicon pMB1, *amp* is transcribed from T7 promoter	69
pGP1-2	Km^r, replicon p15A, encodes for T7 DNA-dependent RNA polymerase under control of λ promoter, temperature-sensitive λ repressor	69
pRI4	pBR322 with *T. pallidum* chromosomal DNA fragment encoding amino acids 10 through 330 of the mature *flaA* gene product	33
pRI4.3	pUC19 with 1.5-kb *Eco*RI-*Pst*I fragment from pRI4 insert	This study
pRI17	PCR product from *T. pallidum* chromosomal DNA with primers oRI17 and oRI7 digested with *Pst*I, cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI18	PCR product from *T. pallidum* chromosomal DNA with primers oRI18 and oRI7 digested with *Pst*I, cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI19	pBluescript II KS- digested with *Bam*HI and *Pst*I, containing 0.3-kb *Bam*HI-*Eco*RI fragment of pRI18 and the 1.5-kb *Eco*RI-*Pst*I fragment from pRI4.3	This study

[a] Abbreviations: Tc^r, Ap^r, and Km^r, resistance to tetracycline, ampicillin, and kanamycin, respectively.

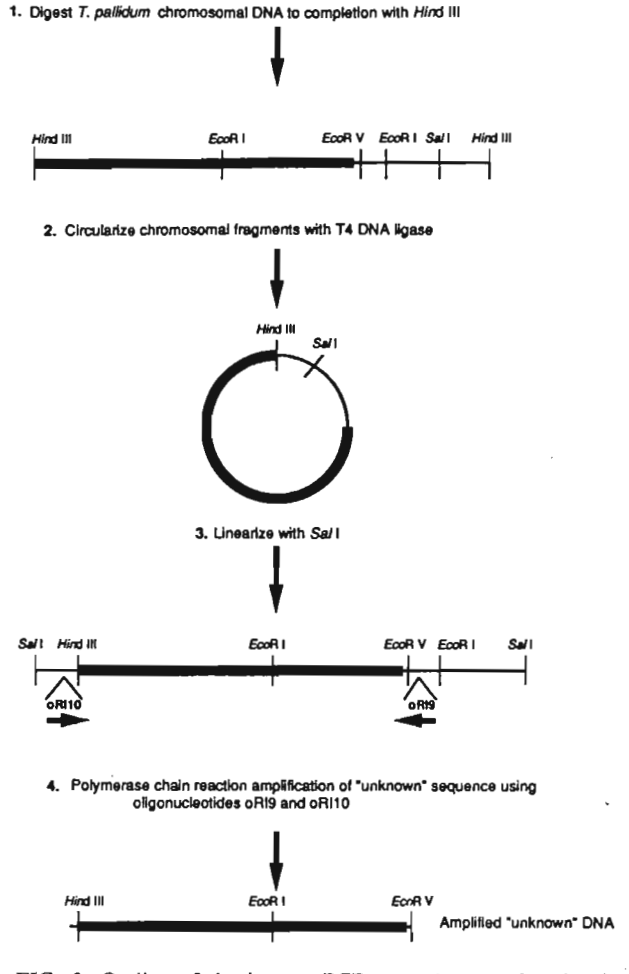

FIG. 2. Outline of the inverse-PCR procedure used to in vitro clone the upstream regions of *flaA*. The region of unknown nucleotide sequence is indicated by a thick line. The arrows under the PCR primers indicate the direction of DNA replication.

gated rabbit anti-mouse immunoglobulin G (Zymed Laboratories, San Francisco, Calif.) was used at a dilution of 1:1,000.

Isolation of *T. pallidum* endoflagella. *T. pallidum* endoflagella were isolated as previously described (33).

PCR DNA amplification. PCR was performed with 2.5 U of recombinant *Taq* polymerase (AmpliTaq; Perkin Elmer Cetus, Norwalk, Conn.) and a Perkin Elmer Cetus Thermocycler. Typically, PCRs were performed in 100-μl volumes each containing 1× PCR reaction buffer (10 mM Tris hydrochloride [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 1 μg of each primer, 200 μM of each deoxynucleoside triphosphate, and 1 μg of chromosomal DNA. Inverse-PCR (73) was performed for 25 cycles by using oligonucleotides oRI9 and oRI10 (Fig. 2); each cycle consisted of denaturation at 94°C for 2 min, annealing at 48°C for 2 min, and extension at 72°C for 3 min. Routine and asymmetric PCRs (24, 41) with oRI1 and either oRI11 or oRI12 were performed for 25 and 40 cycles, respectively, by using the above parameters; 1 pmol of oRI1 and 20 pmol of oRI11 were used for asymmetric PCR. PCRs with oRI7 and either oRI17 or oRI18 were performed for 30 cycles, each consisting of denaturation at 94°C for 2 min, annealing at 66°C for 2 min, and extension at 72°C for 2 min.

