Treponema pallidum Major Sheath Protein Homologue Tpr K Is a Target of Opsonic Antibody and the Protective Immune Response

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Summary

We have identified a family of genes that code for targets for opsonic antibody and protective immunity in *T. pallidum* subspecies *pallidum* using two different approaches, subtraction hybridization and differential immunologic screening of a *T. pallidum* genomic library. Both approaches led to the identification of a polymorphic multicopy gene family with predicted amino acid homology to the major sheath protein of *Treponema denticola*. One of the members of this gene family, *tpr K*, codes for a protein that is predicted to have a cleavable signal peptide and be located in the outer membrane of the bacterium. Reverse transcription polymerase chain reaction analysis of *T. pallidum* reveals that Tpr K is preferentially transcribed in the Nichols strain of *T. pallidum*. Antibodies directed to purified recombinant variable domain of Tpr K can opsonize *T. pallidum*, Nichols strain, for phagocytosis, supporting the hypothesis that this portion of the protein is exposed at the surface of the treponeme. Immunization of rabbits with the purified recombinant variable domain of Tpr K provides significant protection against infection with the Nichols strain of *T. pallidum*. This gene family is hypothesized to be central to pathogenesis and immunity during syphilis infection.

Key words: syphilis • vaccine • subpopulation • surface antigens • antigenic variation

Outer surface molecules of bacterial organisms play a central role in pathogenesis and immunity because they are involved in adherence to host cells and serve as targets of bactericidal or opsonic antibody. Although *Treponema pallidum* adheres to host cells and is susceptible to bactericidal and opsonic antibody, the bacterial molecules involved in those functions have not been defined. Unlike the outer membranes of gram-negative bacteria, the outer membrane of *T. pallidum* is very fragile and easily damaged by physical manipulation (1), which has resulted in the misidentification of several highly immunogenic periplasmic lipoproteins as outer membrane antigens (2). Studies conducted independently in two laboratories (3, 4) have shown by freeze fracture electron microscopy that the surface of *T. pallidum* is relatively devoid of outer membrane proteins compared with other bacteria, and it is hypothesized that this paucity of surface-exposed antigens is responsible for the chronicity of syphilis infection (3). Attempts to identify the rare outer membrane proteins of *T. pallidum* have yielded controversial results (5). Two surface-exposed molecules have been proposed to date, Tromp 1 (6) and Tromp 2 (7). Tromp 1 is reported to have porin activity (6) and appears to be part of an ABC transport operon (8), but its location in the outer membrane is not universally accepted (8, 9), and antiserum directed against recombinant Tromp 1 is not opsonic (9). Independent confirmation of the surface location of Tromp 2 has not been reported. Other searches for outer membrane proteins have been inconclusive (10–14).

Surface-exposed antigens in *T. pallidum* are likely to be important virulence factors, as well as being the molecules that interact with the protective immune response. Several studies have shown that *T. pallidum* infection induces antibodies that inhibit cell attachment (15, 16) and promote macrophage-mediated phagocytosis (17, 18) and complement-mediated neutralization (19–21). Macrophage-mediated phagocytosis of opsonized *T. pallidum* is the major mechanism for clearance of treponemes from primary and secondary syphilis lesions (22–24), and opsonic antibody is required for killing of the treponemes by the macrophages (18).

In this report we describe the identification of a multicopy polymorphic gene family of *T. pallidum*, termed *T.*

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pallidum repeat (*tpr*),[1] that is related to the major surface protein (msp) genes of *Treponema denticola*. The *T. denticola* msps are surface exposed, mediate binding to host cells and extracellular matrix, and function as porins. We show that one member of the paralogous *T. pallidum* gene family, *tpr K*, is preferentially transcribed in the Nichols strain of *T. pallidum*, serves as a target of opsonic antibody, and induces a protective immune response. These results strongly support the exposure of this protein on the surface of the Nichols strain of *T. pallidum*.

Materials and Methods

Treponemal Strains and DNA Extraction. *T. pallidum* subspecies *pallidum*, Nichols strain, originally sent to the University of Washington by Dr. James N. Miller (University of California, Los Angeles, CA) in 1979, was propagated in New Zealand white rabbits as previously described (25). *Treponema paraluiscuniculi* Cuniculi A strain was isolated from an infected rabbit, provided by Dr. Paul Hardy (Johns Hopkins University, Baltimore, MD), and propagated as above. Treponemes were extracted from infected rabbit testes and DNA isolated as previously described (26).

Subtraction Libraries. Subtraction hybridization using PCR technology (representational difference analysis) was performed to enrich for DNA sequences likely to be found in *T. pallidum* subspecies *pallidum*, Nichols strain, but not in *Treponema paraluiscuniculi*. The protocol was followed as described in the manufacturer's instructions (PCR-Select Subtraction Kit; Clontech) except that genomic DNA was substituted for cDNA. All DNA samples were treated with RNAse A before the subtraction experiments. *T. pallidum* subspecies *pallidum* DNA was the "tester" DNA; that is, the DNA containing specific sequences that are left behind after subtraction. A mixture of *T. paraluiscuniculi* DNA plus rabbit genomic DNA was used as the "driver" DNA; that is, the excess DNA used to subtract away the common sequences. Rabbit genomic DNA was included in the driver because treponemes are propagated in rabbits and therefore rabbit DNA contaminates all treponemal samples. After performing the procedure, the resultant PCR products were cloned into the PCR 3.1 T/A cloning vector (Invitrogen).

DNA Sequencing of Clones. Double-stranded plasmid DNA was extracted from clones containing inserts using the Qiagen Plasmid Kit (Qiagen). Full automated sequencing of the inserts was performed by the dye terminator method (Perkin Elmer) according to the manufacturer's instructions, except molecular grade dimethylsulfoxide (Sigma Chemical Co.) was added to give a final concentration of 5% vol/vol. The 5′ and 3′ ends of the plasmid inserts were sequenced with the T7 primer (5′ taatacgactcactataggg) and the PCR3.1 reverse primer (5′ tagaaggcacagtcgag). When necessary, sequencing was completed in both directions by making primers complementary to internal sequence as it became available. Two clones, designated 3 and 33, were found to have predicted amino acid homology with the msp of *T. denticola* (27) and were further evaluated as described below. Comparison with the subsequently released *T. pallidum* genome (28) indicated that they represent *tpr F* and *tpr G*, respectively.

