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Ben Adler *Editor*

Spirochete Biology: The Post Genomic Era

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Spirochete Biology: The Post Genomic Era

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Preface

Spirochetes form a fascinating group of bacteria that are usually considered together because of their common helical cellular morphology. Despite this similarity, their biology is very diverse and they occupy a large array of ecological niches. Some members are free living saprophytes, others exist both in the environment and intimately associated with animal hosts, while some have developed such a close relationship with their animal hosts that they are unable to survive independently. Some spirochetes have simple lifestyles, while others infect vertebrate and invertebrate hosts as part of their life cycle. Accordingly, many spirochetes are pathogens and cause a variety of serious human and animal diseases, including syphilis, leptospirosis, Lyme borreliosis, relapsing fever borreliosis, periodontal diseases, digital dermatitis and dysentery.

The understanding of the biology of these bacteria has lagged some way behind what is known for most other species of bacteria of medical, veterinary, agricultural or environmental importance. This gap in our knowledge was due to largely to the fact that until the last couple of decades, genetic manipulation of spirochetes was not possible. While still relatively inefficient, directed mutation of many species of spirochetes is now much easier and in some cases routine.

A further important advance has been the availability, and now routine determination, of whole genome sequences. Currently many hundreds of spirochete genome sequences are in the public domain. These and other developments have resulted in major improvements in the understanding of spirochete biology in the last 10 years.

This volume is not intended to be a comprehensive treatise on spirochete biology. Clearly that would be impossible in the space available. Rather, some key aspects of the interaction of pathogenic spirochetes with their hosts are presented by a group of international experts in the field. There is of necessity some overlap between chapters. That is unavoidable, but also desirable, in that each chapter can be read on a stand-alone basis, with reference to other chapters where appropriate.

Finally, I would like to express my appreciation to all of the contributors to this volume. You are all valued members of my second family, the spirochete family.

Melbourne, Australia — Ben Adler

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Richard T. Marconi

The *Treponema pallidum* Outer Membrane

Justin D. Radolf and Sanjiv Kumar

Abstract The outer membrane (OM) of *Treponema pallidum*, the uncultivatable agent of venereal syphilis, has long been the subject of misconceptions and controversy. Decades ago, researchers postulated that *T. pallidum*'s poor surface antigenicity is the basis for its ability to cause persistent infection, but they mistakenly attributed this enigmatic property to the presence of a protective outer coat of serum proteins and mucopolysaccharides. Subsequent studies revealed that the OM is the barrier to antibody binding, that it contains a paucity of integral membrane proteins, and that the preponderance of the spirochete's immunogenic lipoproteins is periplasmic. Since the advent of recombinant DNA technology, the fragility of the OM, its low protein content, and the lack of sequence relatedness between *T. pallidum* and Gram-negative outer membrane proteins (OMPs) have complicated efforts to characterize molecules residing at the host–pathogen interface. We have overcome these hurdles using the genomic sequence in concert with computational tools to identify proteins predicted to form β-barrels, the hallmark conformation of OMPs in double-membrane organisms and evolutionarily related eukaryotic organelles. We also have employed diverse methodologies to confirm that some candidate OMPs do, in fact, form amphiphilic β-barrels and are surface-exposed in *T. pallidum.* These studies have led to a structural homology model for BamA and established the bipartite topology of the *T. pallidum* repeat (Tpr) family of proteins. Recent bioinformatics has identified several structural orthologs for well-characterized Gram-negative OMPs, suggesting that the *T. pallidum* OMP repertoire is more Gram-negative-like than previously supposed. Lipoprotein adhesins and proteases on the spirochete surface also may contribute to disease pathogenesis and protective immunity.

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1 Molecular Architecture of the *T. pallidum* Cell Envelope

1.1 Experimental Obstacles

Venereal syphilis is a sexually transmitted infection renowned for its protean clinical manifestations and protracted natural history (Radolf et al. 2014), both of which reflect the extraordinary invasiveness and immunoevasiveness of its etiologic agent, *Treponema pallidum* subsp. *pallidum* (hereafter referred to as *T. pallidum*) (Lafond and Lukehart 2006; Radolf et al. 2016). It is also a disease that well into the genomics era presents extraordinary challenges to investigators attempting to unravel its many enigmas (Ho and Lukehart 2011; Radolf et al. 2016). *T. pallidum* is one of the few major bacterial pathogens of humans that cannot be propagated continuously in artificial medium (Ho and Lukehart 2011; Norris et al. 2001; Radolf et al. 2016). As they have for decades, investigators must employ intratesticular inoculation of rabbits to isolate and propagate the spirochete (Lukehart and Marra 2007). Because *T. pallidum* cannot be genetically manipulated, experimentalists are restricted primarily to protein-based methodologies to confirm findings and evaluate hypotheses originating from genetic and genomic data. Since the advent of recombinant DNA technology, the fragility of the *T. pallidum* outer membrane (OM) and its low protein content have served as twin confounders of efforts to characterize molecules residing at the host–pathogen interface (Cameron 2006; Radolf 1995; Radolf et al. 2016).

1.2 Historical Misconceptions

Researchers have long appreciated the importance of the *T. pallidum* surface in determining the waxing and waning course of syphilis (Radolf et al. 2006). They also have had to overcome a number of misconceptions to clarify its role in disease pathogenesis. Decades ago, investigators recognized that live (i.e., motile) spirochetes react poorly with the antibodies in patient sera (Nelson and Mayer 1949), and they assumed that this property relates to the pathogen's capacity for immune evasion and persistence (Hardy and Nell 1957; Turner and Hollander 1957). To explain the spirochete's poor surface antigenicity, the notion evolved that the bacterium acquires a protective coat of serum proteins and host-derived mucopolysaccharides (Alderete and Baseman 1979; Christiansen 1963; Fitzgerald and Johnson 1979). In 1973, the existence of the OM was established unequivocally by transmission electron microscopy (TEM) of ultra-thin sectioned, plastic-embedded organisms (Johnson et al. 1973). The ability to express *T. pallidum* antigens in *Escherichia coli* (Norgard and Miller 1983; Stamm et al. 1982; Walfield et al. 1982), the major breakthrough of the early 1980s, attracted to the field many talented molecular biologists intent upon using this powerful new technology to develop a syphilis vaccine. However, these investigators naively assumed that the physical properties and protein content of the syphilis spirochete's OM are similar to those of *E. coli* (Radolf et al. 2006). They also incorrectly assumed that treponemal proteins strongly recognized by the human or rabbit syphilitic sera used to screen recombinant libraries were likely to be surface-exposed in *T. pallidum* (Radolf et al. 2006). The result was the discovery of many notable treponemal antigens, mostly lipoproteins of unknown function at the time, but no OMPs (Cameron 2006; Radolf et al. 2006, 2016).

