

Insertion of fluorescent fatty acid probes into the outer membranes of the pathogenic spirochaetes *Treponema pallidum* and *Borrelia burgdorferi*

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The authors examined the ability of octadecanoyl (C₁₈), hexadecanoyl (C₁₆) and dodecanoyl (C₁₂) fatty acid (FA) conjugates of 5-aminofluorescein (OAF, HAF and DAF, respectively) to insert into the outer membranes (OMs) of *Treponema pallidum*, *Borrelia burgdorferi* and *Escherichia coli*. Biophysical studies have demonstrated that these compounds stably insert into phospholipid bilayers with the acyl chain within the hydrophobic interior of the apical leaflet and the hydrophilic fluorescein moiety near the phospholipid head groups. Consistent with the known poor intrinsic permeability of the *E. coli* OM to hydrophobic compounds and surfactants, *E. coli* was not labelled with any of the FA probes. OAF inserted more readily into OMs of *B. burgdorferi* than into those of *T. pallidum*, although both organisms were completely labelled at concentrations at or below 2 µg ml⁻¹. Intact spirochaetes were labelled with OAF but not with antibodies against known periplasmic antigens, thereby confirming that the probe interacted exclusively with the spirochaetal OMs. Separate experiments in which organisms were cooled to 4 °C (i.e. below the OM phase-transition temperatures) indicated that labelling with OAF was due to insertion of the probe into the OMs. *B. burgdorferi*, but not *T. pallidum*, was labelled by relatively high concentrations of HAF and DAF. Taken as a whole, these findings support the prediction that the lack of lipopolysaccharide renders *T. pallidum* and *B. burgdorferi* OMs markedly more permeable to lipophilic compounds than their Gram-negative bacterial counterparts. The data also raise the intriguing possibility that these two pathogenic spirochaetes obtain long-chain FAs, nutrients they are unable to synthesize, by direct permeation of their OMs.

Keywords: flow cytometry, fatty acid metabolism, membrane fluidity

INTRODUCTION

Syphilis, a sexually transmitted disorder, and Lyme disease, the most common tick-borne infection in the United States, are caused by the spirochaetal pathogens *Treponema pallidum* and *Borrelia burgdorferi*, respectively. A number of studies in the last decade have documented major differences in protein and lipid

content between the outer membranes (OMs) of these two pathogenic spirochaetes and those of Gram-negative bacteria (Cox *et al.*, 1992; Belisle *et al.*, 1994; Bourell *et al.*, 1994; Radolf, 1995; Walker *et al.*, 1989, 1991). One principal difference is that LPS, the potent pro-inflammatory glycolipid found in the outer leaflets of Gram-negative bacterial OMs, is absent from the OMs of the two spirochaetes (Fraser *et al.*, 1997, 1998; Radolf *et al.*, 1995a, b). From a physiological standpoint this is potentially important because LPS forms a permeability barrier which limits the penetration of hydrophobic compounds into Gram-negative bacterial OMs (Nikaido, 1996). Indeed, the lack of LPS leads to the

Abbreviations: DAF, dodecanoylamino fluorescein; FA, fatty acid; HAF, hexadecanoyl-aminofluorescein; MFI, mean fluorescence intensity; OAF, octadecanoylamino fluorescein; OM, outer membrane; OspA, outer surface protein A; TpCM, *T. pallidum* cultivation medium.