Hybridization Analysis. 3 μg of genomic DNA from *T. pallidum* subspecies *pallidum*, Nichols strain, and *T. paraluiscuniculi*, Cuniculi A strain, were digested with the restriction enzymes EcoRI, PstI, and BamHI (New England Biolabs), separated in 1% TBE agarose gels, denatured with 0.5 M NaOH, transferred to Hybond N membrane (Amersham Labs.) and probed separately with inserts from clones 3 and 33 inserts labeled with ^{32}P using the Random Priming Labeling Kit (Boehringer Mannheim) according to the manufacturer's protocol. Hybridization and washing were carried out using high stringency conditions, and specific hybridization was detected by autoradiography.

Expression Library Screening. A *T. pallidum* genomic expression library was constructed and differentially screened as previously reported (29). In brief, the library was prepared using the Lambda ZAP® II cloning kit (Stratagene) according to the manufacturer's instructions. Approximately 200,000 plaques (12,500 PFU/plate) were plated and duplicate lifts prepared and screened using established methods (30). Filters were differentially screened with a *T. pallidum*–specific immune rabbit serum depleted of activity against the major known treponemal antigens but still retaining its opsonic capacity (termed opsonic rabbit serum; ORS), and a nonopsonic antiserum prepared using heat-killed *T. pallidum* (termed nonopsonic rabbit serum; non-ORS). The ORS was prepared by sequential adsorption of pooled syphilitic rabbit serum with *T. phagedenis*, biotype Reiter, recombinant *T. pallidum* 47-, 37-, 34.5-, 33-, 30-, 17-, and 15-kD molecules (as designated in Table III in reference 2) and recombinant Tromp 1 (6). In unpublished studies from our laboratory, antisera raised against electroeluted or recombinant forms of these antigens failed to demonstrate opsonic function. The antiserum was further adsorbed with VDRL antigen, a lipid complex that has been shown to be the target of a minor portion of opsonic antibodies (31). These adsorption steps were performed to reduce the number of irrelevant positive clones identified by this antiserum in the expression library screening. Immunoreactive plaques were detected with 1 μCi of ^{125}I-labeled protein A on nitrocellulose filters using established methods (30). Clones showing reactivity with the opsonic antiserum but no reactivity with the nonopsonic antiserum were converted to pBluescript SK phagemids and sequenced.

Tpr Sequences and Alignments. Once the genome sequence of the Nichols strain of *T. pallidum* was released (reference 28 and http://utmmg.med.uth.tmc.edu/treponema/tpall.html), the sequences of the entire open reading frames (ORFs) of the *tpr* genes were located, and the probable coding regions determined based upon ATG and alternative bacterial start codons, putative ribosomal binding sites, and promoters. These may differ slightly from the ORFs identified in the website. The sequences of the variable domains of the 12 *tpr* family members were compared. Transmembrane topology analysis of the predicted amino acid sequence of Tpr K was performed using the TMpred program (http://www.isrec.isb-sib.ch/software/BOX_form.html); prediction of signal sequences and their cleavage was performed using the PSORT program (http://psort.nibb.ac.jp:8800/); and the alignments of the nucleotide and predicted amino acid sequences were performed using the Clustal W program (32).

Transcriptional Analysis of the tpr Genes by Reverse Transcription PCR. Reverse transcription (RT)-PCR systems were developed for semiquantitative and qualitative detection of mRNA for the 12 *tpr* genes of *T. pallidum* subspecies *pallidum*. The DNA sequences of all members were aligned and used to design primers specific for each gene. These primers (Table I) are located in the central variable regions of *tpr* genes from subfamilies I and II (defined in Results), and in the large hydrophilic regions of genes from subfamily III.

Total RNA was extracted from a known number of freshly harvested treponemes followed by treatment of the RNA sample

[1]*Abbreviations used in this paper:* msp, major sheath protein; NRS, normal rabbit serum; ORS, opsonized rabbit serum; RT, reverse transcription; tpr, *Treponema pallidum* repeat.

Table I. *tpr-specific Primers*

	Sense primers	Antisense primers	Amplicon size
			bases
tpr A	5′-tacctaccgggatacgaacagt	5′-tgcaaggcatgggtgtaatcat	315
tpr B	5′-agtcaccaccaggtgtgtgg	5′-gacacaagcttagaaagagaatcgt	370
tpr C/D	5′-caagagagagctatcctcaaag	5′-gtttagcagtgacaactcttgg	289
tpr E	5′-cggcaaagtcctgttcggcaa	5′-gctcaacacgctgtcgtatagta	358
tpr F	5′-gaccctgccgatgcaggtaat*	5′-tcagcaagcacccccctgttc	266
tpr G	5′-gaaggtgttcattaccgaccct	5′-ttgtagcctcagccgtaagctt	359
tpr H	5′-gcagaagctcgatagtgtcaag	5′-gtgtgctcccatacgtaggaaa	280
tpr I	5′-gaccctgccgatgcaggtaat*	5′-taagcacgatgtccgactgact	336
tpr J	5′-tcttcacaccccgcagggaa	5′-cgttatttccgttcgcatcatc	364
tpr K	5′-agtttgcgtctaacaccgactg	5′-tcgcatggccatgttgagaaat	411
tpr L	5′-ggtggtttcccatttggaagg	5′-caagtagtctgtaagctgcctg	295

*Identical sense primers for *tpr* F and I.

with RNAse free DNAse A (GIBCO BRL). First-strand cDNA was made using Superscript II and random primers (GIBCO BRL), and PCR amplification was performed using primers specific for each *tpr*. The PCR reactions were standardized using the same number of treponeme equivalents of cDNA for each primer set. This RT-PCR technique has been optimized so that semiquantitative mRNA results can be obtained when amplification is performed for 35 cycles with 500–1,000 treponeme equivalents of cDNA. In careful experiments using limiting dilutions of Nichols strain DNA, we showed that the efficiency of the primer sets was the following: *msp* 6, 10 > 2, 3, 12 > 1, 4/5, 7, 9, 11 > 8. This control experiment eliminated the possibility that our results showing differential transcription of *tpr K* in the Nichols strain was due simply to differences in primer efficiency. Multiple independent RT-PCR reactions were performed to rule out experiment-to-experiment variation.