1.3 The Outer Membrane Hypothesis

As our work with recombinant *T. pallidum* proteins progressed during the 1980s and early 1990s, several observations led us to question prevailing views about the existence of the outer coat and the nature of the spirochete's OM. First, while clumps of testicular debris often were observed in proximity to organisms in negatively stained preparations viewed by TEM, a continuous outer coat or layer was not discernible (Hovind-Hougen 1983; Radolf et al. 1986). Subsequently, we confirmed these findings by radioimmunoassay of freshly harvested treponemes collected onto low-protein-binding polycarbonate filters; only negligible amounts of surface-adsorbed immunoglobulins or serum proteins were detected (Cox et al. 1992). Second, it was noted by routine negative staining that the OM was easily disrupted

by routine experimental manipulations, such as centrifugation and suspension, or exposure to low concentrations of non-ionic detergents (Cox et al. 1992; Radolf et al. 1988), conditions that do not perturb the OMs of Gram-negative bacteria. Third, removal of OMs using low concentrations of the non-ionic detergent Triton X-114 (TX114) did not result in an appreciable loss of major membrane immunogens detected by immunoblot analysis with syphilitic serum (Fig. 1a) (Radolf et al. 1988). Finally, organisms lacking OMs showed markedly greater reactivity with syphilitic sera than intact treponemes (Cox et al. 1992, 1995; Radolf et al. 1988).

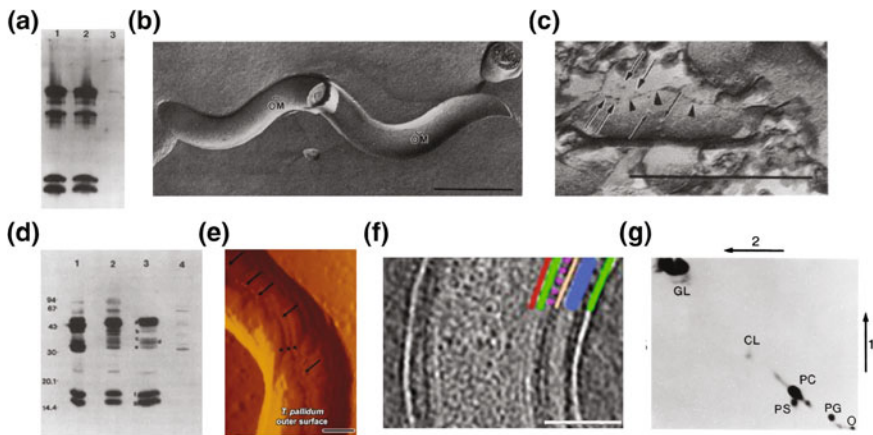

Fig. 1 The *T. pallidum* cell envelope. **a** *T. pallidum*'s major immunogens are associated with the protoplasmic cylinder, not the outer membrane. Reactivity with human syphilitic serum of proteins extracted with Triton X-114 from whole *T. pallidum* cells (*lane 1*), protoplasmic cylinders (*lane 2*), and solubilized outer membranes (*lane 3*); reproduced from reference (Radolf et al. 1988). **b** Freeze-fracture EM reveals scarce intramembranous particles (IMPs) within the *T. pallidum* OM. Convex and concave leaflets of the OM are indicated. Bar, 0.5 µM. Reproduced from reference (Radolf et al. 1994). **c** Deep etching reveals that OM intramembranous particles are surface-exposed. *Arrowheads* indicate the boundaries separating the bacterial surface from the convex fracture face. Particles on the convex fracture face and the treponemal surface are indicated by thin and medium-thickness arrows, respectively. Bar, 0.5 µM. Reproduced from reference (Bourell et al. 1994). **d** TX-114 phase partitioning reveals that the syphilis spirochete's major immunogens (based on reactivity with human syphilitic serum) possess hydrophobic character. Lanes: 1. Percoll-purified *T. pallidum*. 2. TX-114-insoluble material. 3. TX114 detergent-enriched phase proteins. 4. aqueous phase proteins. Reproduced from Reference (Radolf et al. 1988). **e** Scanning probe microscopy reveals rare particles on the *T. pallidum* surface; reproduced with permission from reference (Liu et al. 2010). **f** Cryoelectron microscopy (*longitudinal slice*) showing, from the inside out, cytoplasmic filaments (*red line*), cytoplasmic membrane (*green line*), lipoprotein layer (*purple circles*), peptidoglycan layer (*tan line*), flagellar filament (*thick blue line*), and outer membrane (*green line*). Bar, 50 nM. Reproduced with permission from reference (Liu et al. 2010). **g** [^{3}H]palmitate-labeled lipids were extracted from isolated *T. pallidum* outer membranes and separated by two-dimensional thin layer chromatography. *GL* glycolipids; *CL* cardiolipin; *PC* phosphatidylcholine; *PS* phosphatidylserine; *PG* phosphatidylglycerol; *O* origin. Reproduced from reference (Radolf et al. 1995b)

1.4 Rare Outer Membrane Proteins

Collectively, the above findings led us to hypothesize that the spirochete's fragile OM, not an outer coat, serves as the barrier to antibody binding. Of course, to do so, it would need to have a much lower protein content than conventional Gram-negative bacterial OMs. The question, then, was how to prove this unorthodox idea given how little was known at the time about the molecular architecture and composition of the *T. pallidum* cell envelope. Freeze-fracture EM provided part of the solution. This 'OMP-agnostic' technique revealed that the density of integral membrane proteins (visualized as intramembranous particles, IMPs) in the *T. pallidum* OM is ~100-fold less than that of *E. coli* OMs (Fig. 1b) (Radolf et al. 1989b; Walker et al. 1989). A variant of the freeze-fracture technique, deep etching, showed that these low-abundance particles protrude from the spirochete's surface (Fig. 1c) (Bourell et al. 1994; Radolf et al. 1989b) and, therefore, can interact directly with host cells, tissue components, and circulating molecules, including antibodies. Subsequent efforts to molecularly characterize these morphological entities became known as "the quest for *T. pallidum* outer membrane proteins" (Radolf 1995).