PCR products were analyzed by ethidium bromide agarose gel electrophoresis and purified in one of the following manners: (i) from low-melting-point agarose (Sea-Plaque; FMC Corporation, Marine Colloids Div. Rockland, Maine) gels by using an Elutip-d column (Schleicher & Schuell, Inc., Keene, N.H.) (63), (ii) by electroelution from SeaKem GTG (FMC Corp.) agarose gels, or (iii) by using Centricon-30 microconcentrators (W. R. Grace & Co., Danvers, Mass.) (41). Prior to ligation reactions, the PCR product was phosphorylated by using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass).

DNA-DNA hybridizations. Double-stranded DNA probes for Southern hybridization were labeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Lisle, Ill.) by random primer labeling (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (19). Dephosphorylated oligonucleotide probes were labeled with [γ-^{32}P]ATP (Du Pont Co. Biotechnology Systems, Wilmington, Del.) by using T4 polynucleotide kinase. Colony blot and Southern blot hybridizations with double-stranded DNA probes were performed as previously described (33, 66). When oligonucleotide probes were used, prehybridization and hybridization were performed at 42°C in 6× SSC (1× SSC is 0.15 M NaCl-0.015 M sodium citrate [pH 7.0]), 50 mM sodium phosphate buffer (pH 6.8), 10% (wt/vol) dextran sulfate, and 5× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, 0.02% [wt/vol] bovine serum albumin), containing 0.1 mg of denatured, sheared, salmon sperm DNA per ml. Three washes in 6× SSC at room temperature for 10 min each were performed prior to a stringency wash at 45°C in 6× SSC-0.1% SDS for 30 min.

DNA sequence analysis. DNA sequence analysis was performed with modified T7 DNA polymerase (Sequenase 2.0; U.S. Biochemicals Corp., Cleveland, Ohio) as described previously (33, 62, 70). The University of Wisconsin Genetics Computer Group sequence analysis software package (17) was used in conjunction with Microgenie software (Beckman Instruments, Inc., Palo Alto, Calif.) for DNA sequence analysis.

Expression of FlaA. Bacteria were grown at 30°C to an A_{590} of 1.5 in LB broth containing ampicillin (40 μg/ml) and kanamycin (40 μg/ml). The temperature was shifted to 42°C for 25 min, and then rifampin was added to a final concentration of 200 μg/ml. The culture was incubated for 2 h at 30°C, and the cells were recovered by centrifugation.

Pulse-chase experiments. Bacteria were grown at 30°C to an A_{590} of 0.5 in LB broth containing ampicillin and kanamycin. Cells were recovered by centrifugation, washed in ice-cold M9 medium, and then suspended in M9 medium supplemented with 20 μg of thiamine per ml and 0.01% (wt/vol) amino acids (without methionine and cysteine). The cultures were incubated at 30°C for 30 min and then at 42°C for 15 min. Rifampin was added to a final concentration of 200 μg/ml, and the culture was incubated for an additional 10 min at 42°C. After the culture was incubated for 30 min at 30°C, Trans ^{35}S label (an approximate 80:20 mixture of [^{35}S] methionine and [^{35}S]cysteine; ICN Biomedicals) was added; this was incubated at 30°C for 1 min. The culture was then chased with 0.1% methionine in M9 medium. Samples were removed at various times and immediately mixed with one-half the volume of ice-cold stop solution (0.04% [wt/vol] chloramphenicol, 0.4 M sodium azide, 0.02 M 2,4-dinitrophenol) (23), and the cells were recovered by centrifugation.

Minicell analyses. Bacteria were grown at 30°C for 18 h in LB broth containing ampicillin and kanamycin. Minicells were isolated as previously described (57). Minicells were suspended in M9 medium without glucose (approximately

10^8 minicells per ml) containing D-cycloserine (20 μg/ml) and incubated at 30°C for 15 min followed by incubation at 42°C for 5 min. Rifampin was added to a final concentration of 100 μg/ml, and the minicells were incubated at 42°C for an additional 5 min and then at 30°C for 15 min. Ice-cold 95% ethanol was added to each sample so that the final ethanol concentration was either 0, 1, 3, or 5% (vol/vol). After the addition of Trans ^{35}S label, each sample was incubated at 30°C for 30 min and the cells were then recovered by centrifugation.

Selective release of *E. coli* soluble periplasmic proteins. Bacteria were recovered by centrifugation at 4°C, washed in ice-cold 0.2 M Tris hydrochloride (pH 8), and then suspended in ice-cold 0.2 M Tris hydrochloride (pH 8)-1 M sucrose; EDTA and lysozyme were added sequentially to final concentrations of 0.5 mM and 60 μg/ml, respectively. An equal volume of ice-cold 0.2 M Tris hydrochloride (pH 8) was added, and the mixture was incubated on ice for 30 min. The cell pellet and the supernatant containing the soluble periplasmic fraction were separated by centrifugation at 4°C. The presence of β-lactamase in each fraction was assessed visually by using the colorimetric substrate [1-(thienyl-2-acetamido)]-3-[2-(4-*N*, *N*-dimethylaminophenylazo)pyridium methyl]-3-cepem-4-carboxylic acid (35).