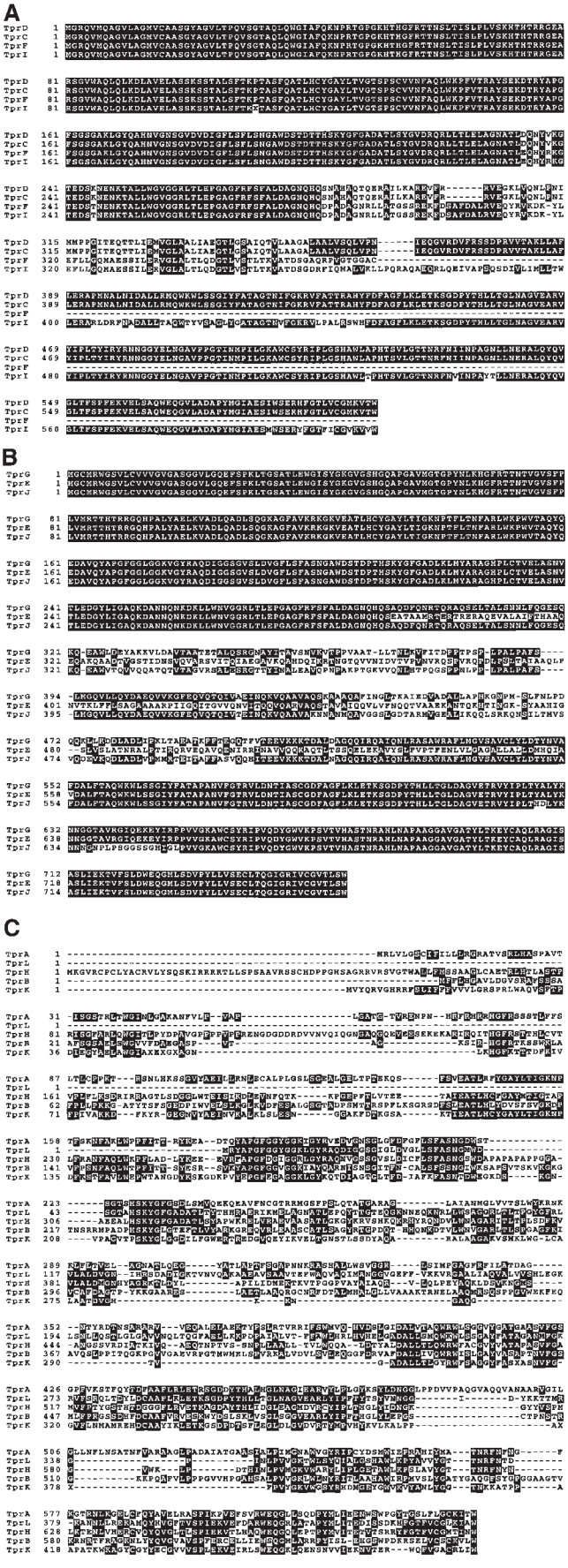
PCR was performed using a 100 μl reaction containing 200 μM dNTPs, 50 mM Tris-HCl (pH 9.0 at 20°C), 200 mM NH_4SO_4, 1 μM of each primer, and 2.5 U of Taq polymerase (Promega Corp.). 2 μl of cDNA containing 500–1,000 treponeme equivalents were used as template. $MgCl_2$ beads (Invitrogen) were added immediately before amplification, giving a final $MgCl_2$ concentration of 1.5 mM. The cycling conditions were as follows: denaturation at 94°C for 3 min, then 94°C for 1 min, 64°C for 2 min, and 72°C for 1 min for 35 cycles. The PCR products were then separated in 3% NuSieve (FMC Bioproducts) agarose gels.

Recombinant Expression of the Tpr K Variable Domain. The variable domain of Tpr K was expressed as a recombinant peptide in *Escherichia coli*. The region of interest (amino acids 37–351) was amplified from Nichols strain DNA using the following primers: sense (5′-atattgaaggctatgcggagctg); antisense (5′-cctcaaggaaagaagtatcagg). The amplicon was cloned directly into the pCR3.1 T/A cloning vector (Invitrogen), sequenced to verify identity and lack of mutations, and subcloned using restriction endonucleases into the pRSET vector (Invitrogen; reference 33). Recombinant expression was induced by 0.4 mM isopropylthiogalactopyranoside during log phase growth at 30°C. Inclusion bodies containing the recombinant protein were isolated and solubilized in 6 M urea, and the recombinant 6-histidine–containing protein was purified by nickel chromatography (33).

Immunization of Rabbits for Antisera and Protection Studies. Five adult male New Zealand white rabbits were immunized with the purified Tpr K recombinant variable domain using a total of 150 μg of recombinant peptide per rabbit in Ribi Adjuvant (MPL + TDM + CWS; Sigma Chemical Co.), divided among intradermal, subcutaneous, intramuscular, and intraperitoneal injections according to the manufacturer's instructions. Two additional booster immunizations were given at 3-wk intervals, and antisera were collected 1 wk after the final immunization.

Opsonization Experiments. The opsonic activity of the antisera raised against the recombinant Tpr K variable domain were tested as previously described (34). In brief, 2×10^6 proteose peptone–induced rabbit peritoneal macrophages were incubated for 4 h on coverslips with 10^7 freshly extracted *T. pallidum*, Nichols strain, and 10% (final concentration) normal rabbit serum (NRS); additionally, 1% (final concentration) anti-Tpr K variable domain or control antisera was added to separate cultures. Control sera included NRS and pooled immune rabbit serum from rabbits infected with *T. pallidum*, Nichols strain. Macrophages were washed, fixed with ethanol, stained by indirect immunofluorescence for *T. pallidum*, and examined by a blinded observer by fluorescence microscopy for the presence of fluorescein-labeled treponemal antigen within typical vacuoles within macrophages. Triplicate specimens were prepared for each condition, and 100 macrophages were counted for each coverslip. The results presented represent four separate experiments, each using a different macrophage donor. Mean values (± SEM) for the percentage of macrophages ingesting *T. pallidum* were calculated and compared for different antisera by Student's *t* test.

Protection Experiments. 3 wk after the last immunization, the five rabbits immunized with recombinant Tpr K variable domain were challenged intradermally at eight sites on the back with 10^5 *T. pallidum*, Nichols strain, per site. Five unimmunized syphilis-seronegative control rabbits were simultaneously challenged to determine the development and evolution of lesions in the absence of immunity. The rabbits were observed daily for the development and character of the lesions. Aspiration of the lesions for examination by darkfield microscopy was performed, and syphilis serologic tests were used to assess the presence of inapparent infection. Transfer of lymph nodes and testes tissue from



challenged rabbits to naive recipients was also used to detect inapparent infection in some challenged rabbits (35).

Results

Identification of a New Gene Family in T. pallidum, Nichols strain, with Homology to the T. denticola msp. The *tpr* gene family was identified in our laboratories by two different approaches, a subtractive library approach and an immunoselective approach. Using the subtractive hybridization technique, we identified several clones of potential interest: two of these clones (clones 3 and 33) were chosen for further study because they had predicted amino acid homology to the msp of *T. denticola* (27). The translated ORFS of the inserts from clones 3 and 33 aligned with the *T. denticola* msp antigen near the NH_2 and COOH termini, respectively. Southern blots of *T. pallidum* subspecies *pallidum* DNA using the clone 3 DNA fragment as a probe demonstrated that at least three different *T. pallidum* DNA fragments hybridized (data not shown), suggesting that the fragments are associated with a multigene family. The inserts from clones 3 and 33 were later identified as representing fragments of *tpr* F and *tpr* G, respectively.

