The TprK Protein of *Treponema pallidum* Is Periplasmic and Is Not a Target of Opsonic Antibody or Protective Immunity

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Abstract

The finding that *Treponema pallidum*, the syphilis spirochete, contains 12 orthologs of the *Treponema denticola* outer membrane major sheath protein has engendered speculation that members of this *T. pallidum* repeat (Tpr) family may be similarly surface exposed. In this regard, the TprK protein was reported to be a target of opsonic antibody and protective immunity and subject to immunologically driven sequence variation. Despite these findings, results from our previous analyses of treponemal outer membranes in concert with computer-based predictions for TprK prompted us to examine the cellular location of this protein. TprK–alkaline phosphatase fusions expressed in *Escherichia coli* demonstrate that TprK contains a signal peptide. However, opsonophagocytosis assays failed to indicate surface exposure of TprK. Moreover, results from three independent methodologies, i.e., (a) indirect immunofluorescence analysis of agarose-encapsulated organisms, (b) proteinase K treatment of intact spirochetes, and (c) Triton X-114 phase partitioning of *T. pallidum* conclusively demonstrated that native TprK is entirely periplasmic. Consistent with this location, immunization with the recombinant protein failed to induce either protective immunity or select for TprK variants in the rabbit model of experimental syphilis. These findings challenge the notion that TprK will be a component of an efficacious syphilis vaccine.

Key words: spirochete • membrane protein • vaccine • antigenic variation • opsonization

Introduction

In recent years, the quest for outer membrane (OM)[1] proteins of *Treponema pallidum* as potential virulence determinants and vaccinogens has become a major focus of syphilis research. The many methodological difficulties inherent in this search, coupled with our limited knowledge of *T. pallidum* OM constituents, has spawned a variety of experimental approaches for identifying these proteins (1–4). However, to date none of these strategies has yielded a polypeptide which is universally accepted as being surface exposed. The availability of the *T. pallidum* genomic sequence has opened new avenues in this search (5). Indeed, one of the most important discoveries is that *T. pallidum* contains 12 orthologs (designated *T. pallidum* repeat [Tpr] proteins) for the surface-exposed major sheath protein (Msp) of the oral commensal spirochete *Treponema denticola* (5), an abundant 55-kD polypeptide which reportedly forms a hexagonal array in the *T. denticola* OM and possesses porin-like activity (6). Consistent with this implied cellular location, Centurion-Lara et al. (7) reported that the *T. pallidum* Tpr paralog designated TprK is a target for opsonic Ab and that immunization with the recombinant protein conferred partial protection in the rabbit model of experimental syphilis. The subsequently reported sequence variability of *tprK* among *T. pallidum* clinical isolates and within "subpopulations" of street and laboratory strains also

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[1]*Abbreviations used in this paper:* GlpQ, glycerophosphodiester phosphodiesterase; IRS, immune rabbit sera; LRT, likelihood ratio test; Msp, major sheath protein; NRS, normal rabbit sera; OM, outer membrane; PK, proteinase K; PP, periplasmic/periplasm; RIT, rabbit infectivity test; RT, reverse transcription; TpEf, endoflagella; Tpr, *Treponema pallidum* repeat.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/05/1015/12 $5.00
Volume 193, Number 9, May 7, 2001 1015–1026
http://www.jem.org/cgi/content/full/193/9/1015

was believed to be consistent with the accessibility of TprK to Ab (8, 9). Moreover, the considerable number of Tpr paralogs has engendered speculation that recombination among these genes provides a means of escaping host immune responses (5, 7, 10).

Despite the above findings, several considerations suggested to us that further examination of the cellular location of TprK was warranted. First, partial protection from challenge with virulent treponemes is not in and of itself indicative of surface location because similar levels of protection have been obtained with Ags which are not surface exposed (11). Second, although opsonophagocytosis assays are theoretically capable of identifying surface-exposed *T. pallidum* Ags, results with this assay have not always correlated with other surface localization methodologies (12, 13). Third, in our own studies (13, 14) using a photoactivatable lipophilic probe (3-(trifluoromethy-)-3-(*m*-[^{125}I]iodophenyl-diazarene) which promiscuously labels integral membrane proteins, we were unable to detect a radiolabeled protein corresponding to TprK when either intact spirochetes or isolated *T. pallidum* OMs were incubated with this compound. Fourth, in a recent study, we found that Msp is predominantly periplasmic in *T. denticola* and does not appear to form OM hexagonal rays as reported previously (15). Lastly, results from computer analyses lend only marginal support to the notion that this polypeptide resides in the OM. PSORT (http://psort.nibb.ac.jp/form.html) predicts that TprK could be either a periplasmic (PP) or an OM protein (7); the localization scores heavily favor a PP location. TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), on the other hand, predicts that TprK contains an α-helical transmembrane domain downstream of the presumptive signal peptide which, if present, would prevent translocation of the nascent polypeptide across the cytoplasmic membrane (16).

Here we used a battery of genetic, biological, and protein-based approaches to study the cellular location of TprK in *T. pallidum*. Our data unambiguously demonstrate that TprK is a periplasmic protein. Consistent with this compartmental assignment, immunization of rabbits with TprK failed to induce either opsonic Abs or protective immunity. Equally important, we found that the *tprK* gene was unaltered in treponemes recovered from TprK-immunized animals, indicating that sequence variation of TprK is not immunologically driven. These findings challenge the notion that TprK will be a component of an efficacious syphilis vaccine.

Materials and Methods

Bacterial Strains

Treponema pallidum subspecies *pallidum* strain was propagated by intratesticular inoculation of adult New Zealand White rabbits as described previously (17). Animal protocols described in this work were approved by the University of Connecticut Health Center Animal Care Committee under the auspices of Animal Welfare Assurance no. A3471-01. *Escherichia coli* strain DH5α was the recipient strain for recombinant constructs and was grown in Luria-Bertani (LB) broth with appropriate antibiotic supplementation.

Transcriptional Analysis of the tpr Genes

Determination of Primer Efficiency. The amplification efficiency of the primer pairs used for reverse transcription (RT)-PCR was determined with limiting dilutions of *T. pallidum* DNA using amplification conditions identical to those used in the RT-PCR experiments (see below). The densitometric signal of the resolved *tpr* PCR products, measured by an Alpha Innotech ChemiImager 4400 (Alpha Innotech Corporation), was divided by the signal of the *fla* PCR product amplified from equivalent amounts of DNA, and expressed as the efficiency of the primer pair (Table I).

RT-PCR. With the following exceptions, RNA isolation and RT-PCR were performed as described previously (18). For each RT-PCR primer pair, four types of reactions were performed: (a) RT-PCR with log-dilutions of RNA ranging from 100 ng to 10 pg, (b) PCR with 100 ng of RNA, (c) RT-PCR with water only, and (d) PCR with 5 ng of DNA. After the RT reaction (50°C for 30 min), PCR was performed using the following parameters: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 63°C for 30 s, 68°C for 45 s, followed by 25 identical cycles incorporating an extension time increase of 5 s/cycle followed by a single terminal extension for 7 min at 68°C. The densitometric signal of the resolved products was divided by the primer pair efficiency; the resulting value was subsequently divided by the signal of the *fla* RT-PCR product amplified from equivalent amounts of *T. pallidum* RNA. Expressed data are therefore quantitatively corrected for primer efficiency and normalized to *flaA* transcript levels.

