Biofilm formation by saprophytic and pathogenic leptospires

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Leptospira spp. exist as saprophytic organisms that are aquatic or as pathogens that are able to survive in water. Leptospirosis is transmitted to humans through environmental surface waters contaminated by the urine of mammals, usually rodents, which are chronically infected by pathogenic strains. The ecology of Leptospira spp. prompted us to evaluate if these spirochaetes were able to form biofilms. This study investigated the characteristics of biofilm development by both saprophytic and pathogenic Leptospira species using microscopic examinations and a polystyrene plate model. Biofilms were formed preferentially on glass and polystyrene surfaces. Electron microscopic images showed cells embedded in an extracellular matrix. The formation of such a biofilm is consistent with the life of saprophytic strains in water and may help pathogenic strains to survive in environmental habitats and to colonize the host.

INTRODUCTION

The traditional paradigm of bacteria living in their planktonic form has been recently revised. Biofilms, which are surface-associated cells enclosed in a matrix of self-synthesized extracellular materials, now appear to be integral components of the prokaryotic life cycle (Hall-Stoodley et al., 2004). In biofilms, micro-organisms become more resistant to killing by biocides and antibiotics. Biofilm formation begins with the adhesion of planktonic bacteria to surfaces, including abiotic surfaces (plastics, glasses, metals and minerals) and biotic surfaces (plant and animal tissues). The attached cells will then produce a matrix, consisting of exopolymeric substances, and multiply, thereby forming a so-called microcolony. From this microcolony, a mature biofilm can arise. In the final stage of biofilm formation, cells may detach and return to planktonic life or die (Hall-Stoodley et al., 2004).

Leptospira spp. belong to the bacterial phylum of Spirochaetes, an evolutionarily and structurally unique group of bacteria. These bacteria are composed of both saprophytic and pathogenic members, such as Leptospira biflexa and Leptospira interrogans, respectively (Faine et al., 1999). Leptospires are motile, obligately aerobic, and slow-growing bacteria that have an optimal growth temperature of 30 °C. They are able to survive in soil and water for long periods (Henry & Johnson, 1978; Trueba et al., 2004). Pathogenic species are the causal agents of leptospirosis, a widespread zoonosis that is a major public health problem in developing countries in South-East Asia and South America. In the animal reservoirs of the disease such as rodents, infection produces chronic and persistent asymptomatic carriage in the renal tubules and bacteria are then excreted in urine. Humans are usually infected through cut or abraded skin contact with water contaminated by the urine of animal reservoirs (McBride et al., 2005). More than 500,000 cases of severe leptospirosis occur each year, with a mortality rate of 5–20 % (WHO, 1999).

Because of the association of both saprophytic and pathogenic leptospires with water sources, we sought to characterize biofilm development by these microorganisms. Although Singh et al. (2003) revealed the presence of Leptospira spp. in biofilms of dental water unit systems by 16S rDNA sequencing, biofilm formation by these organisms has not been characterized to our knowledge. Among the order Spirochaetales, only Treponema denticola, which is phylogenetically distant from leptospires, was shown to form biofilms in vitro (Vesey & Kuramitsu, 2004).

Biofilm formation by Leptospira spp. may play an important role in their ability to survive in diverse
environmental habitats, including in the host. This paper describes, for the first time to our knowledge, the characteristics of *Leptospira* spp. biofilms. We also discuss the possible roles of these biofilms in the lifestyles of these bacteria.

**METHODS**

**Bacterial strains and growth conditions.** The strains used for this study are listed in Table 1. *L. biflexa* serovar Patoc strain Patoc1 (Institut Pasteur) and *L. interrogans* serovar Lai strain Lai 56601 (gift from the National Institute for Communicable Disease Control and Prevention, ICDC China CDC) were used as model bacteria to study biofilm formation. Most of the strains were from the collection maintained by the National Reference Laboratory for leptospirosis at the Institut Pasteur. Additional strains were obtained from humans, dogs and rats (humans, strains L1130, L1 133 and Eco Challenge; dogs, strains Hook and Kito; rats, strains R 59 and R 61) and were provided by Albert I. Ko (Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil). High-passage strains refer to strains that have been subcultured in EMJH liquid medium more than 10 times. Virulence of low-passage strains was maintained by passages in guinea pigs or hamsters. All strains were cultured without shaking at 30 °C in EMJH (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) broth containing 1% BSA.

**Biofilm experiments.** Strains were replicated without shaking at least twice in liquid EMJH before performing biofilm experiments. Tests were performed with a starting bacterial suspension inoculum of $10^6$ bacteria ml$^{-1}$ from a mid-exponential-phase culture.