The immunologic screening method designed to identify opsonic antigens in a *T. pallidum* genomic library (29) also identified a gene encoding a Tpr protein. This clone was distinct from clones 3 and 33, leading us to believe that this was another member of a polymorphic msp-homologue gene family. The sequence of this gene was later shown to correspond to *tpr* K (28).

T. pallidum Tpr Proteins Have Conserved and Variable Domains. The clones identified above are completely homologous with 3 of the 12 polymorphic genes identified in the newly released *T. pallidum* genome (http://utmmg.med.uth.tmc.edu/treponema/tpall.html/; sequence available from EMBL/Genbank/DDBJ under accession No. AE000520; reference 28). Stamm et al. (35a) also submitted the sequence of one of the msp-homologue genes (*tpr* J) to Genbank (sequence available under accession No. U488957). Three Tpr subfamilies can be identified by their predicted AA homology (Fig. 1), although there is some homology in the AA sequences of the conserved domains in all Tpr proteins. Alignment of the AA sequences of the Tprs of subfamily I (*tpr* C, D, F, and I) and subfamily II (*tpr* E, G, and J) shows that the NH_2- and COOH-terminal regions of the predicted proteins are conserved, whereas the central domains are variable in terms of sequence and length. Within subfamily I, Tpr C and D are identical, whereas Tpr F lacks a central variable domain and the

Figure 1. Alignments of predicted amino acid sequences of *tpr* genes of subfamilies I, II, and III using the Clustal W program (41). Dark shaded areas indicate sequence conservation among the Tpr proteins. (A) Alignment of subfamily I (Tpr C, D, F, and I). (B) Alignment of subfamily II (Tpr E, G, and J). (C) Alignment of subfamily III (Tpr A1, A2, B, H, K, and L). The sequences of the genome-derived *tpr* genes can be found in Genbank (under Accession No. AE 000520) or in the *T. pallidum* web site at http://utmmg.med.uth.tmc.edu/treponema/tpall.html

conserved COOH-terminal domain. Subfamily III (Tpr A, B, H, K, and L) is composed of five members that are comparatively poorly homologous to each other or to the other Tpr proteins, but that still retain small areas of conserved sequences at the NH_2 and COOH termini. Potentially cleavable signal peptides were predicted by PSORT analysis (http://psort.nibb.ac.jp:8800/) in all members of subfamily I and Tpr K. Tpr K, F, and I are predicted to be located in the periplasmic space or in the outer membrane (Table II).

Transmembrane Topology Analysis. The predicted AA sequences of the 12 Tpr proteins were analyzed for potential membrane-spanning regions as well as potential hydrophilic regions that might be exposed to the extracellular environment. Subfamilies I and II have potential membrane-spanning regions in the terminal conserved domains, with hydrophilic regions in the central variable domain. For simplicity of terminology, the central hydrophilic regions will be termed "variable domains" even for subfamily III where the conservation of sequence at the termini is less apparent than for subfamilies I and II. The Tpr K sequence was predicted to have two likely hydrophobic transmembrane helices at amino acid positions 10–29 and 303–323, separated by a large hydrophilic domain (Fig. 2). We hypothesize that the hydrophilic portion of Tpr K and perhaps some others of the expressed Tpr proteins are exposed on the surface of the treponeme and are available for interaction with the host and the immune system.

tpr Genes Are Differentially Transcribed by T. pallidum, Nichols Strain. To examine transcription of the *tpr* genes in *T. pallidum*, a group of oligonucleotide primers specific for each of the *tpr* was prepared (Table I). Fig. 3 shows the RT-PCR analysis of *tpr* transcription from *T. pallidum* subspecies *pallidum*, Nichols strain. A strong signal was detectable after 35 cycles for *tpr* K, whereas weak signal was observed for *tpr* E and H. After five more PCR cycles, signal was discernible for all the *tpr* genes (data not shown). The preponderance of *tpr* K product was not due to an increased efficiency of *tpr* K amplification, because the PCR assay for *tpr* K was less efficient with limiting dilutions of genomic DNA than with other *tpr* genes (see Materials and Methods). The PCR products represent amplification of mRNA, and not contaminating genomic DNA because (a) the RNA was pretreated with DNAse, and (b) performing the PCR with the RNA preparation before the cDNA synthesis step yielded no product. RT-PCR on *T. pallidum*, Nichols strain, isolated from rabbit testicles on days 5, 10, and 15 after infection all demonstrated a preponderance of *tpr* K mRNA; smaller amounts of *tpr* B, E, H, or I mRNA were detected weakly and variably (data not shown). We conclude that *tpr* K is transcribed to a higher degree than the other *tpr* genes by *T. pallidum*, Nichols

Table II. *Characteristics of tpr Genes and Predicted Proteins*

tpr*	ORF‡	Predicted MW	Signal peptide§	Predicted cellular location‖
	bp	*kD*		
Subfamily I				
C	1794	64.7	C	IM
D	1794	64.7	C	IM
F	1107	39.3	C	OM/P
I	1827	66.5	C	OM/P
Subfamily II				
E	2286	81.5	U	IM
G	2268	81.3	U	IM
J	2274	81.4	U	IM
Subfamily III				
A1¶	759	27.6	U	IM
A2¶	1167	42.3	N	Cy
B	1932	69.5	N	Cy
H	2079	76.1	N	Cy
K	1440	52.7	C	OM/P
L	1329	48.3	N	IM

*Sequences derived from website http://utmmg.med.uth.tmc.edu/treponema/tpall.html

‡ORF based upon analysis of ATG and alternative bacterial start codons, putative ribosomal binding sites, and putative promoters.

§Signal peptide, predicted by Psort (http://psort.nibb.ac.jp/). U, uncleavable; C, cleavable; N, none.

‖Location of the predicted protein in the bacterial cell, predicted by PSORT. OM, outer membrane; IM, inner membrane; P, periplasmic; Cy, cytoplasmic.

¶In the *T. pallidum* genome (EMBL/GenBank/DDBT accession No. AE 000520), tpr A is a sequence that includes A1 and A2, which are out of frame but appear to be the NH_2 and COOH termini of a single ORF with a small central overlapping region.