Membrane fractionation experiments. Bacteria suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0) were disrupted by French press, and intact cell bodies were removed by centrifugation at 16,000 × *g* for 10 min at 4°C. Inner and outer membranes of *E. coli* were either collected by centrifugation at 240,000 × *g* for 60 min at 4°C or fractionated by using a modification of the procedure of Osborn and coworkers (51) as described by Munford et al. (44). The sucrose gradients were harvested from the top in 250-μl portions.

RESULTS

Nucleotide sequence analysis of the upstream region of *flaA*. pRI4, a chimeric plasmid isolated from a *T. pallidum* chromosomal DNA library (46), provided the starting point for the present investigations; the insert of this plasmid contains the DNA encoding for amino acids 10 through 330 of native FlaA (33). Previously, the nucleotide sequence of the upstream portion of *flaA* could not be determined because exhaustive attempts to clone this region were unsuccessful. As an alternative, we adopted a strategy based upon a variant of PCR called inverse-PCR (73). This technique enables selective amplification of a region of unknown nucleotide sequence adjacent to a region of known sequence.

It was necessary to map the *T. pallidum* chromosome in the vicinity of *flaA* prior to performing inverse-PCR. Oligonucleotide oRI3, derived from the 5′ end of the coding strand of the pRI4 insert, hybridized in Southern analyses with fragments of 2.5, 2.7, and 0.9 kilobase pairs (kb) from *T. pallidum* chromosomal DNA digested to completion with *Hin*dIII, *Sal*I, or *Eco*RI, respectively (data not shown). By combining these data with the restriction map of pRI4 (33), the required genetic map was derived (Fig. 1). The previously unclonable region of *flaA* resides on a 2.5-kb *Hin*dIII genomic DNA fragment. This fragment contains *Sal*I and *Eco*RI sites in the region of known nucleotide sequence and a second *Eco*RI site in the unsequenced region (Fig. 1).

Inverse-PCR was performed as shown in Fig. 2. *T. pallidum* chromosomal DNA was digested to completion with *Hin*dIII, circularized in the presence of T4 DNA ligase, and then linearized with *Sal*I. Oligonucleotides oRI9 and oRI10, which flank the unsequenced area in the linearized chromosomal DNA, were used as the PCR primers. The resulting 1.8-kb product had an internal *Eco*RI site and hybridized with oRI3 (data not shown), confirming that the correct region had been amplified. For reasons as yet unclear, these PCR experiments worked unpredictably and yielded small amounts of product; attempts to reamplify the purified PCR product by using both routine and asymmetric PCR also were unsuccessful. Furthermore, the inverse-PCR product could not be cloned into *E. coli* by using either pUC or pTTQ vectors; pTTQ vectors contain a transcriptional stop downstream of the cloned fragment and are particularly useful for cloning strong promoters (67).

An alternative strategy was then devised for obtaining a limited amount of nucleotide sequence within the unsequenced region of the inverse-PCR product. Routine PCR could then be performed from *T. pallidum* chromosomal DNA, by using a primer derived from this new sequence, with the expectation that this new product could be more easily manipulated than the original inverse-PCR product. Southern hybridization of the inverse-PCR product digested with *Sau*3A I and probed with itself indicated that multiple sites were present (data not shown). The inverse-PCR product, digested with *Sau*3A I, was ligated into M13mp19 digested with *Bam*HI. A clone identified by DNA-DNA hybridization with the entire inverse-PCR product was randomly selected for nucleotide sequencing; the sequence obtained was used to create oRI11. A 1.2-kb product which hybridized with oRI3 was detected when oRI11 was used for PCR with oRI1 (data not shown), a primer derived from the *flaA* sequence downstream of both oRI3 and oRI9. No PCR product was detected when oRI1 was used with oRI12, a primer complementary to oRI11. Since approximately 0.3 kb would encode the N terminus (including a leader peptide) and the promoter, this product was of sufficient size to contain all of the desired upstream portion of *flaA*.

Oligonucleotides oRI1 and oRI11, with oRI11 in excess, were used in asymmetric PCR of chromosomal DNA to generate single-stranded DNA. Nucleotide sequence obtained from the product by using oRI9 as the primer was combined with that previously reported (33) to provide the sequence of the entire *flaA* gene. Three other PCR-derived products containing the 5′ end of the open reading frame of *flaA* had nucleotide sequences identical to those obtained from the asymmetric PCR product (data not shown).

The *flaA* gene contains a consensus *E. coli* promoter (30) and a ribosomal binding site (RBS) (65) (Fig. 3). A 20-amino-acid signal sequence, including a typical signal peptidase I cleavage site (6) (Fig. 3, arrow), immediately precedes the N terminus previously determined by N-terminal sequence analysis of the native antigen (8, 33, 48). The precursor protein (pre-FlaA) has a molecular weight of 38,860. The deduced amino acid sequence of the first 21 amino acids of the mature protein matches precisely that of the native FlaA (8, 33, 48).