Construction of PhoA Fusions

The plasmids pKS/pho and pSK/pho containing the leaderless alkaline phosphatase (*phoA*) gene of *E. coli* in the multiple cloning site of pBluescript have been described previously (19). Using primers K1-5′, K2-5′, K3-5′, and K1-3′, three in-frame *tpr*K–*pho*A fusions were generated by amplifying and cloning DNA containing codons 21 through 61 of the TprK open reading frame (ORF) along with various amounts of upstream DNA into the Xba1 and BamH1 sites of pKS/phoA and pSK/phoA. *E. coli* clones harboring sequenced PhoA constructs were plated on LB agar containing 100 μg/ml of ampicillin and 40 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

Production and Purification of Recombinant Proteins

Two polyhistidine-tagged rTprK expression constructs were generated. The first encodes the full-length TprK protein downstream of a putative signal peptidase I cleavage site (LWA$^{\downarrow}$Q). The second encodes the central region (amino acids 36–350) of TprK as per Centurion-Lara et al. (7). Although this region was originally annotated as the "variable" domain (7), we refer to it as the "central" domain to avoid confusion with the recently described seven discrete regions of variability (V1–V7) present in different TprK alleles (8, 9). DNAs encoding the TprK full-length protein and central domain were amplified from Nichols-Farmington *T. pallidum* DNA using primer pairs K-full-5′/K-full-3′ and K-cen-5′/K-cen-3′, respectively, and cloned into EcoR1/BamH1-digested pProEx-Htb (GIBCO BRL). DNA encoding the mature FlaA protein was amplified with primers FlaA-5′ and FlaA-3′ and cloned into BamH1 cut pProEx-Hta (GIBCO BRL). Recombinant TprK clones were completely sequenced in both directions to verify the identity and fidelity of the cloned PCR products; for both the full-length and central domain fusions, the deduced amino acid sequences were identical to those of *T. pallidum* (Nichols-Farmington) TprK (see Fig. 8).

Table I. *Primers Used in This Study*

Purpose	Name	5′ to 3′ sequence	Efficiency*
RT-PCR	flaA-RT-5′	TGAATTATCCTCATGGTTTGTACGTG	1.00
	flaA-RT-3′	TCAGCACCGCCTTATCATAGATAATC	
	A-RT-5′	TCTCGCTGACGCTTTGTCCACCG	1.33
	A-RT-3′	AAAGTCAAAACCTAGCCCGGAATTC	
	B-RT-5′	ACAGAACTGGACCTGATCAGACGCAT	1.50
	B-RT-3′	AGGCTTTCCCTGCGTTATAGGAGG	
	C/D-RT-5′	TCAGACCCAAGAGAGAGCTATCCTC	0.75
	C/D-RT-3′	AGCAGTGACAACTCTTGGATCGGA	
	E-RT-5′	AGGCTACCGCGGCGATGAGGAC	1.08
	E-RT-3′	GCACTAAGGAAGAAGAGCTTGGTTA	
	F/I-RT-5′	TAATCGCCTTCTGGCAACGGGGAG	0.83
	F-RT-3′	CGTCAGCAAGCACCCCCTGTTC	
	G-RT-5′	GGCAGTGTCAAACGTAAAAGTCACC	0.55
	G-RT-3′	TTTTTGTTGATCCGGAAGGTTGAAG	
	H-RT-5′	GGTCAGCCATAAACAGAAAAGGCAC	1.18
	H-RT-3′	ATTTTGGTTGCAATATCCCGCACAG	
	F/I-RT-5′	TAATCGCCTTCTGGCAACGGGGAG	1.55
	I-RT-3′	AGCACGATGTCCGACTGACTCGG	
	J-RT-5′	ATCTTCACACCCCGCAGGGAAGT	1.18
	J-RT-3′	ATCTTGCACGCTCACCATGGTCAGA	
	K-RT-5′	TGGGAGCCGTGGACCGCGAATG	1.00
	K-RT-3′	TCGGGCTTGGGCATAGTCGCTAG	
	L-RT-5′	ACCGCCTGCTGTGGAGCGCCG	0.44
	L-RT-3′	TGGGTGAGCTGGTTTACCACCGC	
PhoA fusions	K1-5′	TTCTAGTCTAGA*TGA*TTCCCCTGCGGCACGCCTT‡	
	K2-5′	TTCTAGTCTAGACGCAGTTCCGGATTC*TGA*atgATTG	
	K3-5′	GTCTAGAA*TGA*CGGCATCGGAGAAAAgtgGTGTA	
	K1-3′	GCGGGATCCATCCGGGGTGAACGAAACCTGAG	
Protein expression	K-cen-5′	GCGGGATCCGATATTGAAGGCTATGCGGAGCTG	
	K-cen-3′	GCGGAATTC*TCA*ACCCTCAAGGAAAGAAGTATCAGG	
	K-full-5′	GCGGGATCCCAGGTTTCGTTCACCCCGGATATTG	
	K-full-3′	GCGGAATTC*TCA*CCAAATCAAGCGACATGCCCCTAC	
	FlaA-5′	CGGGATCCTGATGAGTCAGTGCTCATCGAC	
	FlaA-3′	CGGGATCCT*CTA*CTGCTGCACTTCCTGCCG	
Amplification of tprK	K1-5′	TTCTAGTCTAGATGATTCCCCTGCGGCACGCCTT	
	Kseq-4	CCACACGCGCCACCTGTGATGGC	
	TprK5′	TTCTGTATTACCTCCGAACCG	
	TprK3′	TGTGATGGCACAAACGTCTAC	
Sequencing of tprK amplicons	K1-5′	TTCTAGTCTAGATGATTCCCCTGCGGCACGCCTT	
	K3-5′	GTCTAGAATGACGGCATCGGAGAAAAGTGGTGTA	
	TprK5′	TTCTGTATTACCTCCGAACCG	
	TprK3′	TGTGATGGCACAAACGTCTAC	
	K-RT-5′	TGGGAGCCGTGGACCGCGAATG	
	K-full-3′	GCGGAATTCTCACCAAATCAAGCGACATGCCCCTAC	
	Kseq-5	GTGCGTACGTACATGCCTGTCC	

*The flaA normalized primer pair efficiencies were determined by PCR amplification with limiting dilutions of *T. pallidum* DNA followed by densitometric analysis of band intensities. The measured efficiency of the tprC/D primer pair (1.5) was divided by two as there are two copies of this template per genome.

‡Underlined sequences indicate restriction sites used for cloning. Naturally occurring and engineered stop codons are italicized, and lowercase codons indicate translational start sites proposed by Fraser et al. (reference 5; K2-5′) and Centurion-Lara et al. (reference 7; K3-5′).

Fusion proteins were expressed by the addition of isopropyl-thiogalactopyranoside to 1 mM and purified on a Ni-NTA matrix (QIAGEN) according to the manufacturer's instructions for insoluble proteins. The identities of the purified rTprK full-length and central domain proteins were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry at the Keck Foundation Biotechnology Resource Laboratory at Yale University.