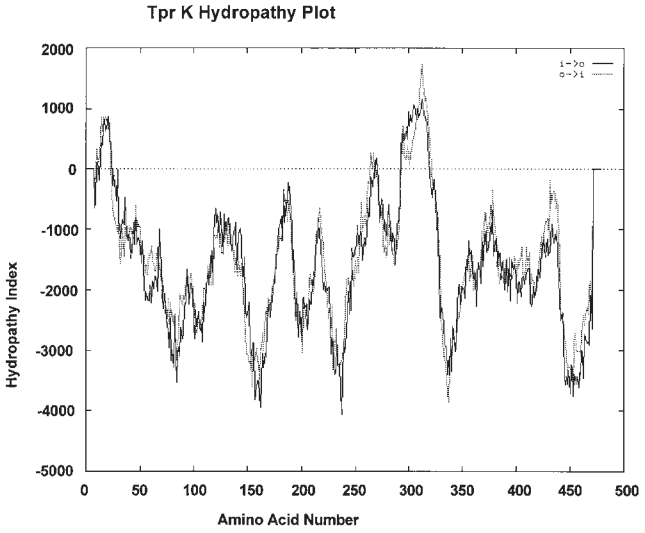


Figure 2. Hydropathy plot for Tpr K determined using the TMpred program (http://ulrec3.unil.ch/software/TMPRED). The x-axis represents the amino acid residues in the sequence, and the y-axis indicates the hydropathy index. Negative values indicate hydrophilic characteristics, and positive values indicate hydrophobic characteristics. This analysis shows two putative transmembrane hydrophobic regions with a large intervening hydrophilic domain.

Figure 3. Ethidium bromide–stained gel showing PCR amplification of Nichols strain cDNA using primers for unique sequences of the variable domains of *tpr* A–L. The lane labeled "+" shows the result of amplification using control primers (demonstrates sufficient amounts of target cDNA in the sample) and lanes 1–12 show the results when amplification was performed using primers specific for each *tpr* gene. Total RNA was extracted from a known number of freshly harvested Nichols strain treponemes, followed by treatment of the RNA sample with RNAse free DNAse A. First-strand cDNA was made by random priming, and PCR amplification was performed using primers specific for each *tpr* gene (Table I). DNAse A treatment of the RNA samples, lack of amplification of RNA without cDNA synthesis, and determination of primer efficiency ruled out the possibility that these results represent either DNA contamination or PCR efficiency artifacts (data not shown).

strain. The preferential expression of Tpr K in the Nichols strain is supported by the selective development of Tpr K–specific antibody and reactive T cells in Nichols-infected rabbits (our unpublished results). Because it is not possible to produce clonal populations of *T. pallidum*, we are uncertain whether the variable transcription of the other *tpr* genes described above occurs in subpopulations of treponemes within our Nichols strain or in all bacterial cells of the Nichols strain.

Tpr K Variable Domain Is the Target of Opsonic Antibodies. The variable domain of Tpr K was examined as a potential target of opsonic antibodies for three reasons: RT-PCR analysis suggests that it is preferentially expressed in the Nichols strain; it is predicted to have a cleavable signal peptide and be localized to the outer membrane; and structural analysis supports the hypothesis that the hydrophilic regions are exposed on the external face of the outer membrane. In addition, the immunologic screening of the expression library identified Tpr K as being reactive with ORS and not with non-ORS. Antisera obtained from the animals immunized with the recombinant Tpr K variable domain were tested in four separate experiments for opsonic activity. As shown in Fig. 4, anti-Tpr K variable domain was significantly opsonic for the Nichols strain of *T. pallidum*, compared with NRS. These results strongly support the hypothesis that Tpr K is expressed in the Nichols strain and that the variable domain is exposed at the surface of the bacterium.

Immunization with Recombinant Tpr K Variable Domain Is Partially Protective Against T. pallidum Challenge. Because Tpr K induces opsonic antibody and because phagocytosis of opsonized bacteria by macrophages is thought to be a major clearance mechanism in syphilis, we investigated the ability of Tpr K to induce protection from challenge with *T. pallidum*, Nichols strain. Five immunized rabbits and five unimmunized controls were challenged intradermally with 10^5 per site at eight injection sites per animal. The lesions that appeared in Tpr K-immunized rabbits were atypical, in that they were flat and nonulcerative, and 95% were devoid of *T. pallidum* by darkfield microscopic examination of aspirates, and healed rapidly compared with control animals (Table III, Fig. 5). In contrast, the lesions that developed in the control rabbits developed large chancres, many of which (76%) progressed to ulceration and were positive for treponemes by darkfield microscopy. Parallel experiments revealed that immunization with unrelated recombinant molecules in adjuvant provided no protection, thus demonstrating that the level of protection induced by Tpr K variable domain is not due to an adjuvant effect (data not shown).

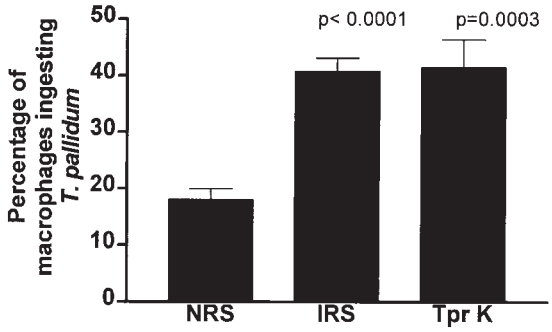


Figure 4. Opsonization of *T. pallidum* by antisera to recombinant Tpr K variable domain. Columns represent the percentage of rabbit peritoneal macrophages ingesting *T. pallidum*, Nichols strain, after 4 h of incubation with viable *T. pallidum* (107 treponemes and 2×10^6 macrophages) in RPMI with 10% final concentration of NRS plus 1% final concentration of test or control antiserum. Ingested treponemes were visualized by indirect immunofluorescence staining. Triplicate cultures were prepared for each experiment and were scored for each condition by a blinded observer. Column values represent means ± SEM of four separate experiments. Significant opsonization is determined by comparison with the NRS values (Student's *t* test) and *P* values are shown. IRS, pooled syphilitic immune rabbit sera; Tpr K, anti-recombinant Tpr K variable domain.

Discussion

The identification of molecules important in syphilis pathogenesis and in protective immunity is critical for understanding the mechanisms by which *T. pallidum* is able to cause a multistage disease and to establish life-long infection. In this study, we demonstrate that *T. pallidum* has a polymorphic, multicopy gene family that codes for proteins homologous to the msp of *T. denticola*. The *T. pallidum tpr*

Table III. *Protection against Challenge after Immunization with Recombinant Tpr K Variable Domain*

	No. of rabbits	Darkfield-positive lesions*	Proportion of ulcerative lesions‡	Status of challenged rabbits
Unimmunized	5	29/38 (76%)	13/40 (33%)	5/5 infected
Tpr K variable domain–immunized	5	2/38 (5%)	0/40 (0%)	5/5 infected

*The denominator indicates the number of lesions that were examined by darkfield microscopy.
‡The denominator indicates the total number of lesions in the treatment group.