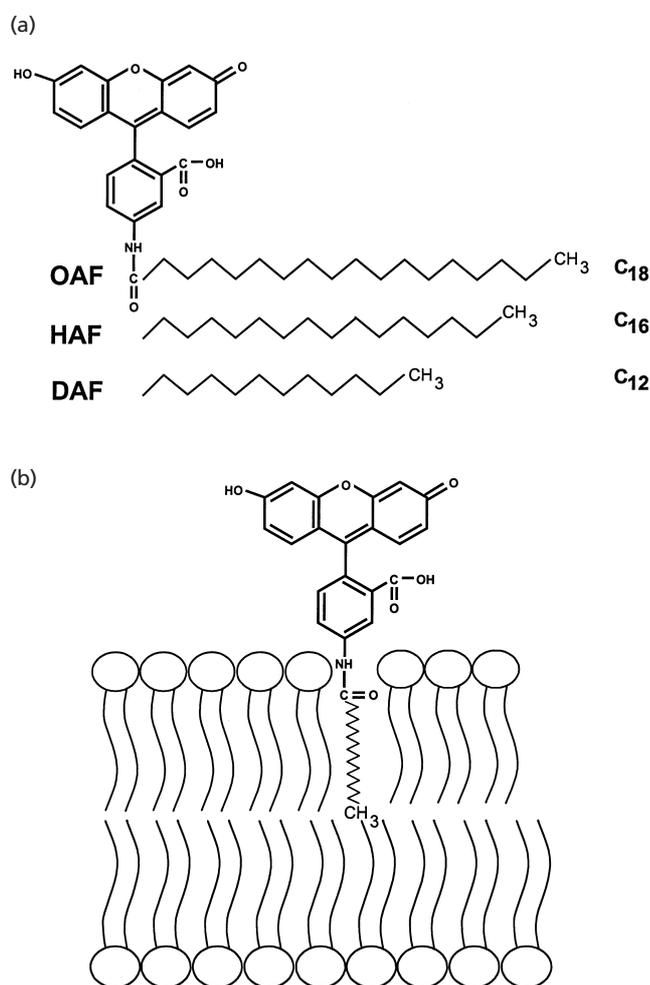


Fig. 1. Probes used in this study. (a) Structures of the C_{18} , C_{16} and C_{12} FA–5-aminofluorescein conjugates: octadecoylamino fluorescein (OAF), hexadecoylamino fluorescein (HAF) and dodecoylamino fluorescein (DAF). (b) Topology following membrane insertion (adapted from the Molecular Probes website, <http://www.probes.com>).

prediction that the OMs of the two spirochaetes are more permeable to lipophilic compounds than are their Gram-negative bacterial counterparts.

In recent years, there has been increasing recognition of the relevance of an improved understanding of the physiological properties of spirochaetal OMs to disease pathogenesis. However, studying the permeability properties of *T. pallidum* and *B. burgdorferi* OMs presents some unique challenges. Unlike *E. coli*, neither of these spirochaetes can be genetically manipulated, so organisms whose OMs are deficient in specific components cannot be created. Moreover, methods used to study the permeability of Gram-negative bacterial OMs to hydrophilic compounds, which also rely upon the ability to genetically transform the bacteria and express periplasmic enzymes (Heine *et al.*, 1988; Roepe *et al.*, 1990; Treptow & Shuman, 1985; Vogele *et al.*, 1993), cannot be applied to these organisms. In contrast,

methods to study the permeability of spirochaetal OMs to hydrophobic molecules are more readily applicable inasmuch as they require only an intact OM and the availability of lipophilic probes whose insertion into the hydrophobic interior of the OM can be temporally and quantitatively evaluated. In order for a compound to passively diffuse across a membrane, it must first penetrate into the bilayer's hydrophobic interior; insertion, therefore, represents a critical step in the diffusion process and can be regarded as an indicator of membrane permeability.

In this study, we took advantage of two technical developments to assess the relative permeability of *T. pallidum*, *B. burgdorferi* and *E. coli* OMs to lipophilic molecules. The first is the commercial availability of a series of fatty acid (FA)–fluorescein conjugates with increasing hydrocarbon chain lengths (C_{12} , C_{16} and C_{18} ; shown Fig. 1a) and thus increasing hydrophobic and amphiphilic properties. Biophysical studies have demonstrated that these compounds stably insert into phospholipid bilayers with the acyl chain within the hydrophobic interior of the apical leaflet and the hydrophilic fluorescein moiety near the leaflet's phospholipid head groups (Fig. 1b) (Christensen *et al.*, 1999; Derzko & Jacobson, 1980; Haugland, 1996; Sklar *et al.*, 1980; Stolz *et al.*, 1992; Tocanne *et al.*, 1989). In systems containing double membranes, the probes insert exclusively with the outermost lipid bilayer (Blanco *et al.*, 1994; Foley *et al.*, 1986; Tocanne *et al.*, 1989). The second technical development is the use of flow cytometry, which offers many advantages over conventional fluorescence microscopy, most notably the ability to rapidly and quantitatively analyse complex bacterial populations (Boye *et al.*, 1983; Martinez *et al.*, 1982; Shapiro, 1988; Waldman *et al.*, 1987). Our finding that long-chain FAs can insert into the OMs of the two spirochaetes, but not that of *E. coli*, directly supports the permeability predictions derived from existing models for *T. pallidum* and *B. burgdorferi* OM architecture (Cox *et al.*, 1992, 1995, 1996; Radolf *et al.*, 1995a, b). Our data also raise the intriguing possibility that these two pathogenic spirochaetes obtain long-chain FAs, molecules they are unable to synthesize, by direct permeation of their OMs.

METHODS

Lipophilic probes. Octadecanoyl (C_{18}), hexadecanoyl (C_{16}) and dodecanoyl (C_{12}) FA conjugates of 5-aminofluorescein (OAF, HAF and DAF, respectively; Fig. 1a) were purchased from Molecular Probes. Unless otherwise noted, stock solutions of the probes were prepared at 1 mg ml^{-1} in DMSO. Stock solutions were usually made fresh for each experiment, but they can be stored at 4°C in the dark. The DMSO will solidify but can be melted at room temperature before dispensing.

Bacterial strains. *Treponema pallidum* subsp. *pallidum* (Nichols strain) was propagated in rabbit testes as previously described (Cox *et al.*, 1990). Low-passage (<5) *Borrelia burgdorferi* (B31 strain) was grown in BSK II broth (Radolf *et al.*, 1994). Spirochaetes were enumerated by dark-field mi-

croscopy and diluted to a concentration of approximately $1 \times 10^8 \text{ ml}^{-1}$ in the appropriate medium prior to use. Mid-exponential-phase *Escherichia coli* C600 (ATCC 23724) was cultivated in tryptone phosphate broth (Difco) at 35 °C.