Immunologic Reagents

Rat anti-TprK antisera were generated by priming Sprague Dawley rats by intraperitoneal injection with 15 μg of full-length rTprK protein in CFA. After 1 mo, animals were boosted four times over 8 wk with a 1:1 mixture of the central domain and full-length rTprK (15 mg of each protein/boost) in IFA. The mAb 1-7H11.1G6, directed against full-length rTprK, was produced at the Hybridoma Center for Agricultural and Biological Sciences at Oklahoma State University. Rabbits were immunized with rTprK as described below; sera were collected 10 d after the final boost. Rat anti-Fla antisera were generated in Sprague-Dawley rats by intraperitoneal injection with 50 μg/rat of rFlaA protein in CFA, followed at weeks 6 and 8 by similarly administered boosts in IFA. Rat anti-Tp47 and rabbit anti-endoflagella (TpEf) antisera have been described previously (13, 17, 20).

ELISA for Detection of Anti-TprK Ab

ELISAs were performed as described previously (13). The wells of microtiter plates coated with dilutions (100 ng/well to 1 pg/well) of full-length, or central domain rTprK were incubated with 1:50 or 1:1,000 dilutions of either NRS, IRS, IRS depleted of anti-TprK Ab (see below), rat, or rabbit anti-TprK antisera.

Immunoblot Analysis

SDS-PAGE of samples followed by transfer to nitrocellulose membranes was preformed as described previously (14). Blots were incubated with 1:1,000 dilutions of rat antisera or 1:10 dilutions of hybridoma supernatant and subsequently developed by either colorimetric (FlaA and Tp47) or chemiluminescent (TprK) methods as described previously (14, 18).

Triton X-114 Phase Partitioning

Freshly extracted *T. pallidum* was solubilized overnight at 4°C in PBS containing 2% Triton X-114 (octylphenoxypolyethoxyethanol). Insoluble material was removed by centrifugation and the supernatant was phase separated as described previously (13). The resulting fractions were analyzed by immunoblot.

Proteinase K Digestion

Proteinase K (PK) digestion was performed as described previously (13, 21). Freshly isolated *T. pallidum* were centrifuged at 20,000 *g* for 15 min and resuspended in PBS containing 1 mM $CaCl_2$ and 5 mM $MgCl_2$. After addition of either PBS, PK to 0.4 mg/ml, or PK plus Triton X-100 (t-octylphenoxypolyethoxyethanol) to 0.01%, spirochetes were incubated at 37°C for 30 min followed by the addition of PMSF to 1 mg/ml. Lysates of untreated, PK-treated, and Triton/PK-treated *T. pallidum* were analyzed by immunoblot.

Indirect Immunofluorescence of T. pallidum Encapsulated in Gel Microdroplets

Preparation of agarose-encapsulated treponemes has been described previously (13, 17). To discriminate between surface and subsurface exposure of TprK by individual spirochetes, encapsulated organisms were simultaneously probed with 1:30 dilutions of both rat anti-TprK and rabbit anti-TpEf antisera in the absence or presence of 0.05% Triton X-100. After a 1-h incubation and three gentle washes, the beads were incubated with 3 μg of biotinylated goat anti–rat IgG, washed, and then incubated with 3 μg of streptavidin-Alexa® 546 conjugate (Molecular Probes) and 3 μg goat anti–rabbit conjugated to Alexa® 488 (Molecular Probes). For each condition, two slides from each of three separate experiments were prepared, and ~100 organisms per slide were scored for labeling with Alexa® 546 (TprK) and Alexa® 488 (endoflagella). Fluorescence emission overlap did not occur as the fluorescence of singly labeled organisms (either TprK or TpEf) was only observed with the appropriate filter.

Opsonophagocytosis Assay

With minor exception, opsonophagocytosis assays were performed as described previously (7, 13, 22). In brief, rabbit peritoneal macrophages were incubated with *T. pallidum* (10 organisms per macrophage) in the presence of 10% heat-inactivated (56°C for 30 min) NRS, IRS, or rabbit anti-TprK antisera. After incubation with *T. pallidum*, macrophages were washed, fixed, and processed for immunofluorescence microscopy using HSS and FITC-conjugated goat anti–human IgG as described previously. In a separate series of experiments, opsonophagocytosis assays were performed using IRS from which anti-TprK Ab had been removed by two sequential rounds of incubation with purified full-length rTprK. In the first round, 40 μg of pelleted, insoluble TprK protein was resuspended in 1 ml of pooled IRS as a colloidal solution and incubated for 2 h followed by two 30-min centrifugations at 100,000 *g* to remove rTprK and bound Ab. The supernatant was further depleted by incubation with 40 μg of rTprK immobilized on a nitrocellulose membrane (Schleicher & Schuell). After centrifugation at 25,000 g, the supernatant was quantitatively recovered. Depletion of TprK immunoreactivity was confirmed by ELISA using both the full-length (see Fig. 3 B, inset) and central domain (data not shown) recombinant proteins and by immunoblot analysis (data not shown).

Intradermal Challenge of TprK-immunized Rabbits

Rabbits were immunized over a 2-mo period with a 1:1 mixture of the TprK central domain (125 μg) and full-length protein (125 μg) per rabbit in Ribi Adjuvant (MPL + TDM + CWS; Corixa Corporation) according to the manufacturer's instructions. It should be noted that, except for the inclusion of full-length protein, this immunization protocol was identical to that of Centurion-Lara et al. (7). The protective capacity of TprK was evaluated in two separate experiments. In the first, two TprK-immunized rabbits and two serologically nonreactive control animals were challenged with 10^3 virulent treponemes by intradermal injection on their shaved backs at each of six sites. In the second experiment, identical challenges were administered to four TprK-immunized and four sham-immunized animals. Animals were examined daily to monitor the development, morphologic appearance, and progression of lesions which were scored for erythema, induration, and ulceration. Lesion aspirates were obtained at designated time points for darkfield microscopy and, in experiment two, for PCR amplification and sequence analysis of the *tprK* gene. At the termination of experiment one (90 d after challenge), the spleens or popliteal nodes were excised for rabbit infectivity testing (RIT; reference 23). After the development of orchitis or seroconversion, recipient animals were killed and treponemes were harvested as described above.

Nucleotide Sequence Analysis

TprK genes were amplified with primers K1-5′/Kseq-4 or TprK5′/TprK3′ (Table I) and the Expand High Fidelity PCR system (Boehringer). PCR was performed as follows: 94°C for 2 min followed by 35 cycles of 94°C for 10 s, 65°C for 10 s, 72°C for 2 min followed by a single terminal extension for 7 min at 72°C; products were TA cloned or purified using the Concert Rapid PCR purification system (Life Technologies). All DNAs were extracted with fresh reagents in a PCR-dedicated clean room. Sequencing was performed using the primers shown in Table I and with an Applied Biosystems Inc. model 377 automated DNA sequencer with the BigDye cycle sequencing kit.

Detection of Molecular Clocks Among Protein Variants

The likelihood ratio test (LRT), a computer algorithm which can detect molecular clocks in phylogenetic reconstructions (24, 25), was applied to alignments of TprK, TprJ, and the P1 protein of *Haemophilis influenzae*. Phylogeny trees were constructed with the maximum likelihood algorithm in PUZZLE (v4.0.2; reference 26) with and without a clock assumption, using the Dayhoff model of substitution (27), gamma-distributed rate heterogeneity, 10,000 puzzling steps, and neighbor-joining reconstruction.