Table 1. Biofilm-forming capacities of *Leptospira* strains grown in static EMJH liquid. *Leptospira* strains were cultured in glass tubes for 2 months at 30 °C as indicated in Methods.

Data are the results of at least three independent observations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Serovar*</th>
<th>Strain†</th>
<th>Biofilm formation‡</th>
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<td>Veldrat Semarang 173</td>
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<tr>
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<td>Castelon3 HP</td>
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<td>Hardjobovis</td>
<td>Sponselee HP</td>
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<td>Hebdomadis</td>
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*When underlined, only the serogroup of the studied strain is indicated (the serovar was not identified). †HP, high-passage strain; LP, low-passage strain. ‡Biofilm formation at the liquid–air interface. Symbols: –, does not form biofilm; +, forms biofilm.
10^6 bacteria ml⁻¹), unless stated in the text. All biofilm experiments were performed without shaking the cultures.

Biofilm formation was tested in glass tubes with 10 ml EMJH liquid medium. Tubes were incubated at 30 °C for a period of 2 months and cultures were observed daily for the formation of surface-associated biofilms at the air–liquid interface and floating biofilms, i.e. floating pellets that cover the liquid medium surface (Table 1). Biofilm production of *L. biflexa* was also assessed in a low-nutrient environment (inoculation of a 1 ml exponential-phase culture grown in EMJH into 9 ml mineral water). For this purpose, *L. biflexa* was cultured in glass tubes with filter-sterilized natural mineral water. Biofilm production in commercially available non-carbonated natural mineral water (pH 6.8; mineral content (mg l⁻¹): Na⁺, 2.7; K⁺, 0.9; Ca²⁺, 7.1; Mg²⁺, 2; Cl⁻, 2; SO₄²⁻, 6.6; NO₃⁻, 2; HCO₃⁻, 24) was investigated over 2 weeks.

Biofilm formation was measured in 12-well polystyrene plates (flat-bottom wells, tissue culture treated; Corning) with 700 μl EMJH liquid medium. Polystyrene plates were sealed during incubation to avoid desiccation. At different time points, the liquid culture was removed by aspiration and the wells were gently rinsed once with distilled water to remove non-adherent planktonic cells. Surface-associated cells were air-dried for 15 min and fixed with 2% sodium acetate. The sodium acetate solution was removed by aspiration and surface-associated cells were allowed to dry again. Cells were then stained with 900 μl 1% crystal violet solution for 20 min. The crystal violet was removed by aspiration, and the wells were carefully rinsed three times in distilled water. Crystal violet remaining in the wells was then dissolved in 1 ml of an ethanol/acetone (v/v 80/20) solution and the absorbance was measured.

**Light microscopy of biofilms.** Glass slides (76 x 26 mm, Menzel-Glaser) were incubated half submerged in a bacterial suspension (initial concentration 10^6 bacteria ml⁻¹) and observed at different times (1, 6, 16, 24, 40, 48, 64, 72, 160 and 190 h). After incubation, slides were rinsed three times in distilled water, air-dried, fixed by heating and observed by phase-contrast microscopy using a Nikon FXA microscope (200 x magnification).

**Electron microscopy of biofilms.** For electron microscopy, glass coverslips (18 x 18 mm, Menzel-Glaser) were placed into wells of 12-well polystyrene plates (Corning) with 2 ml of a bacterial suspension at 10^6 bacteria ml⁻¹ for *L. biflexa* and at 5 x 10^6 bacteria ml⁻¹ for *L. interrogans*, and cultures were incubated for 2 and 8 days, respectively. Coverslips were then removed and rinsed once in sterile distilled water to remove non-adherent planktonic cells. For scanning electron microscopy (SEM), *L. biflexa* and *L. interrogans* biofilms were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer at 4 °C for 1 h and overnight, respectively. The floating biofilm of *L. biflexa* was grown in 10 ml liquid EMJH medium for 72 h at 30 °C, then put on a glass coverslip, dried for 10 min at room temperature and fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer at 4 °C for 1 h. Fixed samples were rinsed three times with 0.2 M cacodylate buffer, post-fixed with 1% osmium tetroxide in 0.2 M cacodylate buffer for 15 min, and washed in water. Samples were treated with 0.2% tannic acid for 20 min, rinsed with water and treated with 0.5% osmium tetroxide in 0.2 M cacodylate buffer for 5 min; this step was done twice. Samples were gradually dehydrated in ethanol baths, desiccated and carbon evaporated. Samples were observed with a secondary electron in-lens (SEI) detector using a JOEL JSM 6700F field emission scanning electron microscope. For cryo-scanning electron microscopy, after washing coverslips in sterile water, they were submerged in liquid nitrogen, cryo-fractured, sublimated for 20 min at −95 °C, and metallized with chrome for 120 s. Samples were observed with a lower electron image (LEI) detector using the above scanning electron microscope. For transmission electron microscopy (TEM), biofilms were fixed in 1.6% glutaraldehyde/0.1 M Sorenson buffer pH 7.2 and rinsed three times for 10 min in Sorenson buffer. Biofilms were post-fixed in 1% osmium tetroxide/0.1 M Sorenson buffer for 1 h, washed in water, dehydrated through a graded series of ethanol baths and embedded in Epon. Ultrathin sections were observed using a JEOL JEM 1010 electron microscope.