Labelling procedures and flow cytometry. For labelling of *T. pallidum* with the FA conjugates, probes were added directly to treponemal suspensions freshly harvested at peak orchitis in *T. pallidum* cultivation medium (TpCM; Cox *et al.*, 1990) containing 10% (v/v) heat-inactivated fetal bovine serum. For labelling of *B. burgdorferi* or *E. coli*, the probes were added directly to mid-exponential-phase cultures in BSKII medium or tryptone phosphate broth, respectively. Unless otherwise noted, organisms were incubated with the probes for 2 h at 34 °C. To examine OM labelling at 4 °C, parallel samples were cooled to 4 °C before the addition of the probe; during the subsequent analysis by flow cytometry, the injection syringe was maintained at 4 °C with an ice pack.

Because of the small size of the spirochaetes, particularly *T. pallidum* ($\leq 0.2 \mu\text{m}$ in diameter), gating on forward light scatter as a means of tracking the total number of cells within a population was extremely difficult. Consequently, we devised an alternative approach in which the total number of organisms in a suspension was determined by labelling with the vital stain Live/Dead BacLight (Molecular Probes), which has the same emission wavelength as fluorescein and labels all bacteria. The percentage of organisms labelled with a particular FA probe was determined by dividing the number of bacteria labelled with the probe by the number of viable organisms detected with Live/Dead BacLight. Because *E. coli* did not label with any of the FA probes (see below), Live/Dead BacLight also was used to confirm that labelled *E. coli* could be detected by the cytometer and to establish gating parameters for this bacterium (data not shown).

Flow cytometry was performed using a Bruker ACR 1400 cytometer (Bruker Instruments). Cells were analysed at a flow rate of $500 \pm 100 \text{ cells s}^{-1}$ until 25 000 events had been collected. Triggering was set routinely for green fluorescence rather than forward light scatter as noted above. During sample analysis, the cytometer generated two-dimensional histograms consisting of forward light scatter (*x* axis) versus fluorescence intensity (*y* axis) for each fluorescent organism detected. Cytometric gates were established to monitor each bacterial population. The accuracy of the 'percentage labelled' determinations was enhanced by the fact that the Bruker cytometer not only counts the number of fluorescent bacteria within a gate, but also monitors the volume of sample injected, so that a bacterial concentration per ml can be calculated. An internal control of 0.9 μm diameter green fluorescent beads (Duke Scientific) was present in each sample to aid in maintaining instrument alignment and to ensure that each sample was analysed under optimal optical conditions. Because of small variations in cell size and the asymmetrical geometry of the spirochaetes, the signals which represent the fluorescence intensity of a population of spirochaetes are scattered over many channels of the fluorescence detectors. The mean fluorescence intensity (MFI), the mean channel of that range for a given population, is provided by the cytometer as the samples are being analysed. Four or five separate analyses were performed on each sample for each time point (100 000 to 125 000 total events) and each experiment was repeated at least twice.

Double-label experiments of spirochaetes encapsulated in gel microdroplets. To confirm that the FA-fluorescein conjugates inserted exclusively into the OMs of intact spirochaetes, double-label experiments were performed as follows.

Spirochaetes were encapsulated into low-melting-point agarose (Sigma, Type IX) gel microdroplets as previously described (Cox *et al.*, 1995, 1996). Briefly, suspensions of bacteria were warmed to 40 °C in a 50 ml round-bottom glass tube and mixed 1:1 with a warmed solution of 2% (w/v) low-melting-point agarose. Unless otherwise noted, the tubes were flushed with 95% N_2 /5% CO_2 (v/v) in each of these steps to protect the spirochaetes from oxygen toxicity. The suspension was vigorously vortexed as 5 ml warm (40 °C) mineral oil was added. The solution was allowed to mix for another 15–20 s until an emulsion formed, and then the tube was set in an ice-water bath (4 °C) for 10 min. The beads were collected by washing the oil/agarose mix twice with 5 ml TpCM containing 10% fetal bovine serum. The aqueous (bottom) layers were pooled from each extraction and washed an additional three times with 10 ml TpCM to remove any remaining oil as described above and 1 ml portions of the pooled bead preparations were placed into several 12 \times 75 mm polypropylene tubes. Two tubes were designated for each antibody used (one with 0.05%, w/v, Triton X-100 and one without). For *T. pallidum*, the beads in each tube were probed for 2 h with OAF and rat antisera against either endoflagella or the 47 kDa lipoprotein (Tp47) (Cox *et al.*, 1995). For *B. burgdorferi*, the beads in each tube were probed with OAF and rat antisera against endoflagella, outer-surface lipoprotein A (OspA) (Cox *et al.*, 1996) or p39 (BmpA) (Simpson *et al.*, 1991). The beads were washed three times with TpCM and then probed with the Alexa 546 conjugate of goat anti-rat antibody (Molecular Probes) for 1.5 h. The beads were again washed twice with TpCM and then the detergent-treated beads were split into two equal aliquots. OAF was added at a concentration of 3 $\mu\text{g ml}^{-1}$ to the beads without detergent and to one aliquot of the beads treated with Triton X-100. To demonstrate that Triton X-100 did not quench fluorescence, 0.05% Triton X-100 was added back with the OAF to the detergent-treated beads. Each experiment was repeated three times.