Expression of FlaA in *E. coli*. It was assumed that the native treponemal promoter would have to be substituted with a controllable *E. coli* promoter to express FlaA in *E. coli*. PCR was the most convenient method for obtaining a clonable fragment containing *flaA* exclusive of its promoter sequences. Three oligonucleotides, oRI7, oRI17, and oRI18, were synthesized for these experiments. oRI7 is complementary to a region downstream of the *flaA* termination codon; it also contains a *Pst*I site near its 5′ end. oRI17 and oRI18 were derived from the sense strand of *flaA* beginning 3 and

```
        10         20         30         40         50         60
TTTGAAAGTGATGGGTAGATGCAGGTCAGTTGACAAAATAGGCGGTGAGCGTATACTCGA
                          -35                    -10

        70         80         90        100        110        120
AGAGTGGTTATCTTATTGTGCGAAAGGAGCGTTTGAATGAAGAAAGCGGTTGTGTTGAGC
                    RBS          M  K  K  A  V  V  L  S

       130        140        150        160        170        180
GCGGTGGCGCTGCTCTCGGGGGTGTGCGCGGTTGCTGATGAGTCAGTGCTCATCGACTTC
 A  V  A  L  L  S  G  V  C  A  V  A  D  E  S  V  L  I  D  F

GCA
 A
```

FIG. 3. Nucleotide and deduced amino acid sequences of the promoter and N-terminal coding region of *flaA*. Putative −35 and −10 *E. coli* promoter and RBS sequences are indicated by double underlining. The start codon and the codon of the first amino acid of the mature protein are indicated by single underlining. The signal peptidase I cleavage site is indicated by the arrow. The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the accession no. M27915.

30 base pairs upstream of the RBS, respectively. oRI18 also contains two mismatches near its 5′ end which overlaps the −10 promoter sequence; these were included to ensure inactivation of the native promoter. PCR experiments with *T. pallidum* chromosomal DNA with oRI7 and either oRI17 or oRI18 produced the predicted 1.2-kb products (data not shown). Phosphorylated, purified PCR product digested with *Pst*I was ligated into pBluescript II KS– digested with *Sma*I and *Pst*I. Plasmids pRI17 and pRI18, which contain *flaA* in the correct orientation for expression from the T7 promoter, were identified by restriction endonuclease mapping and Southern hybridization with oRI3 (data not shown).

Each of these plasmids was used to transform HB101 (pGP1-2) so that expression would be temperature inducible (69). After induction, immunoblot analysis with monoclonal antibody H9-2, a murine monoclonal antibody specific for the sheath protein (33, 40), identified a doublet composed of

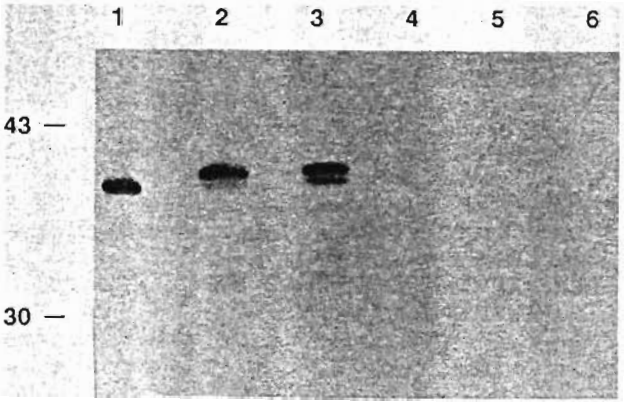

FIG. 4. Antigenic analysis of recombinant clones expressing FlaA. Western blot analysis was performed after sequential incubation in monoclonal antibody H9-2 and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G. Recombinant clones were either expressed as described in Materials and Methods (lanes 2, 3, and 4) or grown at the nonpermissive temperature (lanes 5 and 6). Lanes: 1, *T. pallidum*; 2 and 5, HB101(pRI18)(pGP1-2); 3, HB101(pRI19)(pGP1-2); 4 and 6, HB101(pBluescript II KS−)(pGP1-2). Samples were electrophoresed on an SDS-PAGE (10% polyacrylamide) gel. Molecular weight markers in kilodaltons are indicated on the left.