1.5 Lipoprotein Immunogens

TX114 phase partitioning led to the other major piece of the surface antigenicity riddle, which continues today—the identification and localization of the syphilis spirochete's lipoprotein immunogens (Chamberlain et al. 1989a). This technique, developed by Bordier in the 1980s for isolating membrane-associated proteins (Bordier 1981), exploits the relatively low cloud point (~20 °C) of TX114 (Brusca and Radolf 1994). Above the cloud point, TX114 micelles become too large to remain in suspension and can be collected by centrifugation. Membrane proteins incorporated into TX114 micelles at the low temperatures used for solubilization will pellet with the heavier detergent-enriched phase after warming, leaving water-soluble proteins behind in the lighter, aqueous phase. This simple but extremely powerful method revealed that the syphilis spirochete's major immunogens, as determined by reactivity with syphilitic sera, possess hydrophobic character (*i.e.,* they were recovered in the detergent-enriched phase) (Fig. 1d) (Chamberlain et al. 1989a; Radolf et al. 1988). Within the next several years, DNA sequencing determined that these highly immunogenic membrane proteins are synthesized with signal peptides terminated by lipid modification motifs (Akins et al. 1993; Becker et al. 1994; Purcell et al. 1990; Swancutt et al. 1990; Weigel et al. 1992), now referred to as "lipoboxes" (Setubal et al. 2006). These genetic findings were corroborated at the protein level (i) by radiolabeling of polypeptides in *T. pallidum* and/or in *E. coli* with [^{14}C] or [^{3}H]palmitate (Akins et al. 1993; Chamberlain et al. 1989a, b; Purcell et al. 1990; Swancutt et al. 1990); (ii) in some

cases, by recovery of radiolabeled fatty acids in the expected 2:1 (ester-to-amide) ratio following sequential alkaline and acid hydrolysis (Chamberlain et al. 1989a; Swancutt et al. 1990); and/or (iii) by showing that processing of native lipoproteins in *T. pallidum* or lipoproteins expressed in *E. coli* was prevented by globomycin (Purcell et al. 1990; Swancutt et al. 1990), a specific inhibitor of signal peptidase II, the enzyme that cleaves the signal peptides of lipoproteins at the lipid-modified cysteine residue (Tokunaga et al. 1984). Importantly, in contrast to the lipid-modified proteins, recombinant lipoproteins without their N-terminal acylation signals partitioned into the TX114 aqueous phase (Akins et al. 1993; Chamberlain et al. 1989b; Purcell et al. 1990; Swancutt et al. 1990), demonstrating that the hydrophobic character and membrane association of native lipoproteins were due to their lipid moieties. Parallel freeze-fracture EM experiments showed that, in contrast to proteins with transmembrane domains (e.g., bacteriorhodopsin and bovine rhodopsin), lipoproteins incorporated into liposomes do not form IMPs (Jones et al. 1995). Together, these results indicated that the protein moieties of lipoproteins are extrinsic to the lipid bilayer and, by extrapolation, that the particles observed in freeze-fractured OMs could not be lipoproteins. Immunoelectron microscopy (IEM) and immunofluorescence analysis (IFA) using antisera generated against numerous recombinant lipoproteins (Tpp47 [TP0574], Tpp15 [TP0171], Tpp17 [TP0435], Tpp34 [TP0971], GlpQ [TP0257]) revealed that none of these immunogens could be detected on the spirochete's surface and, instead, were localized to the periplasmic compartment (Cox et al. 1992, 1995; Deka et al. 2007; Shevchenko et al. 1999). Crucial for these localization experiments was our development of the gel microdroplet method (see below) as a means of maintaining the integrity of the fragile *T. pallidum* OM throughout the labeling process (Cox et al. 1995; Luthra et al. 2015b).

1.6 A Model for the T. pallidum *Cell Envelope*

By the mid-1990s, it was possible to integrate the above information into a model for the *T. pallidum* cell envelope that explains the spirochete's poor surface antigenicity in vitro and its stealth pathogenicity in vivo (Cox et al. 1992; Radolf 1995). The model has two basic components: (i) the OM contains a paucity of integral membrane proteins and surface-exposed lipoproteins and (ii) the preponderance of the spirochete's major membrane immunogens are lipoproteins, with most tethered by their N-terminal lipids to the periplasmic leaflet of the CM. Over the past two decades, data obtained using electron microscopy, biochemistry, and structural biology have supported the model's validity. Consistent with the freeze-etch results, scanning probe microscopy of *T. pallidum* directly visualized sporadic particles on an otherwise smooth bacterial surface (Fig. 1e) (Liu et al. 2010). Cryoelectron microscopy (CryoEM) visualized the native *T. pallidum* OM as a simple lipid bilayer (Fig. 1f) (Izard et al. 2009; Liu et al. 2010), quite unlike that of *Borrelia burgdorferi*, whose external surface possesses an easily discernible proteinaceous

layer (Liu et al. 2009). In accord with the notion of a dense array of lipoproteins tethered to the CM's periplasmic leaflet, cryoEM revealed protein "studs" aligned above the CM and below the PG layer (Liu et al. 2010) (Fig. 1f). Tp47, the first *T. pallidum* protein shown to be lipid-modified (Chamberlain et al. 1989b), was found to be a penicillin-binding protein with DD-carboxypeptidase activity involved in PG remodeling (Deka et al. 2002; Weigel et al. 1994), whereas other lipoprotein immunogens are proven substrate-binding proteins (SBPs) for ABC transporters that shuttle a variety of nutrients across the CM (Becker et al. 1994; Brautigam et al. 2016; Deka et al. 2004a, b, 2006, 2013; Machius et al. 2007; Porcella et al. 1996).