Microscopy. Microscopic observations of the labelled suspensions and bead preparations were performed using a Nikon Optiphot (Nikon) fluorescence microscope equipped with a dark-field condenser. Photomicrographs were prepared using a Nikon camera mounted on the fluorescence scope. Fuji 1600 ASA colour film was used to capture images; the exposure time, unless otherwise noted, was 1 s for both dark-field and fluorescence images.

Statistics. All data were analysed using the paired student *t*-test. The analyses were performed using the statistical program Systat (Systat, Evanston, IL, USA) on a Macintosh 8500 computer. Each group was independently compared with other groups in each experiment. The statistical data presented consist of the probability (*P*) for each test. The standard deviation and error bar values were the standard deviations of the mean values from each experiment.

RESULTS AND DISCUSSION

Flow cytometric assessment of labelling with OAF

We began our study by assessing the ability of graded concentrations of OAF, the most lipophilic and amphiphilic of the three conjugates shown in Fig. 1(a), to label *T. pallidum* subsp. *pallidum* (Nichols), low-passage *B. burgdorferi* B31 and *E. coli* C600. Labelling of *E. coli* was not detected at any concentration of OAF (Fig. 2a). In additional experiments, labelling of *E. coli* was not

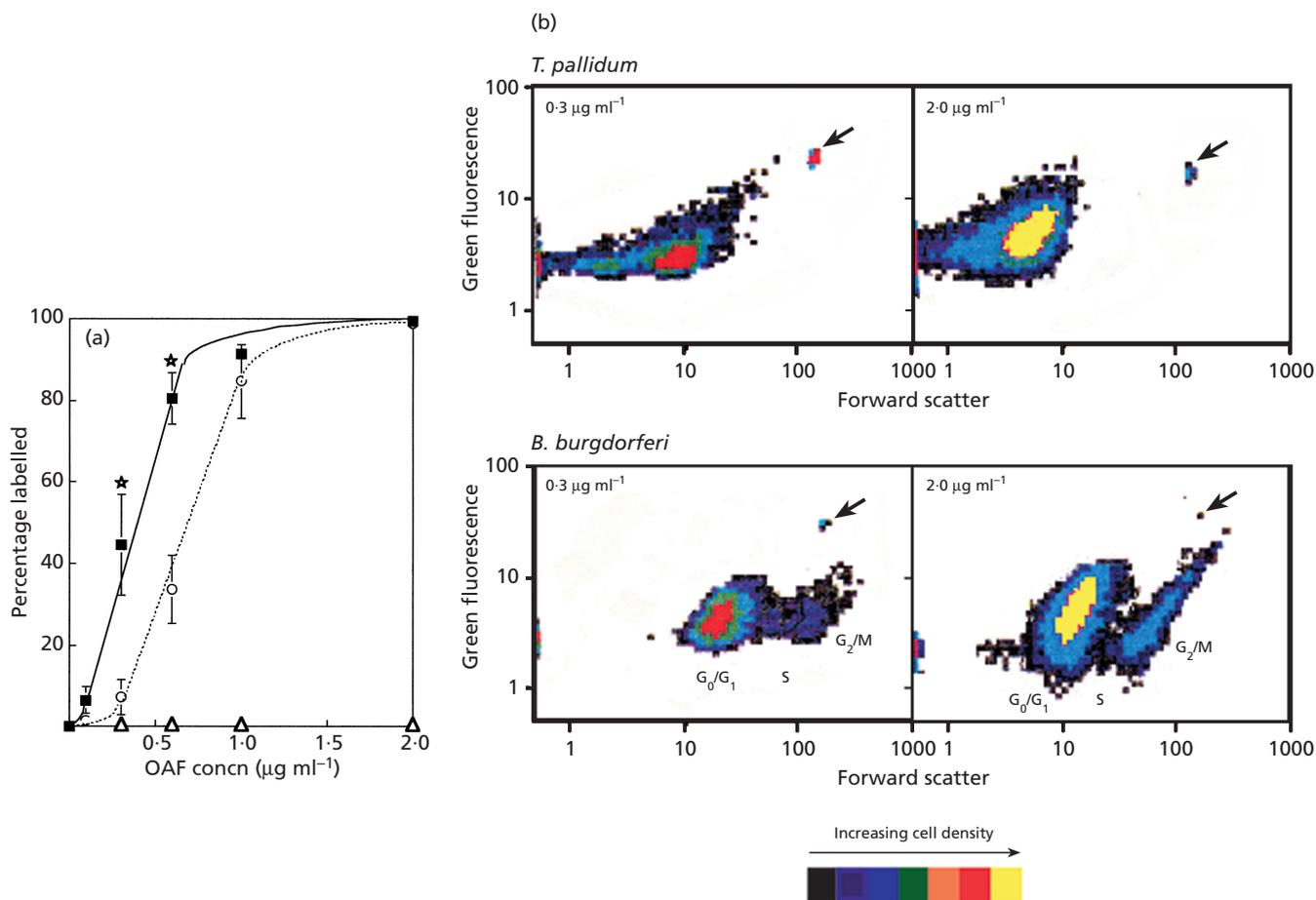


Fig. 2. (a) *T. pallidum* (\circ), *B. burgdorferi* (\blacksquare) and *E. coli* (\triangle) were analysed by flow cytometry following incubation with graded concentrations of OAF. The percentages of organisms labelled are shown as a function of OAF concentration. The percentage of organisms labelled was determined by dividing the number of bacteria labelled with the probe by the number of viable organisms detected with Live/Dead BacLight. Data are expressed as the means of three experiments \pm standard deviation. Asterisks denote values which were significantly different. (b) Representative 2D histograms obtained following incubation of *T. pallidum* and *B. burgdorferi* with 0.3 and 2.0 $\mu\text{g ml}^{-1}$ OAF. Cytometric gates have been removed to simplify the diagrams. The arrow indicates the 0.9 μm green beads used as an internal standard. The *B. burgdorferi* histograms are labelled to indicate the phase of the cell cycle (G_0/G_1 , S and G_2/M) represented by each population.