Results

Simultaneous Transcription of tpr Genes

As a starting point for our study, we used limiting dilution RT-PCR to assess the transcription of *tprK* relative to other members of the *tpr* paralogous gene family and the abundantly expressed *flaA* gene. Before performing these experiments, we determined the amplification efficiency of each primer pair with limiting dilutions of *T. pallidum* DNA template. The resulting efficiency values (Table I) were used to correct the densitometric analyses of the RT-PCR products which were subsequently normalized to *flaA*. As shown in Fig. 1, transcripts for all *tpr* genes were detected, albeit at significantly lower levels than for *flaA* (all P values <0.001). The levels of transcript for *tprs C/D*, *E*, *G*, *H*, *J*, and *K* were not significantly different ($P > 0.05$), whereas transcripts for *tprs A*, *B*, *F*, *I*, and *L* were significantly less abundant than that for *tprK* ($P < 0.05$).

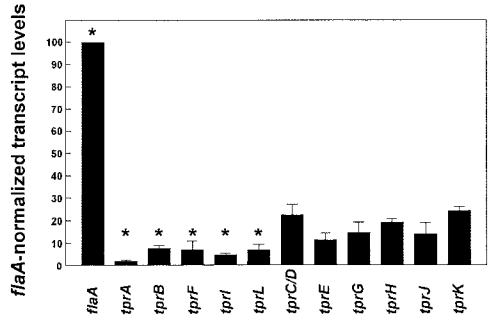

Figure 1. Quantitation of *flaA* and *tpr* transcript levels. Reactions were performed with limiting dilutions of RNA template and the RT-PCR primers listed in Table I; note that *tprC* and *tprD* are identical genes. Negative controls (RT-PCR of H_2O and PCR of RNA template) yielded no signal whereas PCR amplification of DNA produced the same sized product as RT-PCR of RNA (data not shown). Densitometric values of resolved products were obtained within the linear range of amplification, corrected for primer efficiency (Table I), and normalized to the *flaA* value obtained with the same amount of RNA. Column values represent means ± SEM, and asterisks indicate values significantly different than that of *tprK* ($P < 0.05$, Student's *t* test).

Identification of the TprK Translational Start

In the *T. pallidum* genomic sequence (5), the translational start for TprK was assigned to an ATG codon (Fig. 2 A). The predicted 505 residue polypeptide does not possess an NH_2-terminal leader peptide, a prerequisite for OM localization (16), but does contain a potential transmembrane domain at amino acids 36 through 48. Based on this sequence, the PSORT algorithm assigns TprK to the cytoplasmic membrane. Centurion-Lara et al. (7), on the other hand, proposed the GTG codon 75 bases downstream of the ATG as the protein's translational start. The resulting 480 residue protein contains a potential NH_2-terminal export signal with a signal peptidase I cleavage site, consistent with either a PP or OM location. Using this revised sequence, PSORT predicts either a PP or OM location with the former heavily favored (scores of 0.927 and 0.28 for PP and OM, respectively). Scores for known PP and OM proteins, FlaA (PP 0.783, OM 0.221) and *E. coli* OmpF (PP 0.381, OM 0.944), support the algorithm's predictive capacity. As the GTG start would allow TprK to potentially reside in the OM and the ATG start would preclude an OM localization, we next sought to determine the correct translational start of the protein. The low abundance of the native protein precluded obtaining NH_2-terminal sequence by automated Edman degradation. As an alternative strategy, we generated chimeras in which the *tprK* sequence distal to the leader peptide/transmembrane domain was fused in-frame with an alkaline phosphatase reporter construct lacking a leader peptide (19; Fig. 2). The chimeras contained decreasing lengths of upstream sequence such that the shortest insert (*tprK-phoA*3) began downstream of the ATG start predicted by Fraser et al. (5), but upstream of the GTG start predicted by Centurion-Lara et al. (7). Moreover, by cloning these constructs in both orientations relative to the *lac* promoter of pBluescript, we reasoned that we also would be able to identify any autonomously functioning TprK promoter(s). To ensure that the translation of PhoA fusions could initiate only from within TprK sequences, all three constructs contained either naturally occurring or engineered stop codons in-frame with the vector-encoded LacZ α-peptide. In the SK orientation (transcription driven by the *lac* promoter), all of the fusions expressed PhoA activity (Fig. 2 B), strongly supporting the translational start assigned by Centurion-Lara et al. (7) and the presence of an NH_2-terminal leader peptide. In the KS orientation (transcription driven by the native promoter), fusions TprK-PhoA1 and TprK-PhoA2 expressed PhoA activity, whereas fusion TprK-PhoA3, which contains only 15 bp of sequence upstream of the GTG codon, did not. This result indicates that the 78 bp of DNA deleted from TprK-PhoA3 relative to TprK-PhoA2 contains an autonomous promoter. Indeed, consensus −35 and −10 pro-

moter elements with a 17-bp spacer were identified within this stretch; immediately upstream from the GTG start codon is a consensus ribosomal binding site (Fig. 2 B).

Localization of Native TprK

Abs to TprK Do Not Promote Opsonization of Virulent Treponemes. Having shown that TprK contains a functional signal peptide, we next sought evidence that this protein is exposed to the extracellular milieu. To detect surface-exposed TprK, we assayed the ability of rabbit anti-TprK Ab to opsonize spirochetes for phagocytosis by rabbit peritoneal macrophages. In comparison with NRS, IRS significantly ($P < 0.05$) enhanced phagocytosis of motile treponemes, as reported previously (7, 13, 22). In contrast, sera pooled (or tested individually) from TprK-immunized animals did not promote phagocytosis (Fig. 3 A). We considered the possibility that anti-TprK Ab in IRS promote opsonophagocytosis but that these Abs differ qualitatively from those elicited by immunization with rTprK. Consequently, we first determined whether the IRS used for the opsonophagocytosis assays contains anti-TprK Ab. Compared with NRS, significant ($P < 0.05$) immunoreactivity against full-length rTprK was detected in the IRS by ELISA (Fig. 3 A, inset) and by immunoblot (data not shown), indicating that the protein does generate a humoral immune response during infection and that Ab derived from infection recognize rTprK. Not surprisingly, Ab levels were much greater in sera from the TprK-immunized animals ($P < 0.001$). Equivalent results were obtained when the central domain was used as Ag (data not shown). The opsonization assays then were repeated using IRS from which the anti-TprK Ab had been adsorbed (Fig. 3 B, inset). No significant differences in the opsonic activities of IRS and the same IRS depleted of anti-TprK Ab were observed (Fig. 3 B). When decreasing concentrations of sera were used to eliminate the possibility that trace amounts of residual anti-TprK Ab contributed to the opsonic activity of the adsorbed IRS, no significant differences were observed at comparable dilutions (Fig. 3 B).

TprK Is Ab Inaccessible in Intact Treponemes. As our opsonization assays failed to indicate surface exposure of TprK, we used an alternate strategy, immunofluorescence microscopy of spirochetes encapsulated in agarose beads, to examine the cellular location of TprK (13, 17). This highly sensitive technique preserves the integrity of the fragile *T. pallidum* OM during surface immunolabeling studies. Moreover, PP proteins can be detected by permeabilizing the OM to Ab with low concentrations of nonionic detergent. To better distinguish between intact and disrupted organisms, we developed a modified procedure in which encapsulated treponemes were probed simultaneously with rat anti-TprK antisera and rabbit Ab directed against the PP endoflagella (TpEf). In the absence of detergent, 90% of the treponemes were intact (indicated by a lack of labeling with anti-TpEf Ab), whereas all detergent-treated organisms had disrupted OMs. As expected from the low levels of *tprK* mRNA, detection of TprK required the use of two sequential conjugates, whereas TpEf was readily detected with a single conjugate. In three separate experiments involving a total of 1,200 organisms, not a single intact organism was labeled with anti-TprK Ab. Every spirochete labeled by anti-TprK Ab had a disrupted OM. Significantly, every disrupted spirochete was labeled with anti-TprK Ab suggesting that TprK is expressed by all treponemes. Representative micrographs are shown in Fig. 4.