2 The Quest for *T. pallidum* Outer Membrane Proteins

2.1 Isolation of Outer Membranes

Prior to the availability of the genomic sequence, isolation of *T. pallidum* OMs seemed the most straightforward approach to identifying rare OMPs (Blanco et al. 1994; Radolf et al. 1995b). The underlying assumption was that rare OMPs enriched in the OM fraction could be identified by SDS-PAGE in combination with peptide sequencing or mass spectrometry and subsequently cloned. Though rational in concept, the results were profoundly disappointing. The most abundant protein in the OM preparations, originally designated *T. pallidum* rare outer membrane protein 1 (Tromp1) (Blanco et al. 1995), was shown by metal analysis and X-ray crystallography to be the SBP for a transition metal ABC transporter (Deka et al. 1999; Lee et al. 1999, 2002) and, therefore, could not be an OMP. Other OM-enriched proteins were obvious periplasmic contaminants (Shevchenko et al. 1997). Isolation of OMs did, however, yield one valuable dividend—determination of the membrane's lipid composition. The *T. pallidum* OM consists principally of phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, and an uncharacterized, poorly immunogenic glycolipid (Fig. 1g) (Radolf et al. 1995b). This lipid profile differs greatly from those of the *E. coli* (Silhavy et al. 2010) and *B. burgdorferi* OMs (Radolf et al. 1995a). Notably absent was lipopolysaccharide (LPS), the highly proinflammatory glycolipid responsible for creating the OM permeability barrier in Gram-negatives (Nikaido 2003), subsequently confirmed by the genomic sequence (Fraser et al. 1998). The lack of LPS likely explains the relative permeability of the *T. pallidum* OM to long-chain fatty acids (LCFAs) compared to that of *E. coli* (Cox and Radolf 2001). It also helps to explain why Toll-like receptor (TLR)-based innate immune surveillance mechanisms (Kawai and Akira 2010) fail to detect hematogenously disseminating bacteria early during the disease (Radolf et al. 2006) as well as the absence of sepsis-like symptomatology in demonstrably spirochetemic secondary syphilis patients (Cruz et al. 2010; Radolf et al. 2014).

2.2 β-Barrel Predictions

As the 1990s ended, the need for a new line of attack became obvious. The genomic sequence (Fraser et al. 1998) provided the means for this renewed assault, but not without some twists. One surprise was that the spirochete's genome did not encode orthologs for any well-studied OMPs. Equally unexpected was the finding that it encodes a 12-member paralogous family, designated the *T. pallidum* repeat (Tpr) family, whose members have sequence homology to the major outer sheath protein (MOSP) of *Treponema denticola* (Centurion-Lara et al. 1999; Fraser et al. 1998), a known pore-forming protein and adhesin (Anand et al. 2013; Egli et al. 1993; Ellen 2006). The question for investigators, then, was how to take advantage of this powerful new tool to solve the OMP problem. Structural biology eventually provided a solution in the form of the β-barrel, the hallmark conformation of OMPs in all organisms with OMs as well as eukaryotic organelles derived from them (e.g., mitochondria and chloroplasts) (Wimley 2003). By the mid-2000s, algorithms for identifying proteins predicted to form β-barrels with acceptable false-discovery rates were available. As an alternative to unproductive genome mining for sequence orthologs, we devised a consensus computational framework that used a battery of subcellular localization and β-barrel structural prediction tools to identify and rank candidate OMPs (Cox et al. 2010). Recently, using additional β-barrel prediction algorithms (Markov Chain Model for Beta Barrels [MCMBB]) (Bagos et al. 2004b) and Transmembrane β-barrel proteome database [TMBB-DB] (Freeman and Wimley 2012)), along with structural modeling (Swiss-Model and I-TASSER) (Biasini et al. 2014; Yang and Zhang 2015), and domain identification tools (Conserved Domain Database [CDD], pfam, and InterProScan) (Jones et al. 2014), we have modified and expanded the predicted OMPeome of *T. pallidum* using the Nichols strain as our reference genome (see Table 1 for our current list). Of note, this list should apply to all other syphilis spirochete strains, given their remarkably low degree of sequence divergence (Arora et al. 2016; Smajs et al. 2012). The candidates fall into two classes: *T. pallidum* repeat proteins (Tprs) and a group of unrelated proteins, most of which are annotated as hypotheticals. Despite their lack of sequence homology, the 'hypotheticals' appear to be structural and, presumably, functional orthologs of well-characterized Gram-negative bacterial OMPs. Collectively, these findings suggest that *T. pallidum* is more Gram-negative-like than previously supposed and that the appearance of Gram-negative-like OMs predated the evolution of proteobacteria.

2.3 Establishing Authenticity–Biophysical Properties

Bioinformatics is only the starting point for proving that a candidate is a *bona fide* β-barrel-forming OMP. Establishing authenticity requires demonstrating that a protein has the biophysical properties expected of an OMP and is surface-exposed

Table 1 The predicted *Treponema pallidum* OMPeome

TP_ID	Protein annotation	Conserved domains	Structural similarity (PDB ID)	Proposed function	References
TP0011	TPR protein B (TtprB)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013)
TP0117	TPR protein C (TprC)	MOSP^C and MOSP^N	None	Porin	Anand et al. (2012, 2015), Centurion-Lara et al. (1999, 2013), Gray et al. (2006), Sun et al. (2004)
TP0131	TPR protein D (TprD)	MOSP^C and MOSP^N	None	Porin	Anand et al. (2012, 2015), Centurion-Lara et al. (1999, 2013), Gray et al. (2006), Sun et al. (2004)
TP0313	TPR protein E (TprE)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013), Gray et al. (2006), Stamm et al. (1998)
TP0317	TPR protein G (TprG)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013), Gray et al. (2006), Stamm et al. (1998)
TP0610	TPR protein H (TprH)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013)
TP0620	TPR protein I (TprI)	MOSP^C and MOSP^N	None	Porin	Anand et al. (2015), Centurion-Lara et al. (1999, 2013), Gray et al. (2006), Sun et al. (2004)
TP0621	TPR protein J (TprJ)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013), Gray et al. (2006); Stamm et al. (1998)
TP0897	TPR protein K (TprK)	MOSP^C and MOSP^N	None	Unknown	Centurion-Lara et al. (1999), Cox et al. (2010), Giacani et al. (2012), Hazlett et al. (2001), Pinto et al. (2016)
TP1031	TPR protein L (TprL)	MOSP^C and MOSP^N	None	Probable porin	Centurion-Lara et al. (1999, 2013)
TP0126	hypothetical protein	None found	2X27	OmpW-like ion-channel involved in transport of small hydrophobic molecules	Giacani et al. (2015), Hong et al. (2006)
TP0326	outer membrane protein	Surface antigen (Beta-barrel), Polypeptide Transport domains (POTRA)	4K3B	BamA, Outer membrane biogenesis	Cameron et al. (2000), Desrosiers et al. (2011), Luthra et al. (2015a)

(continued)

Table 1 (continued)