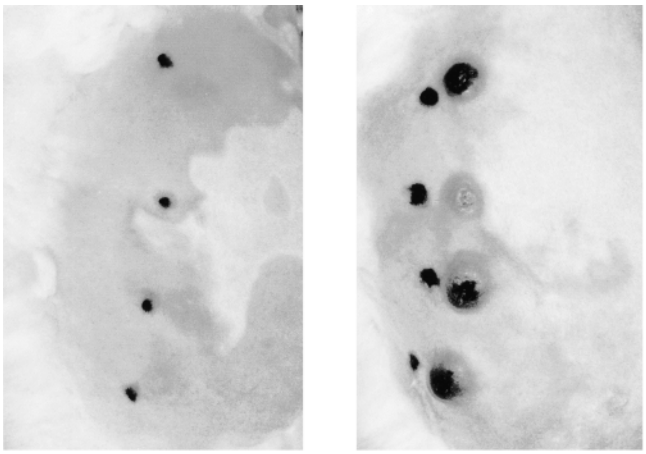


Figure 5. Intradermal challenge in a rabbit immunized with the recombinant Tpr K variable domain (A) and a normal unimmunized (B) rabbit. The immunized rabbit was injected with 125 μg purified recombinant peptide in Ribi adjuvant (MPL + TDM + CWS), divided between intramuscular, intradermal, subcutaneous, and intraperitoneal sites, and similarly boosted at 3 and 6 wk. The rabbits were challenged intradermally with 10^5 *T. pallidum* Nichols strain at each of eight sites 3 wk after the final immunization. Black ink marks are seen to the left of the challenge sites.

gene family represents 2% of the organism's total DNA content, a striking proportion in an organism with an unusually small genome (1.1 Mb). The devotion of this proportion of DNA to a gene family, in an organism that has killed numerous metabolic genes during evolution, suggests that the Tpr proteins may play a critical role in *T. pallidum* survival and virulence.

Several lines of evidence support the hypothesis that at least some of the *T. pallidum* Tpr proteins are located in the outer membrane with surface-exposed variable domains. First, the paralogous msp protein of *T. denticola* is surface exposed. Second, structural analysis of the predicted amino acid sequences of the Tpr proteins found in the *T. pallidum* genome shows central hydrophilic variable domains flanked by amphipathic transmembrane helices, suggesting that the variable domain may be surface-exposed. Further analysis of these molecules identified putative cleavable signal peptides in five of the Tpr molecules, and predicted a possible outer membrane location for Tpr F, I, and K. Finally, and most convincingly, antibodies directed against the variable domain of Tpr K have significant opsonic activity for living *T. pallidum*, thus demonstrating that these variable domains are accessible at the surface of the intact organism.

The critical role that the Tpr family plays during infection is perhaps best illustrated by the immune protection experiments described in this study. Tpr K induced significant protection against homologous challenge with the Nichols strain. Although lesions developed at every site in every animal, the lesions that appeared in the immunized animals were quite atypical in that they were very flat and nonulcerative, lacked demonstrable treponemes by dark-field examination of aspirates, and healed rapidly compared with control animals. It is also noteworthy that it is the protective Tpr K that is predominantly transcribed in the Nichols strain used for challenge.

It is important to emphasize that none of the immunized animals was completely protected from infection, as they all seroconverted after challenge or had *T. pallidum* in their lymph nodes and testes detected by microscopy or tissue transfer to another animals. Nonetheless, the degree of alteration of lesion development after immunization is remarkable. It should be noted that the challenge dose used in these studies (nearly 10^6 treponemes per rabbit) is many logs higher than the rabbit ID_{50} (51 organisms; reference 35). Experiments are currently underway to examine a challenge dose more analogous to those likely to be encountered in nature.

We also provide evidence for the differential transcription (and probable differential expression) of *tpr* K in the Nichols strain of *T. pallidum*. Minor mRNA species were also detected in this strain, and higher levels of amplification reveal mRNA for all *tpr* genes. These results have several implications. First, there is a predominant Tpr antigen that is expressed in a given population of treponemes, rather than uniform expression of all 12 Tpr proteins. This can be predicted to result in a skewing of the immune response toward the predominant antigen. The "minor" *tpr* transcripts that are detected by RT-PCR in any strain may reflect less robust expression of those proteins in the same bacterial cells that are also expressing the predominant msp homologue, or they may represent a small subpopulation of organisms in which the minor Tpr is the predominantly expressed antigen ("subpopulation hypothesis"). To date, there are no direct data that favor either of these possibilities over the other.

The subpopulation hypothesis could explain the natural history of syphilis and some intriguing experimental observations. For example, the majority population is responsible for the development of the primary stage of infection. As the immune response develops to the Tpr antigen expressed on the surface of the majority population, those organism are cleared. This provides a selective advantage for a subpopulation of treponemes that express an alternative surface-localized Tpr antigen and the secondary stage ensues. This cycle can continue again to cause recurrent secondary syphilis, and again in some patients to cause tertiary disease. An analogous situation had been demonstrated for *Borrelia hermsii*, the spirochete causing relapsing fever (36), and may also occur in the Lyme disease spirochete, *Borrelia burgdorferi* (37). The subpopulation hypothesis could also explain our earlier findings (38) that *T. pallidum* harvested from infected rabbit testes in which clearance of the majority of the treponemes has naturally occurred (17 d after infection) are resistant to phagocytosis, whereas treponemes harvested before in vivo clearance occurs are readily opsonized and ingested.

The genetic mechanism responsible for regulation of the expression of *tpr* genes has not yet been defined. The *T. pallidum tpr* genes have a domain structure that resembles the *vsp* and *vlp* gene subfamilies of *B. hermsii*, with 5′ and

3′ terminal conserved domains flanking central variable domains (39). As happens in *B. hermsii*, the conserved domains could serve as sites for recombination for antigenic switch, allowing the expression of previously silent variable domains at transcriptionally active expression sites (40–42). Thus, by analogy to other spirochetes, we propose that the *T. pallidum* subspecies *pallidum* Tpr proteins may undergo antigenic variation (concerted switching of antigen expression within a strain) and that this mechanism is responsible for the multi-stage nature of syphilis.