The OM Protects TprK from PK. Although the above results were most consistent with TprK being PP, an alternative explanation is that our antisera lacked Abs against surface-exposed epitopes. For this reason, we also employed an Ab-independent method, PK accessibility, to as-

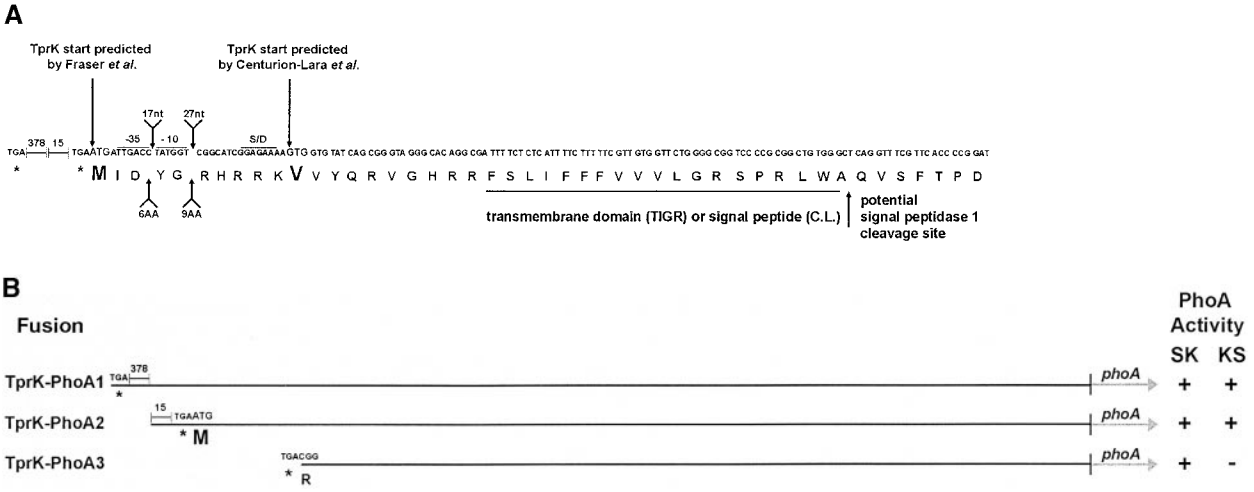

Figure 2. Identification of the TprK translational start and signal peptide. (A) Relevant features of the upstream DNA and 5′ coding region of *tprK* and the resulting NH_2 terminus of the protein. Bold amino acids (M and V) indicate the TprK translational starts predicted by Fraser et al. (reference 5) and Centurion-Lara et al. (reference 7). TIGR, The Institute for Genomic Research; C.L., Centurion-Lara et al. Asterisks indicate stop codons; nucleotide (nt) and amino acid (AA) sequences not shown are indicated by I-bars and two-tailed arrows. (B) Diagrams of the *tprK-phoA* plasmids and the PhoA activities they encode. With the exception of the engineered 5′ TGA stop codon of *tprK-phoA*3, the diagrams in B correspond to the *tprK* sequence shown in A.

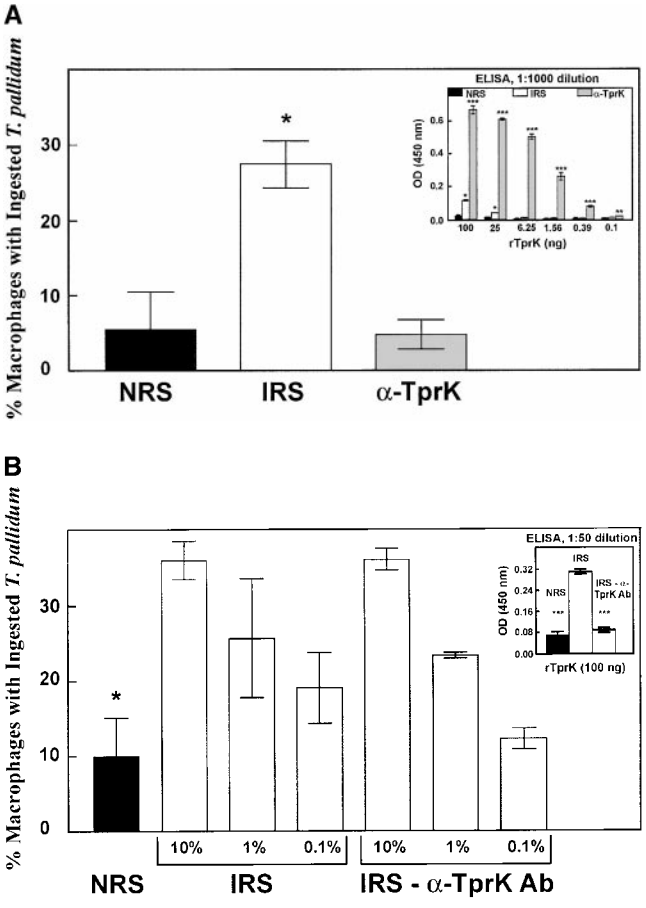

Figure 3. TprK is not an opsonic target in intact *T. pallidum*. (A) Phagocytosis of virulent treponemes by macrophages in the presence of 10% NRS, IRS, and rabbit anti-TprK (anti-TprK) antisera. (B) Opsonic potentials of 10% NRS and decreasing concentrations of IRS and the same IRS depleted of anti-TprK Ab. Asterisks in panel A indicate values significantly different those of NRS; in panel B asterisks indicate values significantly different from those obtained with the same concentration of IRS. In both panels, phagocytosis data are the mean percentage of cells (±SEM) with phagocytosed *T. pallidum* ($n = 3$). Panel insets indicate the levels of seroreactivity (mean OD ± SEM) against rTprK. Differences where considered significant by the Student's *t* test when $P < 0.05$, 0.01, or 0.001 (one, two, or three asterisks, respectively).

sess the cellular location of TprK. As shown in Fig. 5, exposure of motile *T. pallidum* to PK had no discernible effect on the abundance of either TprK or the PP proteins Tp47 and FlaA. In contrast, permeablization of the OM with 0.01% Triton X-100 rendered all three proteins susceptible to proteolytic digestion.

TprK Is Not an Integral Membrane Protein. As noted earlier, PSORT predicts that TprK is PP, whereas TMpred predicts that the protein has a cytoplasmic membrane–spanning domain. Although OM and PP proteins are difficult to distinguish at the sequence level, they possess distinctly different physical properties; membrane proteins are amphiphilic whereas PP proteins are hydrophilic. Therefore, we next used Triton X-114 phase partitioning (13, 28, 29) to determine whether TprK possesses the amphiphilicity characteristic of an integral membrane protein.