**RESULTS AND DISCUSSION**

**Formation of biofilms on abiotic surfaces by saprophytic and pathogenic *Leptospira* species**

The genus *Leptospira* is composed of more than 16 pathogenic and saprophytic species (Levett, 2001). Although these spirochaetes have the ability to survive in diverse environmental habitats, we lack a fundamental understanding of most aspects of their biology.

We initially observed that, under static conditions, the pathogen *L. interrogans* serovar Lai strain Lai 56601 formed a halo attached to the wall of glass tubes at the air–liquid interface in approximately 10 days at 30 °C (Fig. 1). For the saprophyte *L. biflexa* serovar Patoc strain Patoc1, when grown under static conditions, cells were found to strongly attach to the glass surface after 2 days. For *L. biflexa*, we also observed the formation of a floating pellicle at the liquid–air interface after 4–5 days’ incubation (Table 1). We therefore tested a variety of saprophytic and pathogenic strains for their ability to form biofilms attached to glass tubes or floating pellets in nutrient-rich liquid medium in glass tubes (Table 1). A total of 90% of the tested strains, which belong to seven *Leptospira* species, exhibited the ability to form biofilms. Whereas saprophytes formed biofilms in 2–5 days, a mean of 20 days was necessary for biofilm formation by pathogens (data not shown). This is correlated with the growth rates of saprophytes and pathogens, respectively (Faine et al., 1999). Only a minority of strains were also able to form floating biofilms. Finally, three strains did not form biofilms on glass tubes (Table 1).
Pathogenic leptospires are known to show reduced virulence phenotype after repeated in vitro cultures (Haake et al., 1991). It has also been reported that colony morphology variants (leptospires grow into solid medium in subsurface colonies) may arise after continuous in vitro passages (Wood et al., 1981). We therefore assessed the ability of low- and high-passage strains to form biofilms. Pathogenic strains recovered from experimentally infected guinea pigs, or low-passage strains, retained the biofilm formation phenotype. Similarly, the biofilm formation phenotype was maintained when strains were passaged in liquid cultures. Generally, low-passage strains showed a higher level of biofilm formation (reduced time for the appearance of biofilm and increased biomass) than high-passage strains (data not shown).

The saprophyte *L. biflexa* serovar Patoc strain Patoc1 and the pathogen *L. interrogans* serovar Lai strain 56601 were chosen for further biofilm analysis because these strains have been well characterized and both their genomes have been sequenced (M. Picardeau, unpublished; Ren et al., 2003).

We assessed biofilm formation under static conditions at different temperatures. In this *in vitro* model of biofilm formation in tubes, the temperature of incubation did not influence the development of *L. biflexa* biofilms, as we observed formation of similar surface-attached biofilms at 21, 30 and 37 °C. The ability to form biofilms was also assessed in a low-nutrient environment (see Methods). Again, *L. biflexa* was observed to form biofilms in glass tubes with natural mineral water. However, the strain showed a delay in biofilm production, which correlated with slower growth in comparison to growth in EMJH medium (data not shown).

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**Fig. 1.** Visualization of biofilms formed by *Leptospira* spp. (A) Surface-attached biofilm of *L. interrogans* serovar Lai was found at the air–liquid interface in glass tubes containing 10 ml EMJH liquid medium incubated at 30 °C for 10 days in a stationary batch culture (1 ml liquid medium was removed from the glass tube before taking the picture). (B) Crystal violet staining of biofilms of *L. biflexa* serovar Patoc attached to polystyrene plates at 2 days.

**Fig. 2.** Light micrographs of *L. biflexa* biofilms on glass slides. (A) Single bacteria attached to glass at 1 h at the air–liquid interface. (B) Cell aggregates forming intricate network structures at 24 h at the air–liquid interface. (C) Attached bacteria form microcolonies covered by an amorphous matrix at 2 days. (D) Biofilms are less dense below the liquid–air interface at 2 days. (E) Cellular aggregates are interspersed with areas devoid of cells or matrix at 6 days at the air–liquid interface. (F) Cells from a pellicle (floating biofilm) form a dense zone of bacteria at 4 days.
For *L. interrogans*, biofilms were only observed at 30 and 37 °C, but bacterial growth was not optimal at 21 °C (data not shown).

