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Ben Adler *Editor*

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

Contents

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).

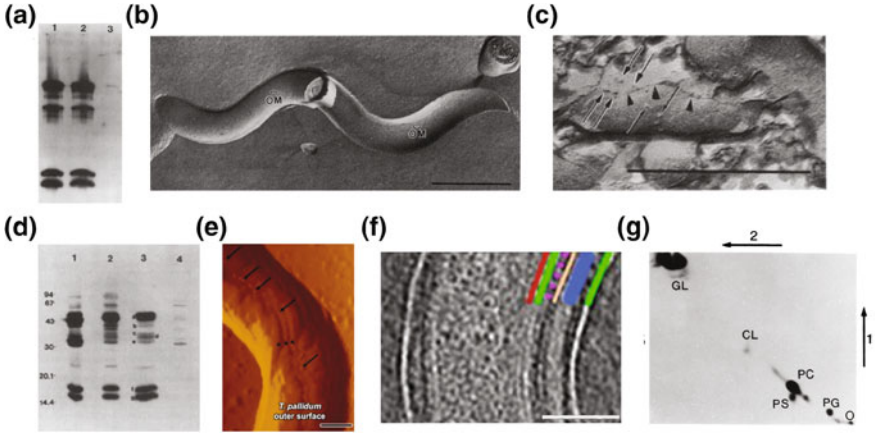

Fig. 1 The *T. pallidum* cell envelope. **a** *T. pallidum*'s major immunogens are associated with the protoplasmic cylinder, not the outer membrane. Reactivity with human syphilitic serum of proteins extracted with Triton X-114 from whole *T. pallidum* cells (*lane 1*), protoplasmic cylinders (*lane 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).