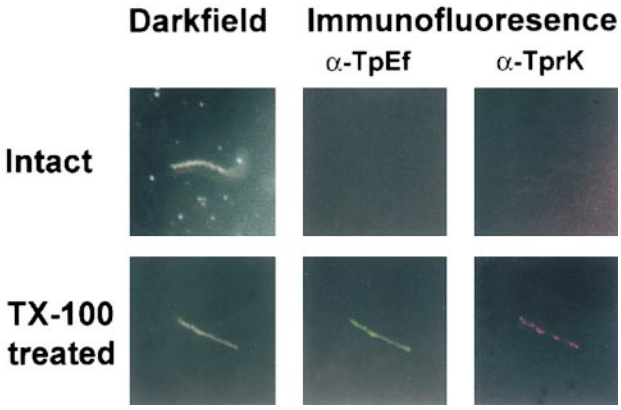

Figure 4. TprK is inaccessible to anti-TprK Ab in intact *T. pallidum*. Agarose-encapsulated spirochetes were simultaneously probed with rat anti-TprK and rabbit anti-TpEf Ab in the absence or presence of 0.05% Triton X-100. Treponemes were subsequently incubated with biotinylated goat anti–rat IgG followed by incubation with streptavidin-Alexa® 488 and goat anti–rabbit conjugated to Alexa® 546. Panels on the left show encapsulated spirochetes by darkfield microscopy; panels on the right show immunofluorescence of the same treponemes.

Freshly harvested *T. pallidum,* solubilized in 2% Triton X-114, was phase partitioned and the resulting fractions were analyzed by immunoblot with Ab directed against either TprK or Tp47. As reported previously (13, 29), approximately half of the Tp47 remained associated with the insoluble material, whereas the remainder was detected exclusively in the detergent-enriched phase (Fig. 6). In contrast, both rat anti-TprK antisera (Fig. 6) and the anti-TprK mAb 1-7H11.1G6 (data not shown) recognized a 47-kD polypeptide in the aqueous phase (Fig. 6), indicating that TprK is not an integral membrane protein. This result also indicates that the TprK leader peptide is cleaved because proteins with uncleaved leader peptides partition into the detergent-enriched phase (1, 14).

Immunization with TprK Does Not Confer Protective Immunity or Select for TprK Sequence Variants

Despite the evidence that TprK is periplasmic, an examination of its protective capacity appeared to be warranted in light of the previous report of TprK-induced partial protection (7). In two independent experiments involving a total of 12 rabbits, the time course for lesion development and resolution and the gross appearance of lesions were indistinguishable between 6 TprK-immunized, and 6 control animals. Typical examples of lesion development are shown in Fig. 7. TprK immunization also had no discernible effect on the presence of spirochetes within tissues. In the first experiment, spirochetes were recovered by RIT from spleen and/or lymph nodes from one of the two TprK-immunized and one of the two unimmunized rabbits. In the second experiment, lesion aspirates from two to three sites on each animal were obtained for darkfield microscopy 24 and 31 d after challenge. On day 24, seven of eight aspirates from the TprK-immunized animals were darkfield positive as opposed to eight of eight from the sham-immunized controls. All aspirates were darkfield positive on day 31.

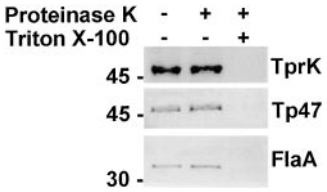

Figure 5. TprK is inaccessible to PK in intact *T. pallidum*. Spirochetes were left untreated, or treated with PK (0.4 mg/ml) with or without Triton X-100 (0.01%). Lysates were probed with rat anti-TprK, anti-Tp47, and anti-FlaA antiseras followed by development using enhanced chemiluminescence (TprK) or colorimetric (Tp47 and FlaA) methods. Molecular masses (kD) are indicated on the left.

As noted earlier, the discovery of variant *tprK* genes among geographically disparate *T. pallidum* strains and within some treponemal subpopulations has fueled speculation that host immune responses select for TprK sequence variants (8, 9). If this were the case, then TprK "escape variants" should be overrepresented among treponemes recovered from TprK-immunized animals. To examine this issue, we PCR amplified and sequenced *tprK* genes from our challenge strain (Nichols-Farmington), the isolates recovered by RIT in experiment one, and the spirochetes in the lesion aspirates from experiment two. As described in Materials and Methods, extensive precautions were taken to avoid PCR artifacts which would confound this analysis. As is the case for all TprK proteins reported to date, the Nichols-Farmington TprK was not an exact match for any other polypeptide in the databases (Fig. 8 A, and not shown). However, it was most similar to the Nichols-Seattle and Nichols-Houston TprKs with 97% amino acid identity to each. The 12 amino acid differences (10 substitutions and 2 deletions) between the Farmington and Seattle orthologs are located in four of the seven recently described TprK variable regions (i.e., V1, V3, V6, and V7; Fig. 8 A; references 8 and 9). The amino acid identity of the Nichols-Farmington TprK with the remaining orthologs in the databases ranged from 87 to 91% with the differences mapping to the seven variable regions. It is noteworthy that the PSORT algorithm heavily favors a PP location for all TprK orthologs (Fig. 8 A and data not shown). Downstream of the *tprK* coding region, the Nichols-Farmington strain contained the same 67-bp deletion reported for the Nichols-Seattle and Bal7 strains but which is absent from all other strains examined thus far (data not shown; references 8 and 9). In contrast to the heterogeneity among the geographically distinct TprK proteins, the TprK sequences of treponemes recovered from five different TprK-immunized animals were identical to that of Nichols-Farmington (Fig. 8 B). The sole sequence variant, with two amino acid changes in region V7, was recovered from an unimmunized control rabbit (Fig. 8 B).

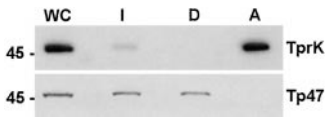

Figure 6. TprK does not possess the amphiphilicity typical of integral membrane proteins. *T. pallidum* was phase partitioned using Triton X-114. Whole cells (WC), Triton X-114–insoluble material (I), the detergent-enriched (D), and the aqueous phases (A) were subjected to immunoblot analysis with rat anti-TprK and anti-Tp47 antisera. Molecular masses (kD) are indicated on the left.

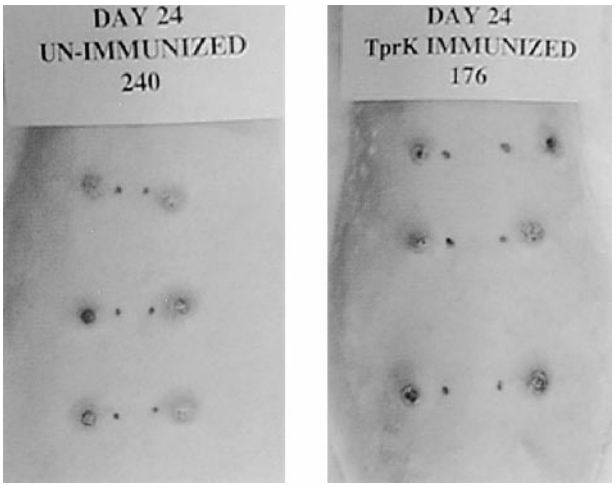

Figure 7. Immunization with TprK does not protect against experimental syphilis. Unimmunized and TprK-immunized rabbits were challenged by intradermal inoculation at six sites with 10^3 *T. pallidum* per site. Black ink marks are visible between the erythemous lesions. Photographs depict typical animals in one of two separate experiments involving a total of 12 rabbits.