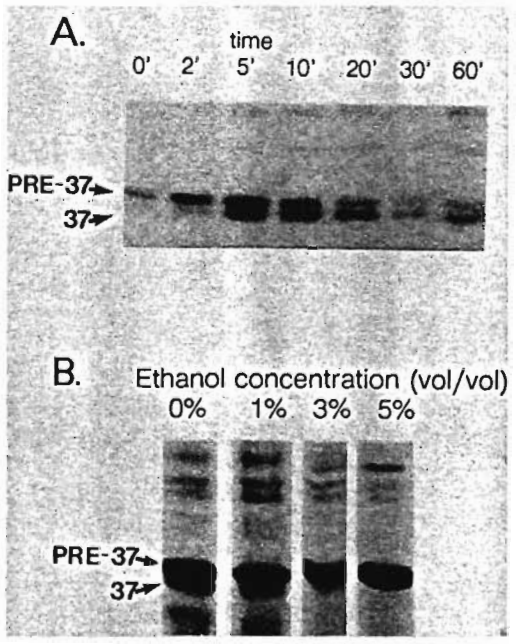

FIG. 5. Processing of FlaA in *E. coli*. The 37-kDa sheath protein (protein 37) and its precursor (PRE-37) are indicated on the left. (A) HB101(pRI18)(pGP1-2) were pulsed with Trans ^{35}S label for 1 min and then chased with 0.1% (wt/vol) methionine. Samples were removed at the indicated times, electrophoresed on an SDS-PAGE (10% polyacrylamide) gel, and subjected to autoradiography. (B) ORN103(pRI18)(pGP1-2) minicells were labeled with Trans ^{35}S label in the presence of different concentrations of ethanol, electrophoresed on an SDS-PAGE (10% polyacrylamide) gel, and subjected to autoradiography. Ethanol concentrations are shown above each lane.

39- and 37-kDa proteins in *E. coli* HB101(pRI18)(pGP1-2) (Fig. 4, lane 2) but not in HB101(pRI17)(pGP1-2) (data not shown). No H9-2 immunoreactive material was detected when the cultures were grown only at the nonpermissive temperature (Fig. 4, lanes 5 and 6). Corresponding bands were not readily seen on Coomassie blue-stained gels (data not shown).

FlaA is processed inefficiently in *E. coli*. In pulse-chase experiments with HB101(pRI18)(pGP1-2), FlaA was synthesized as a 39-kDa precursor which was subsequently processed to the 37-kDa mature protein (Fig. 5A); even after a 60-min chase, a significant amount of precursor was still present (Fig. 5A). In a parallel experiment with HB101(pT7-3)(pGP1-2) incubated under identical conditions, virtually no β-lactamase precursor was detected at 60 min (data not shown). Ethanol, a nonspecific inhibitor of membrane translocation by secretory proteins (54), was used in experiments with ORN103(pRI18)(pGP1-2) minicells. Although substantial accumulation of precursor occurred even in the absence of ethanol, inhibition of processing was ethanol concentration dependent (Fig. 5B).

Comparative 2D-PAGE analysis of recombinant and native FlaA. Because *Taq* polymerase lacks proofreading function, misincorporation of nucleotides may occur during PCR (20, 22, 60). For this reason, 2D-PAGE immunoblotting was used to confirm that the native and recombinant FlaA proteins were identical and that the 39-kDa H9-2-reactive recombinant protein was the pre-FlaA. First, ^{35}S-labeled HB101 (pRI18)(pGP1-2) was coelectrophoresed with isolated *T. pallidum* endoflagella. Immunoblot analysis with monoclo-

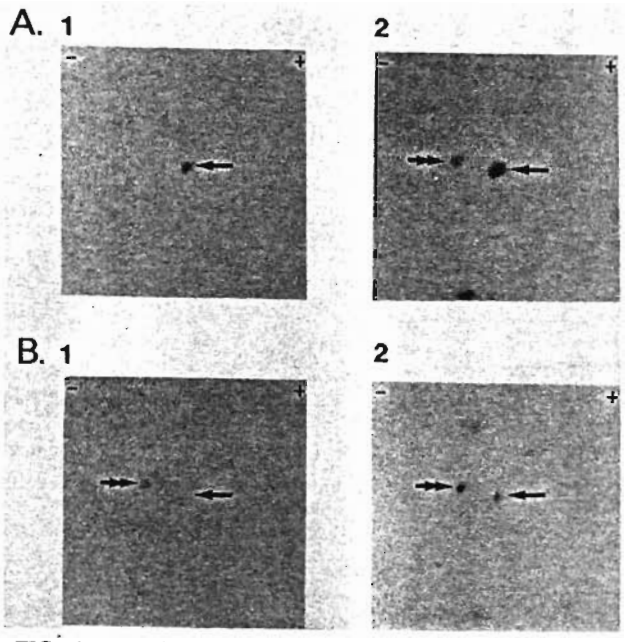

FIG. 6. Two-dimensional electrophoretic analyses of recombinant FlaA. Samples were separated by isoelectric focusing in the first dimension and by SDS-PAGE on a 10% gel in the second dimension. After transfer to nitrocellulose, the blots were incubated sequentially with H9-2 and peroxidase-conjugated rabbit anti-mouse immunoglobulin (panel 1) and then were subjected to autoradiography (panel 2). FlaA (single arrow) and pre-FlaA (double arrow) are indicated. The basic and acidic ends of the isoelectric focusing gel are indicated by − and +, respectively. (A) ^{35}S-labeled HB101 (pRI18)(pGP1-2) coelectrophoresed with isolated *T. pallidum* endoflagella. (B) ^{35}S-labeled HB101(pRI18)(pGP1-2) coelectrophoresed with hyperexpressed HB101(pRI18)(pGP1-2).