TP_ID	Protein annotation	Conserved domains	Structural similarity (PDB ID)	Proposed function	References
TP0515	hypothetical protein	LPS-assembly outer membrane protein LptD, Organic solvent tolerance protein OstA	4Q35	LPS-assembly protein LptD, substrate unknown	This chapter, Botos et al. (2016), Gu et al. (2015)
TP0548	hypothetical protein	Uncharacterized protein family (UPF0164)	3BRY	TbuX/FadL-like, long-chain fatty acid transport protein	Cox et al. (2010), van den Berg et al. (2004)
TP0733	hypothetical protein	None found	2MHL	OprG/OmpW-like ion-channel involved in transport of small hydrophobic molecules	This chapter, Hong et al. (2006)
TP0856	hypothetical protein	Uncharacterized protein family (UPF0164)	3BS0	TodX/FadL-like long-chain fatty acid transporter	This chapter, van den Berg et al. (2004, 2005)
TP0858	hypothetical protein	Uncharacterized protein family (UPF0164)	3DWO	TbuX/FadL-like long-chain fatty acid transporter	Cox et al. (2010), van den Berg et al. (2004), van den Berg (2005)
TP0859	hypothetical protein	Uncharacterized protein family (UPF0164)	3BRZ	FadL-like long-chain fatty acid transport protein	This chapter, van den Berg et al. (2004), van den Berg (2005)
TP0865	hypothetical protein	Uncharacterized protein family (UPF0164)	3BRY	TbuX/FadL-like long-chain fatty acid transport protein	This chapter, van den Berg et al. (2004), van den Berg (2005)
TP0966	hypothetical protein	None found	5AZS	OprJ-like outer membrane efflux protein	This chapter, Yonehara et al. (2016)
TP0967	hypothetical protein	None found	5AZO	OprN-like outer membrane efflux protein	This chapter, Yonehara et al. (2016)
TP0969	hypothetical protein	OEP (outer membrane efflux protein) family	2VDE	TolC-like outer membrane efflux protein	Bavro et al. (2008), Cox et al. (2010)

NA = Not available
Signal peptide and transmembrane helixes were predicted by TOPCONS (Tsirigos et al. 2015), LipoP (Juncker et al. 2003), Phobius (Kall et al. 2007), TMHMM (Krogh et al. 2001), and SignalP (Petersen et al. 2011)
Subcellular localizations were predicted by Cello (Yu et al. 2006) and PsortB (Yu et al. 2010)
Conserved domain identification was done by Conserved Domain Database (CDD) (Marchler-Bauer et al. 2015), pfam (Finn et al. 2016), and InterProScan (Jones et al. 2014)
β-barrel outer membrane protein predictions were performed using TMBETA-RBF (Ou et al. 2008), Markov Chain Model for β-barrels (MCMBB)(Bagos et al. 2004a), and transmembrane β-barrel proteome database (TMBB-DB) (Freeman and Wimley 2012)

in *T. pallidum* (Anand et al. 2012, 2013, 2015; Desrosiers et al. 2011; Luthra et al. 2011). The three essential biophysical properties of an OM-spanning β-barrel are (i) amphiphilicity (*i.e.*, ability to insert into a lipid bilayer), (ii) extensive β-sheet secondary structure, and (iii) adoption of a closed conformation (Wimley 2003). To examine amphiphilicity, we use TX114 phase partitioning of native (i.e., immunoblotting from phase-partitioned cell lysates) and folded recombinant proteins, and we assess the ability of the folded recombinant protein to insert into liposomes. For the latter, we typically use liposomes with a phospholipid composition simulating that of the *T. pallidum* OM (Radolf et al. 1995b). Proteins that do insert into liposomes can be examined for porin activity; channel formation is a strong evidence for β-barrel formation as well as functional activity (Zeth and Thein 2010). β-sheet content can be assessed quantitatively by far UV circular dichroism (CD) spectroscopy (Shao et al. 1996). Heat modifiability is a technically simple, but powerful, indicator of β-barrel formation; β-barrels are very stable structures that typically migrate faster by SDS-PAGE without than with boiling in final sample buffer (Conlan and Bayley 2003). TEM is an additional means of demonstrating that the folded recombinant protein forms a closed circular structure (Dorset et al. 1983). Incorporation of the folded protein into nanodiscs (a protein scaffold that encloses a lipid bilayer) (Nath et al. 2007) enables one to simultaneously confirm amphiphilicity and ability to circularize (Anand et al. 2015).

2.4 Establishing Authenticity–Surface Exposure in **T. pallidum**

Because of the many pitfalls inherent in surface labeling spirochetes, particularly an organism with as fragile an OM as *T. pallidum*, complementary methods always should be used before drawing conclusions about surface-exposure. We typically employ (i) IFA in our gel microdroplet system (see reference (Luthra et al. 2015b) for a detailed description), (ii) proteinase K (PK) accessibility, and (iii) opsonophagocytosis assay using rabbit peritoneal macrophages (Anand et al. 2012, 2013, 2015; Desrosiers et al. 2011; Hazlett et al. 2001, 2005; Lukehart and Miller 1978; Luthra et al. 2011). Each method has strengths and weaknesses and presents its own set of technical challenges. The importance of including control antisera for proteins or protein domains whose locations on the surface or in the periplasm are universally accepted cannot be over-emphasized. Antibodies against the flagellar sheath protein FlaA are often used for this purpose. Opsonophagocytosis is sensitive and, because it uses live organisms, surface-specific. However, background levels of internalization can be high and, as with any complex bioassay, reproducibility can be a problem. With PK accessibility experiments, use of motile organisms is extremely important; we use videomicroscopy to document motility throughout the PK incubation period (Desrosiers et al. 2011). Importantly, opsonophagocytosis assay and PK accessibility only determine whether an antigen

is surface-exposed. In addition to being able to detect proteins expressed on the *T. pallidum* surface in low copy numbers, the gel microdroplet assay allows localization of periplasmic proteins or the periplasmic domains of bipartite OMPs following controlled removal of OMs. Moreover, when performed in a double-labeling format with an antibody directed against FlaA or another periplasmic marker, the method enables one to assess the intactness of individual organisms thought to be surface-labeled (Cox et al. 2010; Hazlett et al. 2005). This is important because even under optimal circumstances, a small percentage of organisms (usually ~5%) have disrupted OMs.