As the above results suggested that *tprK* is not subject to immunological pressure, we next used the LRT to determine whether the variation among TprK sequences is consistent with evolutionary drift. LRT, a phylogenetic tool used in molecular evolutionary biology, can determine whether variation among related sequences has occurred at a constant or at a discontinuous rate (24, 25). The hypothesis underlying our LRT analysis of TprK was that proteins subject to immunological pressure would show a discontinuous rate of change and therefore not display a molecular clock. To assess this hypothesis, we tested for the presence of molecular clocks in TprK, TprJ, and the *H. influenzae* P1 protein. TprJ is predicted by PSORT to be anchored to the cytoplasmic membrane via an uncleaved leader peptide (5, 7); a protein sequestered in this manner should not be subject to immunological pressure. In this regard, TprJ has been shown to be stable in *T. pallidum* Nichols for >1 yr despite repeated passage in rabbits (30). P1 is an OM protein with surface-exposed hypervariable domains which are known to be subject to immunological selection (31). Consistent with our hypothesis, the molecular clock was rejected for the immunologically driven variation of P1 (Table II). Both TprJ and TprK displayed a molecular clock (Table II) indicating constant rates of change consistent with evolutionary drift.

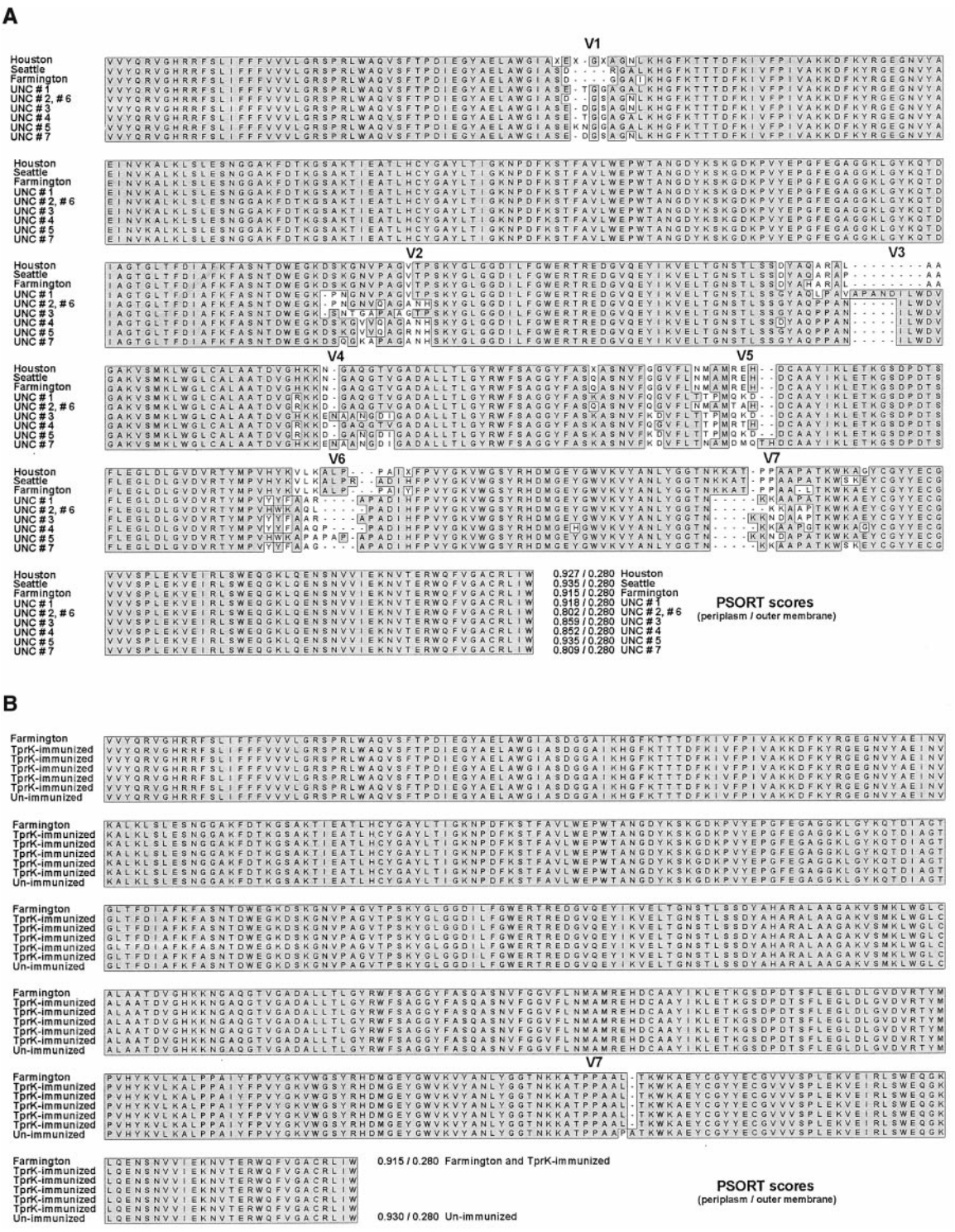

Figure 8. Immunization with TprK does not select for TprK sequence variants. (A) Alignment of geographically distinct Nichols strain TprKs. UNC indicates the University of North Carolina. The seven discrete regions of variability (V1–V7) are shown above the aligned proteins. (B) Alignment of Nichols-Farmington TprK with the TprKs of treponemes recovered from five different TprK-immunized rabbits and one unimmunized rabbit which had been challenged with Nichols-Farmington *T. pallidum*. The TprK of spirochetes recovered from an unimmunized rabbit displayed variability in the V7 region. PSORT scores are shown at the COOH termini. Sequences of the *tprK* gene of Nichols-Farmington *T. pallidum* and of treponemes recovered from challenged rabbits are available from GenBank/EMBL/DDBJ under accession nos. AF343915–AF343921.

Table II. *Likelihood Ratio Analysis for Molecular Clocks Among Protein Variants*

Protein	Variants*	Log L_0‡	Log L_1‡	−2 Log Λ‡	df§	*P* value	Molecular clock‖
T. pallidum TprJ	Nichols-Houston SS14 nos. 1–3	−2213.24	−2213.01	0.47	2	0.7923	Accepted
H. influenzae P1	BCH-3 BCH-2 1-H-1085 22-H-1154 4-H-1094 13-H-1157 4-H-1093	−2440.01	−2428.80	22.43	7	0.0004	Rejected
T. pallidum TprK	Nichols-Houston SS14 nos. 1–7 Bal 73-1 no. 134 Bal 7 no. 124, no. 126 Sea 81-4 no. 120, no. 121 Nichols-UNC nos. 1–7	−3580.59	−3571.28	18.63	17	0.3500	Accepted

*For the *T. pallidum* alignments, the Nichols-Houston sequences were used as out-groups, whereas the BCH-3 sequence was used as an out-group for the *H. influenzae* P1 alignment.
‡As a molecular clock hypothesis is nested within a more general assumption of nonclock-like evolution, the LRT equation can be resolved to the test value; −2(Log Λ), where Log Λ = Log L_0 − Log L_1. Log L_0 is the maximum likelihood value derived for a tree that assumes a molecular clock, whereas Log L_1 is the comparable value derived for a tree that allowed for nonclock-like rates of change.
§Degrees of freedom equals the number of sequences minus two.
‖Clock-like evolution was rejected at a statistical significance of $P < 0.05$.