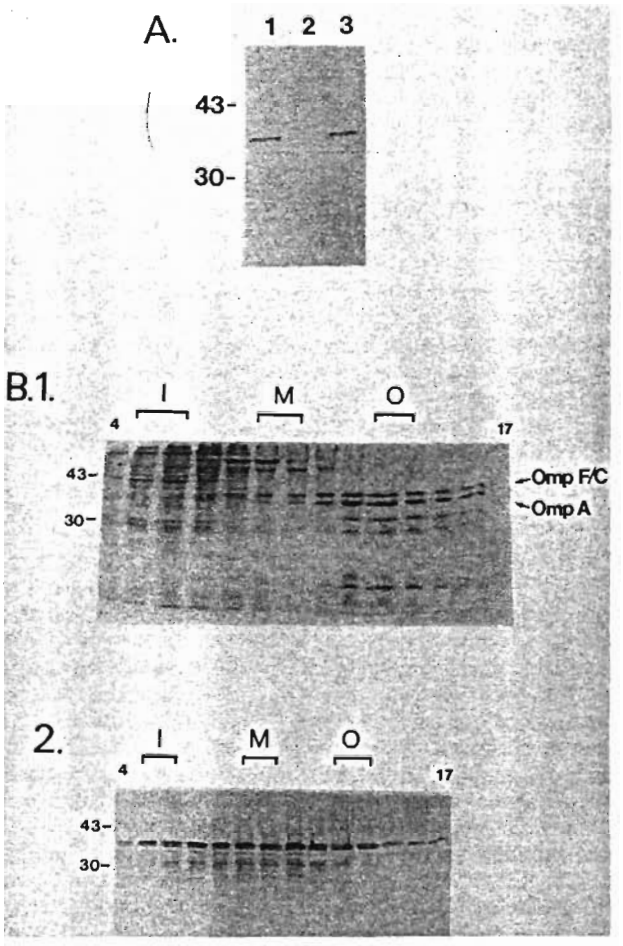

FIG. 7. Fractionation of *E. coli* clones expressing FlaA. (A) Immunoblot analysis with monoclonal antibody H9-2 of soluble periplasmic proteins released from HB101(pRI18)(pGP1-2) spheroplasts. Lanes: 1, starting material; 2, supernatant following osmotic shock of spheroplasts; 3, insoluble pellet. Samples were analyzed on an SDS-PAGE (12.5% polyacrylamide) gel. Molecular weight markers in kilodaltons are indicated on the left. (B) Coomassie blue-stained SDS-PAGE (12.5% polyacrylamide) gel (panel 1) and corresponding immunoblot probed with H9-2 (panel 2) of cell envelopes of HB101(pRI18)(pGP1-2) separated by sucrose density gradient centrifugation into inner (I) and outer (O) membranes and an intermediate membrane fraction (M). Fractions 4 to 17 are shown. Molecular weight markers in kilodaltons are indicated on the left. The *E. coli* outer membrane proteins, OmpA and OmpF/C, are indicated.

nal antibody H9-2 demonstrated that the native 37-kDa antigen (Fig. 6A, panel 1) comigrated with the 37-kDa radiolabeled protein (Fig. 6A, panel 2). In a second experiment, radiolabeled HB101(pRI18)(pGP1-2) from processing experiments was coelectrophoresed with unlabeled HB101 (pRI18)(pGP1-2) whole-cell lysates (Fig. 6B). As predicted by the presence of two lysines in the signal sequence, the pre-FlaA was significantly more basic than the mature protein during isoelectric focusing (Fig. 6A, panel 2, double arrow). The radiolabeled pre-FlaA (Fig. 6B, panel 2) comigrated with the H9-2-reactive 39-kDa antigen identified by immunoblotting (Fig. 6B, panel 1).

Construction of pRI19. Despite the above results from 2D-PAGE, the possibility still existed that the recombinant antigen contained an amino acid substitution which did not affect its pI. Such a substitution(s) could adversely impact future structure-function analyses of FlaA. As an alternative to sequencing the entire insert of pRI18 for comparison with the known sequence, an additional chimeric plasmid was subsequently constructed from fragments of known nucleotide sequence. Nucleotide sequence analysis of pRI18 showed that the 5′ sequence matched the expected sequence up to the *Eco*RI site (data not shown). pRI19 was constructed in pBluescript II KS− from the 0.3-kb *Bam*HI-*Eco*RI fragment of pRI18 (which encodes the RBS, the leader and amino acids 1 through 62 of the mature protein), and the 1.5-kb *Eco*RI-*Pst*I fragment of pRI4.3 (which encodes the remainder of the mature protein). Immunoblots of SDS-polyacrylamide (Fig. 4, lane 3) and 2D-polyacrylamide (data not shown) gels showed that HB101(pRI19)(pGP1-2) produced a H9-2-reactive 39- and 37-kDa doublet identical to that of HB101(pRI18)(pGP1-2). Processing of pre-FlaA in HB101(pRI19)(pGP1-2) was also incomplete (data not shown).