3 The Expanding Repertoire of Rare Outer Membrane Proteins

3.1 T. pallidum *Repeat Proteins (Tprs)*

3.1.1 TprC/D, TprI, and TprF

Among the Tprs, our original consensus computational matrix (Cox et al. 2010) identified TprC/D (TprC [TP0117] and TprD [TP0131] are identical in the Nichols strain) and TprI (TP0620) as the strongest candidate OMPs. We verified these predictions for the native and recombinant proteins using the methods described above (Anand et al. 2012, 2015). In *T. pallidum*, native TprC/D and TprI are low abundance (~200 copies each per cell), trimeric, amphiphilic, and surface-exposed, while the folded recombinants form β-sheet-rich, heat-modifiable trimers that partition into the TX114 detergent-enriched phase and insert readily into artificial membranes. As with *T. denticola* MOSP (Anand et al. 2013; Egli et al. 1993), integration of TprC/D and TprI into liposomes results in increases in permeability comparable to those produced by the archetypal porin, *E. coli* OmpF (Anand et al. 2012, 2015; Nikaido 2003). With classical porins, the entire polypeptide forms the β-barrel (Nikaido 2003). However, to our surprise, the NCBI conserved domain database (CDD) revealed that Tpr C/D and TprI contain N- and C-terminal regions related to the corresponding domains of *T. denticola* MOSP (Fig. 2a) (Anand et al. 2012, 2015). When examined separately as recombinant proteins, the $MOSP^C$ domains of TprC and TprI formed amphiphilic β-barrels with porin activity in vitro (Fig. 2b) and were surface-exposed in *T. pallidum* (Fig. 2c). The $MOSP^N$ domains, in contrast, were a mixture of α-helix and β-sheet, lacked amphiphilic character, and were periplasmic in *T. pallidum* (Fig. 2c). Consistent with these results, TprF, a truncated protein which contains only a $MOSP^N$ domain (Fig. 2a), lacked amphiphilicity (Fig. 2b), failed to increase liposome permeability (Fig. 2b) and was found by small-angle X-ray scattering (SAXS) analysis to have an elongated structure (Anand et al. 2015). Because TprF is identical to the $MOSP^N$ domains of TprC and TprI along most of its length, this elongated structure almost certainly applies to the N-terminal halves of TprC and TprI as well (Anand et al. 2015).

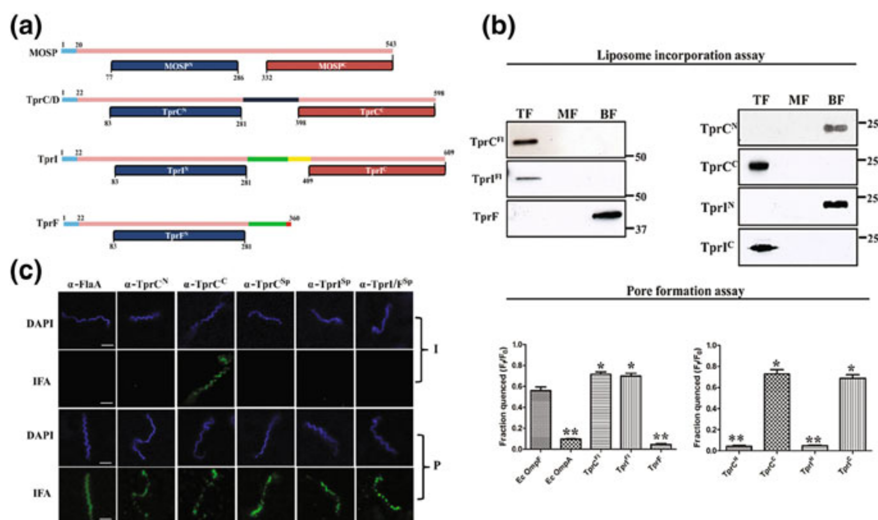

Fig. 2 Bipartite topology of Tpr C/D and I (Nichols strain). **a** Domain architectures of *T. denticola* major outer surface protein (MOSP) and TprC/D/I/F subfamily members. The signal sequences of all three proteins are shown in *blue*. The portions of TprC and TprI colored in *black* and *yellow*, respectively, denote the TprC- and TprI-specific regions of each protein ($TprC^{Sp}$ and $TprI^{Sp}$). The *green* regions in TprI and TprF denote regions present in TprI and TprF but not TprC ($TprI/F^{Sp}$). Reproduced with permission from reference (Anand et al. 2015)). **b** The $MOSP^{C}$ domains of TrpC and TprI are solely responsible for membrane insertion and pore formation by the full-length proteins. Liposomes were reconstituted with folded, full-length recombinant proteins ($TprC^{Fl}$ and $TprI^{Fl}$), TprF, or the $MOSP^{C}$ ($TprC^{C}$ and $TprI^{C}$) or $MOSP^{N}$ ($TprC^{N}$ and $TprI^{N}$) domains of TprC and TprI followed by sucrose density gradient ultracentrifugation and immunoblot analysis. The top fractions (TF) contain liposome-incorporated material, whereas the middle and bottom fractions (MF and BF, respectively) contain unincorporated material. The *bar graphs* show pore formation by the same proteins, along with *E. coli* OmpF (positive) and OmpA (negative) controls, measured by efflux of $Tb(DPA)_3^{3-}$ encapsulated into liposomes (100% efflux = the degree of quenching obtained by detergent lysis). Statistical significance compared with *E. coli* OmpF was assigned according to the following scheme: * $P < 0.05$; ** $P < 0.0001$. Reproduced from Reference (Anand et al. 2015). **c** Bipartite topology of native TprC and TprI in live treponemes. Motile *T. pallidum* were encapsulated in gel microdroplets and probed with 1:100 dilutions of rat antisera against $TprC^{N}$, $TprC^{C}$, or FlaA without (intact I) or with the removal of OMs (Permeabilization P) by pre-incubation with 0.10% Triton X-100. Antibody binding was detected with goat anti-rat Alexa Fluor 488 (*green*) conjugate. Given that $TprC^{C}$ antibodies are highly cross-reactive with $TprI^{C}$, both TprC and TprI are being labeled. Reproduced from reference (Anand et al. 2015)

By conventional TX114 phase partitioning of *T. pallidum*, both native TprC/D and TprI fractionate with the detergent-insoluble material (Fig. 3a), which contains the peptidoglycan sacculus (Fig. 3b) (Radolf et al. 1989a). However, if TprC and TprI were first dissociated from the sacculus by extraction with the detergent n-dodecyl-β-D-maltoside (DDM), they then partitioned into the TX114 detergent-enriched phase (Fig. 3a). These results, in combination with those described above for the recombinant proteins, indicate that, with both Tprs, the

C-terminal β-barrels insert into the OM, while the N-terminal portions extend downward, anchoring the barrels to the PG sacculus within the periplasm (Fig. 3c); structural modeling predicts that the β-barrels contain 10 transmembrane strands (Fig. 3d). Interestingly, pre-extraction with DDM did not release TprF (Fig. 3a), indicating that it is even more tightly bound to the sacculus than TprC and TprI. Heterologous expression studies performed with *E. coli* surrogates provided additional support for the bipartite model. When TprC/D, TprI, and TprF were placed downstream of PelB leader sequences, TprC/D and TprI were OM-associated with only their C-terminal β-barrels surface-exposed, while TprF was exclusively periplasmic (Fig. 4). Finally, since MOSP is considered the parental Tpr ortholog, we also examined its domain architecture (Anand et al. 2013). Our finding that only $MOSP^C$ forms a β-barrel with porin activity and is surface-exposed in *T. denticola* strongly suggests that the bipartite architectural model applies to the entire Tpr family.