observed at OAF concentrations as high as 100 $\mu\text{g ml}^{-1}$ or when the coliforms were washed and resuspended in phosphate-buffered saline (pH 7.4) prior to incubation with the probe (data not shown). These results are consistent with the poor intrinsic permeability of the *E. coli* OM to hydrophobic compounds and surfactants (Nikaido, 1996). In contrast, both *T. pallidum* and *B. burgdorferi* showed concentration-dependent increases in labelling, with significantly greater labelling of *B. burgdorferi* ($P < 0.001$) at 0.3 and 0.6 $\mu\text{g ml}^{-1}$ (Fig. 2a). The forward light scatter signal (shift to the right) for *B. burgdorferi* was stronger than that for *T. pallidum* because of the Lyme disease spirochaete's larger size. Time-course studies showed that, at each concentration, labelling of the spirochaete populations reached steady state by 20 min (data not shown); the failure of organisms to label, therefore, was not due to an insufficient period of incubation with the probe.

Interestingly, for both spirochaetes, concentration-dependent increases in the proportion of labelled cells occurred without significant increases in MFI. Had the MFI increased, the position of the spirochaetal populations in the histograms would have shifted upwards; no shift, however, was ever observed. Representative histograms (Fig. 2b) are shown for 0.3 $\mu\text{g ml}^{-1}$, the lowest OAF concentration at which labelling was detected in both organisms, and 2.0 $\mu\text{g ml}^{-1}$, a concentration at which both populations were completely labelled (Fig. 2a). We believe this phenomenon reflects (i) heterogeneity in OM lipid composition within the spirochaetal population such that individual organisms have different 'threshold concentrations' for labelling and (ii) the fact that once a threshold concentration for labelling is reached for a particular organism, a saturating amount of label is taken up by its OM. That labelled organisms were indeed saturated was supported by the finding that