Localization of recombinant FlaA in *E. coli*. Spheroplasts derived from HB101(pRI18)(pGP1-2) were osmotically shocked to release selectively soluble periplasmic proteins. All of the FlaA remained associated with the cell pellet (Fig. 7A), whereas β-lactamase was easily detectable in the supernatant (data not shown). In a separate experiment, HB101(pRI18)(pGP1-2) whole cells were disrupted in a French press and membranes were collected by differential ultracentrifugation. The supernatant, comprising the periplasmic and cytoplasmic fractions, did not contain H9-2-reactive material (data not shown). SDS-PAGE of the

membranes fractionated on a sucrose gradient revealed protein profiles characteristic of *E. coli* inner and outer membranes (Fig. 7B, panel 1). The buoyant densities of the outer and inner membrane fractions were 1.230 g/ml and 1.167 g/ml, respectively. Immunoblot analysis of the gradient fractions (Fig. 7B, panel 2) revealed the surprising result that both the precursor and the mature protein were present in all fractions. Fractionation studies with HB101(pRI19)(pGP1-2) showed identical results (data not shown).

Diminished growth of *E. coli* clones expressing FlaA. Growth curves of HB101(pRI18)(pGP1-2) and HB101(pRI19)(pGP1-2) were compared to that of HB101(pBluescript II KS−)(pGP1-2). At the permissive temperature, a small difference in the growth rates for both HB101(pRI18)(pGP1-2) and HB101(pRI19)(pGP1-2) was consistently observed (data not shown). Viable colonies were produced from samples taken at all time points (data not shown).

DISCUSSION

For nearly a decade, recombinant DNA methodologies have been used to clone *T. pallidum* proteins in *E. coli* (68). Virtually all of these proteins were identified in genomic libraries by using monoclonal or polyclonal antibodies (68). It is clear, however, that only a limited number of recombinant *T. pallidum* proteins can be identified by using techniques dependent upon expression from native treponemal promoters. The difficulties experienced by a number of investigators in cloning the endoflagellar proteins (3, 33, 53, 74) have emphasized the need to employ alternative strategies. Pallesen and Hindersson (53) used degenerate oligonucleotide probes derived from the N-terminal amino acid sequence of the endoflagellar core proteins to identify a gene, designated *flaB2*. DNA sequence analysis revealed that *flaB2* lacked a consensus promoter, a finding which most likely explained the lack of its expression in *E. coli*.

Cloning of the 37-kDa endoflagellar sheath protein (33) was particularly problematic. Exhaustive attempts to clone the entire gene or any portion that contained the putative promoter and the N terminus were unsuccessful. A chimeric plasmid, pRI4, encoding all but the N-terminal nine amino acids of FlaA, was identified by screening a library with a nondegenerate DNA probe derived from a λgt11 clone expressing an epitope of the protein (33). To complete the nucleotide sequence of *flaA*, a strategy based on inverse-PCR was employed. Initially, we planned to use the inverse-PCR product as a template for nucleotide sequence analysis. However, a product was detected in only a few experiments and could not be used as a template for subsequent PCR reactions. Complementarity between oRI9 and oRI10 (Fig. 1), with resultant primer dimer formation during PCR (60) and/or variability in circularization of the chromosomal DNA may have contributed to this problem. Our inability to clone the relevant portion of the inverse-PCR product was not surprising in view of previous failures to clone the upstream regions of *flaA* (33). By using a sequence derived from a *Sau*3A I fragment cloned from the inverse-PCR product, the complete nucleotide sequence of *flaA* was finally obtained by asymmetric PCR.

The inability to clone putative bacterial virulence factors in *E. coli* is not unique to FlaA. Examples include the porin proteins of *Haemophilus influenzae* (29, 45) and *Neisseria gonorrhoeae* (12, 21), both of which are presumed to be toxic when expressed in *E. coli* from their native promoters. Nucleotide sequence analysis of such putatively toxic proteins often has been accomplished by generating overlapping clones, one of which encoded the putative promoter and N terminus (12, 21, 45). It is noteworthy that the nucleotide sequence of *flaA* could not be completed in this manner (33). The strategy we employed will be useful for obtaining sequences from other unclonable portions of genes.

Expression of FlaA in *E. coli* was achieved by using PCR-derived constructs in a temperature-inducible T7 expression system (69). The insert of the first construct, pRI17, encoded for only three base pairs upstream of the RBS and did not express FlaA. Analysis of the mRNA transcript suggested that a stem-loop could form between the *Sma*I site of the vector polylinker and the RBS of *flaA*, leading to inactivation of the RBS. Expression was achieved with an alternative construct, pRI18, whose insert encoded for 30 base pairs upstream of the RBS.