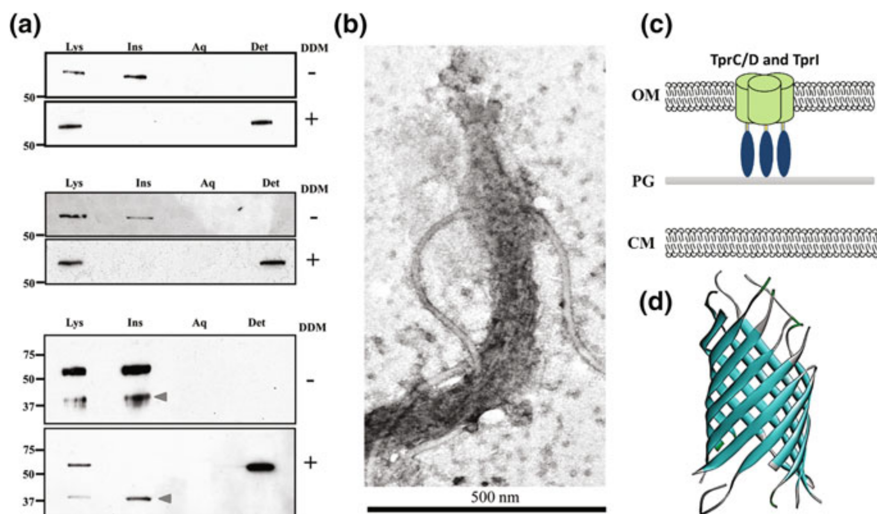

Fig. 3 Native TprC and TprI are amphiphilic but tethered to the peptidoglycan sacculus, whereas TprF is tightly bound to the peptidoglycan sacculus. **a** Triton X-114 phase partitioning of *T. pallidum* lysates without (−) or with (+) pre-solubilization with 2% DDM. Whole cells (WC), Triton X-114-insoluble material (Ins), and aqueous and Triton X-114-enriched phases (Aq and Det, respectively) were separated by SDS-PAGE followed by immunoblotting using antisera specific for TprC (*top*), TprI (*middle*), or TprI and F (*bottom*). *Arrowheads* in bottom panel indicate TprF; TprI is the larger protein. Reproduced from reference (Anand et al. 2015). **b** Extensively washed Triton X-114-insoluble material visualized in negatively stained whole mounts by transmission electron microscopy. Previous studies have shown that this material contains the peptidoglycan sacculus (Radolf et al. 1989a). Reproduced from reference (Anand et al. 2015). **c** Bipartite model for Tpr C/D and TprI. **d** Structural model of TprC (Nichols) generated using TMBpro (Randall et al. 2008) predicts a 10-stranded β-barrel

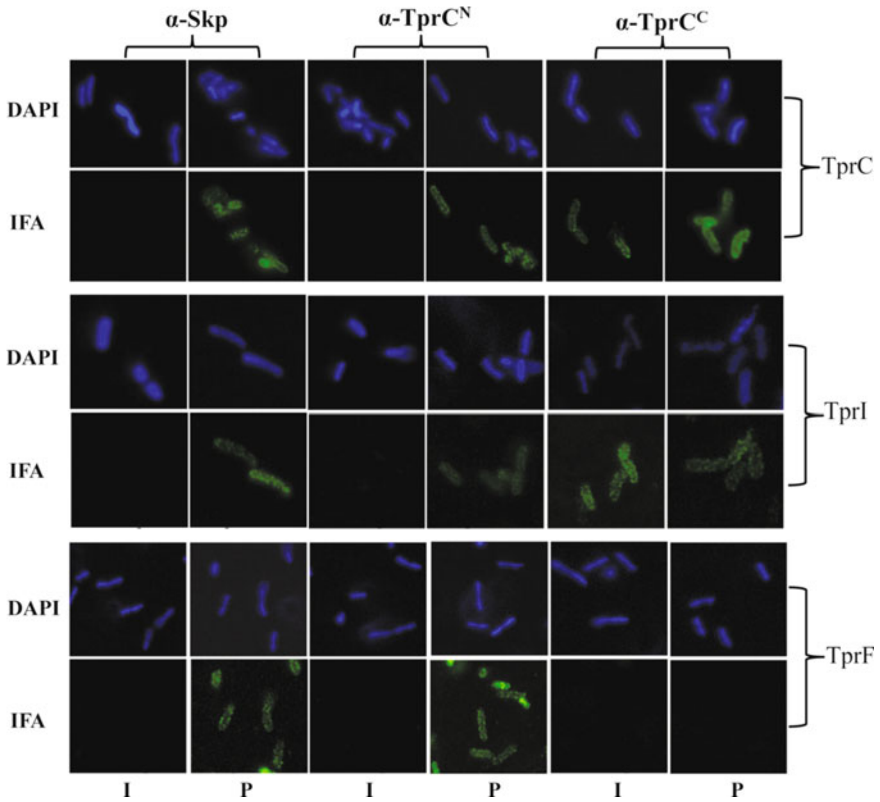

Fig. 4 TprC and TprI, but not TprF, expressed in E. coli with PelB signal sequences display bipartite topology. IFA of intact (I) or permeabilized (P) *E. coli* C41 (DE3) expressing TprC, TprI, or TprF with a PelB signal sequence were probed with rat antisera against TprC^{N}, TprC^{C}, and Skp (periplasmic control). Antibody binding was detected with goat anti-rat Alexa Fluor 488 conjugate. Reproduced from reference (Anand et al. 2015)

3.1.2 TprK

When the *T. pallidum* Nichols genomic sequence became available and the existence of the Tpr family came to light (Fraser et al. 1998), TprK drew immediate attention because of its sequence relatedness to *T. denticola* MOSP and its relatively high level of expression, determined by semi-quantitative qRT-PCR, among *tpr* genes (Centurion-Lara et al. 1999). In their landmark study, Centurion-Lara et al. (1999) reported that antibodies against TprK promote opsonophagocytosis of treponemes by rabbit peritoneal macrophages and that immunization with a large N-terminal fragment of TprK induces partial protection against *T. pallidum* challenge. The subsequent discovery that *TprK* undergoes intra-strain variation, generating subpopulations of organisms with variant *TprK* sequences as infection proceeds (Centurion-Lara et al. 2000; LaFond et al. 2003, 2006a; Stamm and