It is also possible that *T. pallidum* undergoes phase variation (switching gene expression on and off) of the *tpr* genes. This mechanism would provide an alternative explanation for the development and survival of a subpopulation of treponemes that can resist opsonization and phagocytosis (38) and survive to cause persistent infection. For example, if Tpr molecules are necessary for attachment to host cells, they may be expressed during establishment of infection, but may be unnecessary and turned off during the prolonged latent stage. Appearance of the secondary and tertiary manifestations may be due to renewed Tpr expression (of a different antigenic specificity than during the primary stage) in organisms from latent infection, or alternatively, due to outgrowth of a pre-existing Tpr-expressing subpopulation that has not yet been recognized by the immune system (antigenic variation). Furthermore, expression of specific Tpr molecules may confer tissue tropism on *T. pallidum*, analogous to the situation in *Borrelia turicatae* (43) in which a single *vsp* gene is expressed in neurotropic organisms.

Yet another form of antigenic diversity results from antigenic drift (slower minor changes in epitope composition that occur during strain divergence in evolution and lead to strain-to-strain antigenic differences). This may occur by random mutational events or by recombination within the *tpr* gene family. There are already data to suggest that antigenic drift of *tpr* occurs. Restriction fragment length polymorphism analysis of a *tpr* gene has revealed different restriction patterns between strains of *T. pallidum* subspecies *pallidum* (44 and our unpublished results). Furthermore, sequence heterogeneity and the presence of multiple *tpr* K alleles in other strains has been demonstrated in our laboratory (unpublished results).

In summary, we describe the identification of a polymorphic, multi-membered gene family in *T. pallidum* subspecies *pallidum*. *Tpr* K is preferentially transcribed in the Nichols strain, and its encoded protein serves as a target of opsonic antibody, induces significant protection against infectious challenge, and is likely to be surface exposed. This gene family appears to be central to the pathogenesis of syphilis and may contribute to antigenic diversity of *T. pallidum*.

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References

1. Cox, D.L., P. Chang, A.W. McDowall, and J.D. Radolf. 1992. The outer membrane, not a coat of host proteins, limits antigenicity of virulent *Treponema pallidum*. *Infect. Immun.* 60:1076–1083.
2. Norris, S.J., J.F. Alderete, N.H. Axelsen, M.J. Bailey, S.A. Baker-Zander, J.B. Baseman, P.J. Bassford, R.E. Baughn, A. Cockayne, P.A. Hanff, et al. 1987. Identity of *Treponema pallidum* subsp. *pallidum* polypeptides: correlation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis results from different laboratories. *Electrophoresis*. 8:77–92.
3. Radolf, J.D., M.V. Norgard, and W.W. Schultz. 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. *Proc. Natl. Acad. Sci. USA*. 86:2051–2055.
4. Walker, E.M., G.A. Zampighi, D.R. Blanco, J.N. Miller, and M.A. Lovett. 1989. Demonstration of rare protein in the outer membrane of *Treponema pallidum* subsp. *pallidum* by freeze-fracture analysis. *J. Bacteriol.* 171:5005–5011.
5. Radolf, J.D. 1995. *Treponema pallidum* and the quest for outer membrane proteins. *Mol. Microbiol.* 16:1067–1073.
6. Blanco, D.R., C.I. Champion, M.M. Exner, E.S. Shang, J.T. Skare, R.E. Hancock, J.N. Miller, and M.A. Lovett. 1996. Recombinant *Treponema pallidum* rare outer membrane protein 1 (Tromp1) expressed in *Escherichia coli* has porin activity and surface antigen exposure. *J. Bacteriol.* 178:6685–6692.
7. Champion, C.I., D.R. Blanco, M.M. Exner, H. Erdjument-Bromage, R.E.W. Hancock, P. Tempst, J.N. Miller, and M.A. Lovett. 1997. Sequence analysis and recombinant expression of a 28-kilodalton *Treponema pallidum* subsp. *pallidum* rare outer membrane protein (Tromp2). *J. Bacteriol.* 179: 1230–1238.
8. Hardham, J.M., L.V. Stamm, S.F. Porcella, J.G. Frye, N.Y. Barnes, J.K. Howel, S.L. Muller, J.D. Radolf, G.M. Weinstock, and S.J. Norris. 1997. Identification and transcriptional