Nucleotide sequence analysis indicated that mature FlaA arises from cleavage of a signal peptide. Immunoblot analysis with monoclonal antibody H9-2 revealed a 37- and 39-kDa doublet in the *E. coli* transformants. The 37-kDa protein comigrated with the native FlaA by both SDS-PAGE and 2D-PAGE analyses; similar analyses with ^{35}S-labeled pre-FlaA confirmed that the 39-kDa antigen was the accumulated precursor. Although the reason(s) for inefficient processing is unclear, several possibilities were excluded. First, it is unlikely that the temperature shock required for FlaA expression contributed to the abnormal processing. In the pulse-chase experiments, bacteria were induced at 42°C for 25 min, equilibrated at 30°C prior to the addition of the radiolabel, and then chased at the same temperature. Further, β-lactamase in controls was efficiently processed under the same conditions. Second, although the amino acid sequence of the signal peptide was derived entirely from PCR products, PCR conditions associated with the lowest misincorporation rates were chosen to minimize the possibility of errors introduced by the infidelity of *Taq* polymerase (20, 22, 60). In addition, the nucleotide sequences of the signal peptides of several PCR-derived constructs were identical. Third, pre-FlaA also accumulated when expressed from pRI19, indicating that unidentified mutations downstream of the cleavage site in pRI18 were not responsible for the inefficient processing. Finally, the observations that the cleavage site so closely matches the consensus site for *E. coli* signal peptidase I (6) and that the native and recombinant FlaA had identical electrophoretic mobilities strongly argue against the possibility that a difference in cleavage site specificity exists between the *E. coli* and *T. pallidum* signal peptidases.

Overloading of the *E. coli* export machinery is the most likely explanation for inefficient processing of FlaA. This phenomenon is well recognized in *E. coli* clones hyperexpressing native *E. coli* secretory proteins (1, 2, 34, 43, 52). Analyses of other cloned treponemal secretory proteins further support this contention. The treponemal basic membrane protein (16), TmpB (28), and the 34-kDa protein (68) are all processed slowly in *E. coli*, and precursors for both TmpA and TmpB accumulate when they are hyperexpressed in *E. coli* (28, 64). In combination with the FlaA processing data, these findings suggest that *T. pallidum* secretory proteins are, in general, inefficiently processed in *E. coli*. One might speculate that toxicity resulting from progressive accumulation of pre-FlaA during unregulated expression explains our inability to clone *flaA* with its native promoter. The diminished growth of *E. coli* clones expressing FlaA at the relatively low levels achieved in our study supports this contention, although there are alternative explanations for

this finding (e.g., the increased metabolic burden of transcription).

The processing experiments indicated that recombinant FlaA should be detectable within the periplasmic space. However, FlaA was not detected in the supernatants from either osmotically shocked spheroplasts or disrupted whole cells. Instead, both precursor and mature protein were found in association with the inner and outer membrane fractions. FlaA is likely to be present as a polymer in *T. pallidum* but not so in *E. coli*. Therefore, recombinant FlaA may be relatively insoluble and have aggregated onto both the inner and outer membranes following either translocation and processing or cell disruption; proteins are not released during osmotic shock and/or spheroplasting unless they are soluble within the periplasmic space (7). Unexported pre-FlaA that accumulated within the cytoplasm most likely aggregated onto the membranes after cell disruption (72).

A number of cloned *T. pallidum* proteins have been localized putatively to the outer membrane in *E. coli* by use of methodologies similar to those employed in this study (14, 18, 28, 64, 68). These data have often been used to support an outer membrane location for the native antigens in *T. pallidum*. There are now significant reasons to question the validity of this approach. Given the ultrastructural differences between the outer membranes of *T. pallidum* and *E. coli* (59, 75), it is unclear whether location in the outer membrane of *E. coli* is relevant to the native antigen in *T. pallidum*. Furthermore, fractionation results in *E. coli* may not even be representative of the true subcellular location of the cloned antigen (72). Aberrant outer membrane localization is well recognized in *E. coli* clones expressing genetically manipulated native or hybrid secretory proteins analyzed by using the same techniques (for a review, see reference 72). The FlaA fractionation data emphasize the need for caution in extrapolating *E. coli* fractionation data to *T. pallidum*.

In *E. coli*, flagellin monomers are exported across the cell membrane by a poorly characterized, flagellum-specific mechanism which does not involve cleavage of an N-terminal signal (36). It has been proposed that monomers are transported via a channel within the growing flagellin to its distal end. The nucleotide sequences of FlaA and FlaB2 now make it possible to apply this model to the assembly of *T. pallidum* endoflagella. FlaB2 does not have a signal sequence and shares significant sequence homology with other flagellins (48, 53); presumably the endoflagellar core assembles in a manner similar to that of *E. coli* flagella. Secretion by a signal peptide-dependent pathway is consistent with the hypothesis that FlaA forms the sheath by polymerizing around the growing core. The studies reported here will provide a basis for future investigations into the genetics of endoflagella assembly and the role of the endoflagella in the pathogenesis of syphilis.

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