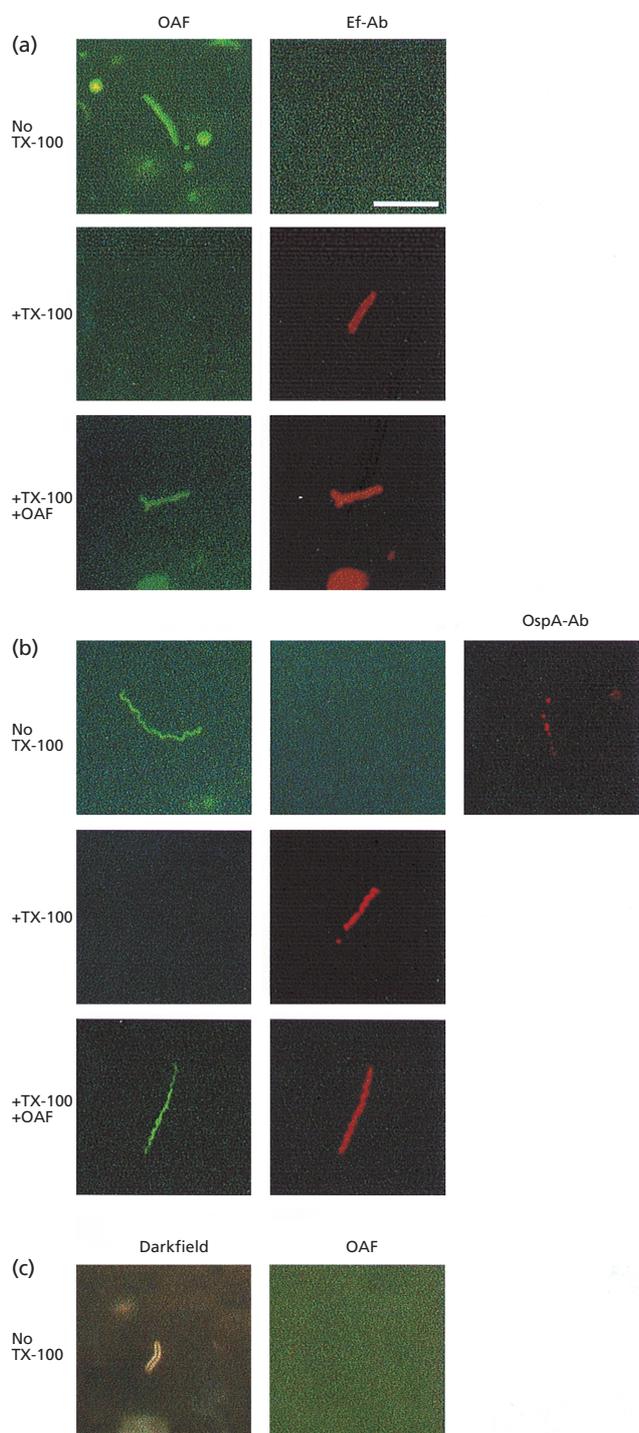


Fig. 3. (a, b) OM localization of OAF in *T. pallidum* (a) and *B. burgdorferi* (b). Organisms encapsulated in agarose gel microdroplets were probed in the absence or presence of 0.05% Triton X-100 with OAF ($2 \mu\text{g ml}^{-1}$) and rat antibodies against endoflagella (Ef-Ab) for 2 h at 34°C . One set of *B. burgdorferi* was also probed with OAF and antibodies against OspA (OspA-Ab). Bound antibodies were detected by probing with the Alexa 546 conjugate of goat anti-rat IgG. OAF ($2 \mu\text{g ml}^{-1}$) was added back to one portion of the beads treated with the detergent. To demonstrate that Triton X-100 did not quench fluorescence, 0.05% Triton X-100 was added back with the OAF to the detergent-treated beads; under these

increases in MFI did not occur at even greater OAF concentrations (up to $10 \mu\text{g ml}^{-1}$; data not shown). Analysis of non-synchronized growing cultures of bacteria by flow cytometry commonly reveals a distinct, large population of average-sized cells on the left (G_0/G_1); a small population of larger cells undergoing cell division on the right (G_2/M), and a much smaller population of intermediate-sized cells about to enter cell division (S phase) (Boye *et al.*, 1983; Shapiro, 1988). Also noteworthy was that the histograms of *B. burgdorferi* fit this model for typical cell-cycle distribution (Fig. 2b). The absence of comparably shifted treponemal subpopulations is consistent with the syphilis spirochaete's slower doubling time and the fact that treponemes harvested at peak orchitis are most likely at or near stationary phase (Akins *et al.*, 1998; Magnuson *et al.*, 1948).

FA-fluorescein conjugates insert into the OMs of intact spirochaetes

The biophysical properties of OAF lead to the prediction that it will insert into and remain trapped within the first bilayer it encounters (Christensen *et al.*, 1999; Foley *et al.*, 1986; Haugland, 1996; Stolz *et al.*, 1992), which, in the case of intact *T. pallidum* and *B. burgdorferi*, would be the spirochaetal OMs (Blanco *et al.*, 1994). Two experimental approaches were pursued to confirm this important contention.

In one series of experiments, organisms encapsulated in agarose gel microdroplets were probed simultaneously with OAF ($2 \mu\text{g ml}^{-1}$ for 2 h at 34°C) and with specific antibodies in the absence or presence of 0.05% Triton X-100, a detergent concentration which selectively removes the OMs of both spirochaetes (Akins *et al.*, 1998; Caimano *et al.*, 1999; Cox *et al.*, 1995, 1996; Lahdenne *et al.*, 1997; Shevchenko *et al.*, 1997, 1999). The antibodies were directed against targets whose cellular locations in *T. pallidum* and/or *B. burgdorferi* have been well documented (Cox *et al.*, 1996; Radolf *et al.*, 1995a, b; Simpson *et al.*, 1991). Representative micrographs are shown in Fig. 3. Consistent with the above flow-cytometry studies, all of the encapsulated spirochaetes were labelled by OAF at the concentration tested (Fig. 3a, b, top row, left panels), whereas labelling of *E. coli* was not discernible under the same conditions (Fig. 3c). No binding of anti-flagellar antibodies to either *T. pallidum* or *B. burgdorferi* was observed in the absence of detergent, confirming that the OMs of the two spirochaetes were intact (Fig. 3a, b, top row, right panels). Further evidence for this was provided by the observation that antibodies also failed to bind to two other *T. pallidum* and *B. burgdorferi* periplasmic antigens, Tp47 and p39 (BmpA), respectively (data not shown). In the absence of detergent, however, *B.*

conditions, the cytoplasmic membranes become labelled with the OAF. (c) *E. coli* was probed with only OAF ($2 \mu\text{g ml}^{-1}$ for 2 h at 34°C). Representative micrographs are shown. Bar, $10 \mu\text{m}$.