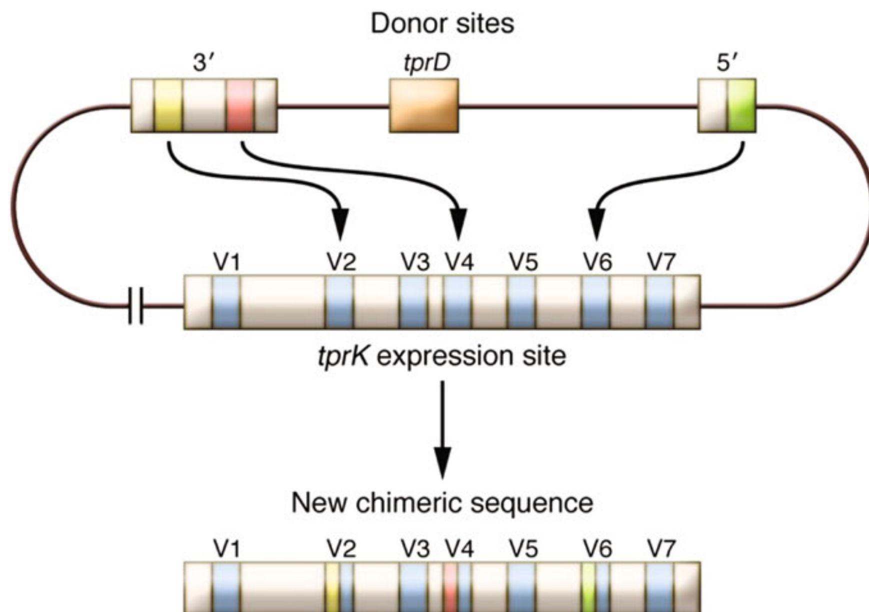

Fig. 5 Variation in TprK is attributed to gene conversion wherein variant DNA segments adjacent to *tprD* recombine with variable regions (V1–V7) of *tprK* to generate new TprK mosaics. Reproduced with permission from reference (Ho and Lukehart 2011)

Bergen 2000), suggested that the protein plays a fundamental role in immune evasion by *T. pallidum* (Deitsch et al. 2009; Ho and Lukehart 2011; Lafond and Lukehart 2006). Importantly, accumulation of TprK sequence variants occurs in syphilis patients as well as infected rabbits (LaFond et al. 2003, 2006a; Myint et al. 2004; Pinto et al. 2016). Indeed, an extraordinary recent study from Portugal in which DNAs for genomic sequencing were 'captured' from genital ulcer swabs described "rampant" sequence variation in *tprK* genes (Pinto et al. 2016). Variability in *tprK*/TprK sequences is not randomly distributed. It occurs in seven discrete variable (V) regions separated by stretches of conserved sequences (Fig. 5), with some V-regions displaying greater sequence diversity than others (Deitsch et al. 2009; LaFond et al. 2003; Pinto et al. 2016). DNA sequence cassettes that correspond to V-region sequences were discovered in an area of the *T. pallidum* chromosome located away from the *tprK* gene (Centurion-Lara et al. 2004). The authors proposed that these cassettes serve as unidirectional donor sites for the generation of variable regions by nonreciprocal gene conversion (Fig. 5) (Deitsch et al. 2009; Ho and Lukehart 2011; Lafond and Lukehart 2006). Generation of TprK variants differs widely among *T. pallidum* strains and, surprisingly, appears to occur at particularly low frequency in the Nichols reference strain (Giacani et al. 2012; LaFond et al. 2006a), perhaps because of extensive passage in rabbits. Sequence variability in the *tprk* donor and expression sites may also explain these

strain differences (Giacani et al. 2012). It is important to note that the Lisbon genomic sequences described above were obtained in a relatively confined locale (i.e., Lisbon) and, thus, may be derived from the same *T. pallidum* strain circulating in a circumscribed social network.

The TprK protein elicits both cellular and humoral immunity in infected animals (LaFond et al. 2006b; Morgan et al. 2002a, 2003). TprK antibodies are specifically targeted to the V-regions (Morgan et al. 2002b, 2003), which are thought to be located on extracellular loops (Centurion-Lara et al. 2013); slight changes in the amino acid sequence of a V-region can abrogate antibody binding (LaFond et al. 2006b). Consistent with the notion that immune pressure selects for variants, immunization of rabbits with peptides to V6 resulted in enhanced sequence variability (Giacani et al. 2010). The finding in a rabbit model of secondary syphilis that TprK variability is significantly greater at sites of dissemination compared to the inoculum is further evidence that immune pressure selects for variants and that sequence variation facilitates immune evasion (Reid et al. 2014).

Nevertheless, TprK presents something of a conundrum. While the genetic data collectively constitute a powerful argument that TprK is an authentic OMP, the information available about the protein does not agree with this assertion. In contrast to TprC/D and TprI, both recombinant and native TprK (Nichols) are hydrophilic by TX114 phase partitioning (Cox et al. 2010; Hazlett et al. 2001). In accord with these results, localization data obtained by proteinase K susceptibility and IFA in the Nichols strain place the native protein in the periplasm (Cox et al. 2010; Hazlett et al. 2001). Like other full-length Tprs, TprK is predicted to have a bipartite domain architecture (Anand et al. 2013). Three of the V domains are predicted to be upstream of the $MOSP^C$ domain, a location that would be of no value for immune evasion. Also worth noting is that the portions of the protein reported to confer partial protection (Centurion-Lara et al. 1999; Morgan et al. 2002a) are from the hydrophilic N-terminal half one would expect to be periplasmic based on our bipartite model for the Tprs; indeed, in other hands (Hazlett et al. 2001), this portion of the protein is not protective. However, why a periplasmic protein would undergo such extensive sequence variation is, without question, perplexing. Definitive structural and topologic analyses of intra- and interstrain TprK variants, including localization of epitopes subject to sequence variation, are needed to resolve these discordances.

3.1.3 Why a Family?

Finally, why so many Tprs? One obvious answer is that sequence diversity in the extracellular loops of the β-barrel domains of the full-length Tprs, in conjunction with differential expression, enhances the spirochete's capacity for immune evasion (see below). A second, non-mutually exclusive possibility is physiological. Sequence diversity in the strands that form the walls of the barrel lumen would be expected to affect the conductance properties and substrate specificities of the channel (Nikaido 2003; van den Berg 2012). Indeed, in addition to a role in immune evasion, we propose that the Tprs function collectively as a family of OM