analysis of a *Treponema pallidum* operon encoding a putative ABC transport system, an iron-activated repressor protein homolog, and a glycolytic pathway enzyme homolog. *Gene*. 197:47–64.
9. Akins, D.R., E. Robinson, D. Shevchenko, C. Elkins, D.L. Cox, and J.D. Radolf. 1997. Tromp1, a putative rare outer membrane protein, is anchored by an uncleaved signal sequence to the *Treponema pallidum* cytoplasmic membrane. *J. Bacteriol*. 179:5076–5086.
10. Shevchenko, D.V., D.R. Akins, E.J. Robinson, M. Li, O.V. Shevchenko, and J.D. Radolf. 1997. Identification of homologs for thioredoxin, peptidyl prolyl *cis-trans* isomerase, and glycerophosphodieter phosphodiesterase in outer membrane fractions from *Treponema pallidum*, the syphilis spirochete. *Infect. Immun*. 65:4179–4189.
11. Radolf, J.D., E.J. Robinson, K.W. Bourell, D.R. Akins, S.F. Porcella, L.M. Weigel, J.D. Jones, and M.V. Norgard. 1995. Characterization of outer membranes isolated from *Treponema pallidum*, the syphilis spirochete. *Infect. Immun*. 63: 4244–4252.
12. Radolf, J.D., N.R. Chamberlain, A. Clausell, and M.V. Norgard. 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. *Infect. Immun*. 56:490–498.
13. Blanco, D.R., K. Reimann, J. Skare, C.I. Champion, D. Foley, M.M. Exner, R.E.W. Hancock, J.M. Miller, and M.A. Lovett. 1994. Isolation of the outer membranes from *Treponema pallidum* and *Treponema vincentii*. *J. Bacteriol*. 176:6088–6099.
14. Blanco, D.R., M. Giladi, C.I. Champion, D.A. Haake, G.K. Chikami, J.N. Miller, and M.A. Lovett. 1991. Identification of *Treponema pallidum* subspecies *pallidum* genes encoding signal peptides and membrane-spanning sequences using a novel alkaline phosphatase expression vector. *Mol. Microbiol*. 5:2405–2415.
15. Wong, G.H., B. Steiner, and S. Graves. 1983. Effect of syphilitic rabbit sera taken at different periods after infection on treponemal motility, treponemal attachment to mammalian cells in vitro, and treponemal infection in rabbits. *Br. J. Vener. Dis*. 59:220–224.
16. Fitzgerald, T.J., L.A. Replesh, D.R. Blanco, and J.N. Miller. 1984. Attachment of *Treponema pallidum* to fibronectin, laminin, collagen IV, and collagen I, and blockage of attachment by immune rabbit IgG. *Br. J. Vener. Dis*. 60:357–363.
17. Lukehart, S.A., and J.N. Miller. 1978. Demonstration of the in vitro phagocytosis of *Treponema pallidum* by rabbit peritoneal macrophages. *J. Immunol*. 121:2014–2024.
18. Baker-Zander, S.A., and S.A. Lukehart. 1992. Macrophage-mediated killing of opsonized *Treponema pallidum*. *J. Infect. Dis*. 165:69–74.
19. Bishop, N.H., and J.N. Miller. 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. *J. Immunol*. 117: 197–207.
20. Blanco, D.R., J.N. Miller, and P.A. Hanff. 1984. Humoral immunity in experimental syphilis: the demonstration of IgG as a treponemicidal factor in immune rabbit serum. *J. Immunol*. 133:2693–2697.
21. Blanco, D.R., E.M. Walker, D.A. Haake, C.I. Champion, J.N. Miller, and M.A. Lovett. 1990. Complement activation limits the rate of in vitro treponemicidal activity and correlates with antibody-mediated aggregation of *Treponema pallidum* rare outer membrane protein. *J. Immunol*. 144:1914–1921.
22. Lukehart, S.A., S.A. Baker-Zander, R.M.C. Lloyd, and S. Sell. 1980. Characterization of lymphocyte responsiveness in early experimental syphilis. II. Nature of cellular infiltrations and *Treponema pallidum* distribution in testicular infection. *J. Immunol*. 124:461–467.
23. Lukehart, S.A. 1992. Immunology and pathogenesis of syphilis. *In* Advances in Host Defense Mechanisms, vol. 8: Sexually Transmitted Diseases. T.C. Quinn, J.I. Gallin, and A.S. Fauci, editors. Raven Press, New York. 141–163.
24. Van Voorhis, W.C., L.K. Barrett, D.M. Koelle, J.M. Nasio, F.A. Plummer, and S.A. Lukehart. 1996. Primary and secondary syphilis lesions contain mRNA for Th1 cytokines. *J. Infect. Dis*. 173:491–495.
25. Lukehart, S.A., S.A. Baker-Zander, and S. Sell. 1980. Characterization of lymphocyte responsiveness in early experimental syphilis. I. *In vitro* response to mitogens and *Treponema pallidum* antigens. *J. Immunol*. 124:454–460.
26. Centurion-Lara, A., C. Castro, W.C. Van Voorhis, and S.A. Lukehart. 1997. Two 16S-23S ribosomal DNA intergenic regions in different *Treponema pallidum* subspecies contain *tRNA* genes. *FEMS Microbiol. Lett*. 143:235–240.
27. Fenno, J.C., K.H. Muller, and B.C. McBride. Sequence analysis, expression, and binding activity of recombinant major outer sheath protein (Msp) of *Treponema denticola*. *J. Bacteriol*. 178:2489–2497.
28. Fraser, C.M., S.J. Norris, G.M. Weinstock, O. White, G.G. Sutto, R. Dodson, M. Gwinn, E.K. Hickey, R. Clayton, K.A. Ketchum, et al. 1998. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science*. 281:375–388.
29. Stebeck, C.E., J.M. Shaffer, T.W. Arrol, S.A. Lukehart, and W.C. Van Voorhis. 1997. Identification of the *Treponema pallidum* subsp. *pallidum* glycerophosphodiester phosphodiesterase homologue. *FEMS Microbiol. Lett*. 154:303–310.
30. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. Baker-Zander, S.A., J.M. Shaffer, and S.A. Lukehart. 1993. VDRL antibodies enhance phagocytosis of *Treponema pallidum* by macrophages. *J. Infect. Dis*. 167:1100–1105.
32. Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties, and weight matrix choice. *Nucleic Acids Res*. 22:4673–4680.
33. Kroll, D.J., H. Abdel-Malek Abdel-Hafiz, T. Marcell, S. Simpson, C.Y. Chen, A. Guiterrez-Hartmann, J.W. Lustbader, and J.P. Hoeffler. 1993. A multifunctional prokaryotic protein expression system: overproduction, affinity purification, and selective detection. *DNA Cell Biol*. 12:441–453.
34. Shaffer, J.M., S.A. Baker-Zander, and S.A. Lukehart. 1993. Opsonization of *Treponema pallidum* is mediated by IgG antibodies induced only by pathogenic treponemes. *Infect. Immun*. 61:781–784.
35. Turner, T.B., P.H. Hardy, and B. Newman. 1969. Infectivity tests in syphilis. *Br. J. Vener. Dis*. 45:183–196.
35a. Stamm, L.V., S.R. Greene, H.L. Bergen, J.M. Hardham, and N.Y. Barnes. 1998. Identification and sequence analysis of *Treponema pallidum tprJ*, a member of a polymorphic multigene family. *FEMS Microbiol. Lett*. 169:155–163.
36. Donelson, J.E. 1995. Mechanisms of antigenic variation in *Borrelia hermsii* and African trypanosomes. *J. Biol. Chem*. 270:

7783–7786.
37. Zhang, J.-R., J.M. Hardham, A.G. Barbour, and S.J. Norris. 1997. Antigenic variation in Lyme disease Borreliae by promiscuous recombination of VMP-like sequence cassettes. *Cell*. 89:275–285.
38. Lukehart, S.A., J.M. Shaffer, and S.A. Baker-Zander. 1992. A subpopulation of *Treponema pallidum* is resistant to phagocytosis: possible mechanisms of persistence. *J. Infect. Dis.* 166: 1449–1453.
39. Hinnebusch, B.J., A.G. Barbour, B.I. Restrepo, and T.G. Schwan. 1998. Population structure of the relapsing fever spirochete *Borrelia hermsii* as indicated by polymorphism of two multigene families that encode immunogenic outer surface lipoproteins. *Infect. Immun.* 66:432–440.
40. Barbour, A.G., N. Burman, C.J. Carter, T. Kitten, and S. Bergstrom. 1991. Variable antigen genes of the relapsing fever agent *Borrelia hermsii* are activated by promoter addition. *Mol. Microbiol.* 5:489–493.
41. Meier, J.T., M.I. Simon, and A.G. Barbour. 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia*. *Cell*. 41:403–409.
42. Plasterk, R.H.A., M.I. Simon, and A.G. Barbour. 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. *Nature*. 318:257–263.
43. Cadavid, D., D.D. Thomas, R. Crawley, and A.G. Barbour. 1994. Variability of a bacterial surface protein and disease expression in a possible mouse model of systemic Lyme Borreliosis. *J. Exp. Med.* 179:631–642.
44. Pillay, A., H. Liu, C.Y. Chen, B. Holloway, A.W. Sturm, B. Steiner, and S.A. Morse. 1997. Molecular subtyping of *T. pallidum* subsp. *pallidum*. *Sex. Transm. Dis.* 25:408–414.