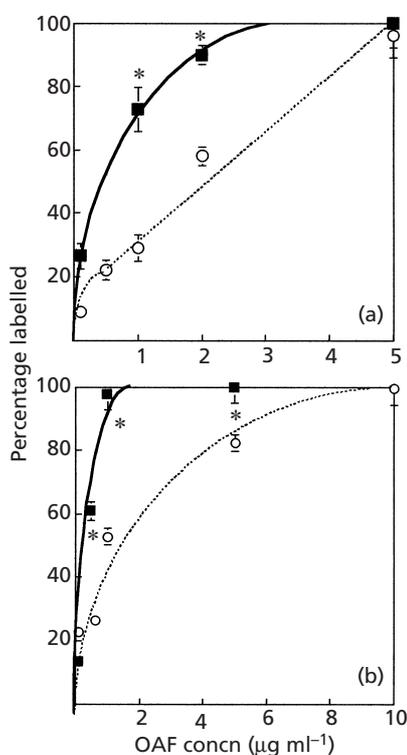


Fig. 4. Insertion of OAF into spirochaetal OMs is temperature-dependent. *T. pallidum* (a) and *B. burgdorferi* (b) were incubated with graded concentrations of OAF at 37 °C (■) or 4 °C (○). The percentage of labelled organisms is shown as a function of the concentration of OAF (note the different concentration scales in panels a and b). Data are expressed as the means of three experiments \pm standard deviation. Asterisks denote values which were significantly different ($P < 0.001$).

burgdorferi was labelled simultaneously with antibodies directed against OspA (Fig. 3b, top row, far right panel). Comparable labelling experiments could not be performed with *T. pallidum* because its surface antigens have not yet been definitively identified (Fraser *et al.*, 1998). In contrast, OAF labelling was lost when spirochaetes were incubated with detergent (Fig. 3a, b, middle row, left panels). Moreover, detergent-treated organisms were readily labelled with antibodies directed against flagellin (Fig. 3a, b, middle row, right panels), Tp47 (not shown) and p39 (not shown), thereby demonstrating that the OMs had indeed been removed by the detergent treatment. When OAF was added back to the detergent-treated spirochaetes, they once more became labelled (Fig. 3a, b, bottom row, left panels). These findings demonstrate that OAF can insert into cytoplasmic membranes once OMs are removed and that Triton X-100 does not quench the fluorescence of the probe.

Having established that the probe was associated exclusively with the OMs of both *T. pallidum* and *B. burgdorferi*, we next sought additional evidence that labelling involved actual insertion into the bilayer. A second series of experiments took advantage of the fact

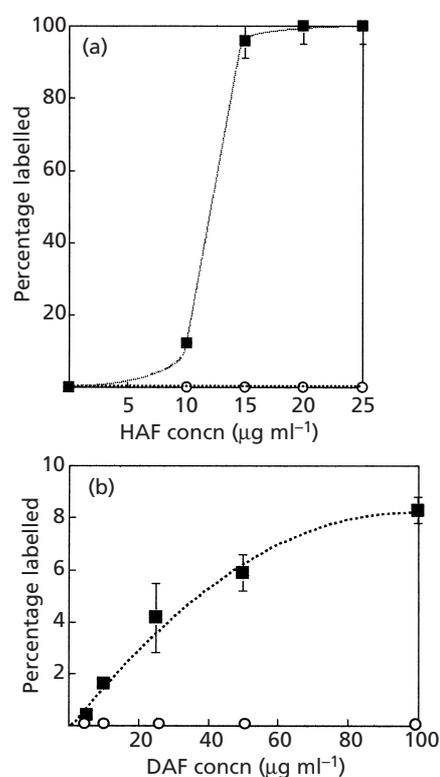


Fig. 5. HAF and DAF insert into OMs of some *B. burgdorferi*, but not *T. pallidum*. (a) The percentages of labelled spirochaetes (■, *B. burgdorferi*; ○, *T. pallidum*) are shown as a function of HAF concentration (a) or DAF concentration (b). Data are expressed as the means of three experiments \pm standard deviation. *E. coli* did not label with either probe and is not shown.

that penetration of lipophilic compounds is retarded at temperatures below a membrane's liquid crystalline to gel phase-transition temperature, while simple adsorption to the bacterial surface tends to be independent of membrane fluidity (Gennis, 1989a, b). As shown in Fig. 4, a marked decrease in concentration-dependent labelling of both spirochaetes was observed at 4 °C, a temperature below the phase-transition temperatures for both OMs (Bourell *et al.*, 1994; Radolf *et al.*, 1994). Interestingly, even at the lower temperature, OAF penetrated more efficiently into the *B. burgdorferi* OM.