Discussion

Several years ago, in an effort to facilitate the identification of syphilis vaccine candidates, we enumerated the fundamental properties which bona fide *T. pallidum* rare OM proteins ought to possess (32). Many of these characteristics are intrinsic to gram-negative bacterial OM proteins: a signal peptidase I–cleavable export signal, an absence of long stretches of hydrophobic residues within the mature polypeptide, an overall amphiphilic nature, and susceptibility to surface-specific labeling techniques. The remaining properties, low abundance and poor immunogenicity, are unique to *T. pallidum* and reflect the limited reactivity of the spirochete's surface with the specific Abs in human and rabbit syphilitic sera (17, 20) and the paucity of intramembranous particles observed when the *T. pallidum* OM is examined by freeze-fracture electron microscopy (33). With the availability of the bacterium's genomic sequence and the aid of computer algorithms, *T. pallidum* polypeptides now can be rapidly screened for potential OM proteins. Nevertheless, such predictions still must be confirmed empirically using techniques which examine both the protein's physical properties and its cellular location (32).

TprK meets some of these criteria and, therefore, is a viable candidate OM protein. It is predicted (7), and has been shown here, to have a signal peptide which is likely cleaved. The mature polypeptide does not contain significant stretches of hydrophobic residues. The relatively low levels of transcript detected by RT-PCR, coupled with the need for enhanced chemiluminescence in order to detect the native protein on immunoblots, point to its low abundance in *T. pallidum*. Also noteworthy was the meager fourfold increase in rTprK-ELISA reactivity of IRS compared with NRS, a result indicating that the protein is poorly immunogenic during acquired experimental infection. Significantly, TprK is related to the *T. denticola* OM protein, Msp (7). Despite these findings, TprK has both computer-predicted and empirically revealed properties which are irreconcilable with an OM location. As noted earlier, PSORT scores for the protein heavily favor a PP location. The observations that the protein has a functional signal peptide, partitions into the Triton X-114 aqueous phase, and becomes susceptible to Ab and PK only after perturbation of the OM strongly indicate that TprK resides entirely within the PP space.

To maximize the potential for observing TprK-mediated protection, we immunized rabbits with the central domain as well as an equivalent amount of the full-length protein. Additionally, we used a 100-fold lower challenge dose (i.e., 10^3 treponemes per site) than was used by Centurion-Lara et al. (7), which should have biased the experiment in favor of a protective effect. Nevertheless, in two independent experiments we failed to observe any alteration of lesion development after challenge of TprK-immunized rabbits. How, then, can these observations be reconciled with the previously reported partial protection results (7)? We can only speculate that minor differences in challenge protocol

resulted in these conflicting levels of protection. Precedent for such disparate findings is provided by another PP protein, the treponemal glycerophosphodiester phosphodiesterase (GlpQ) homologue. Like TprK, GlpQ was initially identified as a candidate opsonic target by immunologically screening an expression library in duplicate with IRS and with a nonopsonic sera raised against heat-killed *T. pallidum* (4, 7). Immunization with rGlpQ was reported to confer partial protection to homologous *T. pallidum* challenge, although, contrary to expectations, anti-rGlpQ Ab were not opsonic (12). Subsequently, using techniques similar to those described here, we demonstrated that native GlpQ is a lipoprotein anchored to the PP leaflet of the cytoplasmic membrane and that immunization with rGlpQ failed to induce either opsonic Ab or protective immunity (13). Thus, we can only reiterate that, in our hands, for two different proteins, both the lack of opsonic activity and protective immunity correlated with Ab inaccessibility.

As Abs do not have access to TprK within live *T. pallidum*, the *tprK* gene should not be subject to the immunological pressure applied to genes encoding surface-exposed proteins. Consistent with this notion, the *tprK* gene remained stable after challenge of rabbits with preexisting humoral immunity to TprK. Significantly, variation of TprK displays a molecular clock, whereas the immunologically driven variation of the *H. influenzae* OM protein P1 does not. As variation of TprK does not appear to be immunologically driven, the significance of the TprK variable regions (V1 to V7) remains to be determined. In light of our findings, we postulate that this variability represents evolutionary change in the regions of TprK in which strict sequence conservation is not critical for maintenance of the protein's physiological function(s).

As much of the thinking concerning TprK has been guided by the protein's homology with Msp, the finding that Tprk is periplasmic raises an apparent conceptual inconsistency. How can orthologs reside in different cellular compartments? Prior studies held that Msp was porin like, extensively surface exposed, and formed hexagonal arrays in the *T. denticola* OM (6). However, prompted by the discovery of the Tpr family in *T. pallidum*, we recently reexamined the membrane topology of Msp. In contrast to previous reports, we found that (a) the *T. denticola* OM does not contain a hexagonal array, (b) the array-like structure visualized in previously published electron micrographs (6) was most likely the spirochete's peptidoglycan sacculus, and (c) the majority of the Msp molecule is periplasmic with only limited regions protruding through the OM to the treponemal surface (15). Based on these more recent findings, one can envision that the significant amino acid differences between Msp and TprK (26% identical, 39% similar) allow one or more small domains of Msp to adopt the β-strand structure required for OM insertion whereas TprK would lack the comparable sequences. Moreover, this minor variance in localization could allow the presumably similar PP functions of Msp and TprK to be maintained. Despite our findings for TprK, this formulation suggests that other members of the Tpr family merit further investigation as potential rare OM proteins. Although preliminary PhoA fusion analyses indicates that all but TprA, TprB, and TprH possess NH_2-terminal export signals (data not shown), most of the remaining Tpr proteins are predicted to reside in the PP either free or anchored to the cytoplasmic membrane by an uncleaved leader peptide (5). TprF and TprI appear to be notable exceptions in that they have favorable PSORT scores for OM localization.

The regions of homology among the *tpr* genes, coupled with the putative surface exposure of a subset of Tpr proteins, has invited speculation that the Tprs represent a recombination-based system of antigenic variation which contributes to the relapsing nature of syphilis (5, 7). However, the existing data do not support this notion. Unlike other recombination-based antigenic variation systems in which a single allele is expressed at a given time, both we and others (10) have found that all the *tpr* genes are simultaneously expressed albeit at different levels. Although one could postulate that this represents a mixture of subpopulations each expressing a different Tpr, our finding that every disrupted spirochete examined by immunofluorescence was labeled by anti-TprK Abs is inconsistent with this hypothesis. Moreover, although recombination between subsets of the *tprs* is not conceptually impossible, multiple sequences of *tprJ* and *tprK*, which share homology with *tprs G* and *E*, and *tprs A, B, H, L*, respectively, show no evidence of recombination among these genes (8, 9). Furthermore, we have shown that *tprK* remains entirely unaltered despite propagation in TprK-immunized animals. To search for chimeric *tpr* genes, we have begun using long-range PCR with primers specific for the upstream and internal sequences in a checkerboard pattern (i.e., *tprA* upstream forward with *tprB* internal reverse). With a sensitivity of 1 recombinant gene in 10,000 spirochetes, preliminary results offer no evidence for *tpr* recombination in either Nichols-Farmington *T. pallidum* or in treponemes recovered from challenged animals (unpublished observations).

We are grateful to Dmitriy Shevchenko and Donald Ammerman for technical assistance with the RT-PCR and PhoA fusion analyses.

This research was supported in part by U.S. Public Health Service grants AI-26756 and AI-38894 to J.D. Radolf. K.R.O. Hazlett was supported by National Research Service Award (NRSA) postdoctoral fellowship AI-09973. T.J. Sellati was supported by NRSA postdoctoral fellowship AI-10573 and a research fellowship from the Arthritis Foundation.

Submitted: 6 February 2001
Revised: 21 March 2001
Accepted: 26 March 2001

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