Relatively high concentrations of HAF and DAF can label intact *B. burgdorferi* but not *T. pallidum*

Neither *T. pallidum* nor *E. coli* was labelled by HAF at concentrations up to 100 $\mu\text{g ml}^{-1}$ (Fig. 5a and data not shown). *B. burgdorferi*, in contrast, was labelled by this reagent. Appreciable labelling with HAF, however, required a concentration greater than 5 $\mu\text{g ml}^{-1}$, while 50% and 100% labelling occurred at approximately 12 $\mu\text{g ml}^{-1}$ and 20 $\mu\text{g ml}^{-1}$, respectively (Fig. 5a). Also noteworthy was the slope of the HAF curve in comparison to that for OAF (compare Figs 2a and 5a), indicating that borrelian OMs have a wider threshold

(10–15 $\mu\text{g ml}^{-1}$) for labelling with HAF as opposed to the narrower range of concentrations (0.1–1.0 $\mu\text{g ml}^{-1}$) at which OAF inserts into the OMs. As with OAF, the MFI of the labelled organisms did not show a concentration-dependent increase. To determine if the lack of labelling of *T. pallidum* with HAF reflected an intrinsic property of the treponemal OM that was constant throughout the cell cycle, HAF at 100 $\mu\text{g ml}^{-1}$ was added to growing cultures of *T. pallidum* co-cultivated with Sf1Ep cells (Cox *et al.*, 1990). Although the epithelial cells avidly took up the dye, no labelling of *T. pallidum* was observed during a 7 d period in which the treponemal cell density increased approximately 40-fold (data not shown). The lack of permeability of the treponemal OM to HAF was surprising in light of previous studies showing that *T. pallidum* can assimilate radiolabelled palmitate into cellular lipids and lipoproteins (Belisle *et al.*, 1994; Chamberlain *et al.*, 1989). This apparent inconsistency can be explained by the fact that steric hindrance caused by the fluorescein moiety reduces the length of hydrocarbon chain effectively available for insertion into the membrane (Molecular Probes, personal communication). Finally, we also examined the permeability of *T. pallidum* and *B. burgdorferi* to DAF, the least lipophilic and amphiphilic of the three FA conjugates. As with HAF, no labelling of *T. pallidum* or *E. coli* was detected with concentrations up to 100 $\mu\text{g ml}^{-1}$, whereas *B. burgdorferi* was slightly permeable. Approximately 8% of the borreliae were labelled at the maximum DAF concentration tested (Fig. 5b).

Spirochaetal OMs and lipophilic molecules – implications for FA uptake

In this study, we examined the ability of FA–fluorescein conjugates to insert into the OMs of *T. pallidum* and *B. burgdorferi*, but not into that of *E. coli*. Although these amphiphilic probes do not extend beyond the outer leaflet of the bilayer, insertion into the hydrophobic interior of the membrane is a prerequisite for passive diffusion and can be considered an indicator of the membrane's relative permeability to these compounds under investigation. Our findings that *T. pallidum* and *B. burgdorferi*, but not *E. coli*, were labelled by OAF and/or HAF and DAF support the prediction that the absence of LPS renders the OMs of these two pathogenic spirochaetes relatively permeable to lipophilic compounds. Two other findings also are noteworthy. The first is that labelling spirochaetes within a population occurred over a range of OAF and HAF concentrations, a result suggesting variability of OM lipid constituents among individual bacteria. Christensen *et al.* (1999) also noted heterogeneous labelling of mycobacterial populations incubated with the same probe, indicating that organism-to-organism variability in lipid constituents is not limited to spirochaetes. The second noteworthy finding is the relatively greater ability of lipophilic compounds to insert into *B. burgdorferi* OMs as compared to those of *T. pallidum*. The disparate properties of the two spirochaetal OMs presumably

reflect previously noted differences in their lipid constituents and the greater relative abundance of unsaturated FAs in borrelial cellular lipids (Belisle *et al.*, 1994; Radolf *et al.*, 1995a, b). Biophysical studies of natural and artificial membranes have shown that increased levels of unsaturated FAs can be particularly important for enhancing membrane fluidity (Araiso *et al.*, 1990).

Because the *E. coli* OM is impermeable to long-chain FAs, a specialized transport protein, FadL, is needed to transport these compounds across the OM; they then passively diffuse across the cytoplasmic membrane (Clark & Cronan, 1996). Both *T. pallidum* and *B. burgdorferi* are incapable of elongating, β -oxidizing or desaturating long-chain FAs and therefore are entirely dependent upon environmental sources for these molecules (Belisle *et al.*, 1994; Fraser *et al.*, 1997, 1998; Schiller & Cox, 1977). The absence of an identifiable FA transporter in the genomes of both spirochaetes (Fraser *et al.*, 1997, 1998) appears to necessitate an alternative mechanism for OM transport of long-chain FAs. The studies presented here enable us to postulate that *T. pallidum* and *B. burgdorferi* obtain these essential nutrients, most likely in their uncharged protonated forms, by passive diffusion across their OMs. Such a process is also consistent with the slow growth rates of both parasites (Akins *et al.*, 1998; Magnuson *et al.*, 1948). The presumptive impermeability of the *T. pallidum* OM to FAs with shorter hydrocarbon chain lengths may be the result of an adaptation to the range of FAs found in human tissues; oleic acid (C_{18}), palmitic (C_{16}) and linoleic (C_{18}) acids are, in that order, the most prevalent FAs in human fat (Cramer & Brown, 1943). The ability of *B. burgdorferi* to take up a wider range of FAs than *T. pallidum*, an obligate pathogen of humans, may reflect the Lyme disease spirochaete's need to adapt to more diverse environments found in both arthropod and mammalian hosts.

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