We also assessed biofilm formation of *L. biflexa* on different plastic surfaces by visual and direct microscopic examination after repeated washes. *L. biflexa* formed biofilms after 2 days growth not only on polystyrene plates (Fig. 1B), but also on polyolefin polymers (data not shown). Although we observed slight adherence of *L. interrogans* to polystyrene flasks and plates, biofilms were not resistant to washes. The fastidious growth of pathogenic leptospires may affect the rate of bacterial attachment and biofilm formation in static conditions. A continuous-culture biofilm system may be more suitable for cultivating and characterizing biofilms of pathogenic leptospires.

**Microscopy analysis of biofilms on abiotic surfaces**

As a first approach, we used light microscopy to study biofilm formation by *L. biflexa* over time. Cells were allowed to adhere to and form biofilms on glass slides and were observed by phase-contrast light microscopy at intervals. The adherence of *L. biflexa* and subsequent biofilm formation on a glass surface is illustrated in Fig. 2.

*L. biflexa* formed a dense layer on glass slides, which increased in a time-dependent manner, reaching maximal levels at 48 h. Fig. 2(C) shows typical surface coverage of the biofilm. As documented for other biofilm-forming bacteria, the developmental process of *L. biflexa* biofilm formation can be characterized as a three-step process: (i) adherence of planktonic cells to the surface (Fig. 2A), (ii) biofilm maturation (Fig. 2C), and (iii) complete or partial disintegration of the biofilm (Fig. 2E). The microscopic aspect of the pellicular biofilm resembles the biofilm attached to a glass surface (Fig. 2). *L. biflexa* cells form denser biofilms at the air–liquid interface than below this interface (Fig. 2). This is probably due to the fact that leptospires are motile and obligate aerobic spirochaetes.

We used SEM to confirm the results obtained by light microscopy and to elucidate the biofilm architecture. We observed thick biofilms consisting of bacteria associated with amorphous material at the air–liquid interface (Figs 3 and 4). For *L. biflexa*, there were intertwined networks of attached cells that served as scaffolding for further biofilm development over time. At 48 h, we observed the formation of microcolonies (Fig. 3A) and, at a higher magnification, large clusters of cells surrounded by components of the matrix (Figs 3B and C). For *L. interrogans*, at 8 days, microcolonies appeared as large,
distinct mound-shaped colonies, with the top cell layer covered with a matrix-like material (Fig. 4A). Two types of biofilm architecture were observed: one (L. interrogans) consisting of large, distinct mound-shaped microcolonies, and the other (L. biflexa) showing smaller microcolonies with a flatter structure that were linked together by a complex network of bacteria. In SEM, samples are prepared by fixation, staining, drying and coating prior to imaging under high vacuum. In other bacterial biofilms, the matrix is essentially composed of water, but also includes polysaccharides, proteins and nucleic acids. Desiccation of the samples may therefore alter the biofilm morphology. We therefore used TEM, which stabilizes the biofilms by embedding samples in a resin, and cryo-scanning electron microscopy. In this latter technique, samples are not treated chemically as in conventional scanning, but are immediately frozen in liquid nitrogen. This technique is recommended for fragile and highly hydrated samples. By cryo-scanning electron microscopy and TEM, Leptospira biofilms appear to form multicellular and multilayered structures, ranging from 5 to 14 μm and from 10 to 30 μm in thickness for L. biflexa and L. interrogans, respectively (Fig. 5). Bacteria were separated by void spaces that may correspond to channel-like spaces that provide oxygen and nutrients to embedded cells. For planktonic bacteria (culture grown under agitation with no formation of biofilm), SEM identified only a few spirochaetes on the surface and no multilayered bacteria (data not shown).

The constituents of the matrix of leptospirae remain to be determined. In other bacteria, polysaccharides, after water, are major components of the matrix (Hall-Stoodley et al., 2004). The annotation of the genomes, including the genome of the saprophyte L. biflexa serovar Patoc strain Patoc1 (M. Picardeau, unpublished), suggested that several genes are involved in the biosynthesis of polysaccharides (Bulach et al., 2006; Nascimento et al., 2004; Ren et al., 2003). Approximately 30 genes encoding putative glycosyltransferases, which are capable of synthesizing levans and dextrans, are found in the genomes of both saprophytic and pathogenic Leptospira species. Interestingly, genes involved in alginate biosynthesis are present in both L. biflexa (11 genes) and L. interrogans (8 genes), but are absent in Leptospira borgpetersenii, a finding consistent with the reduced environmental survival of L. borgpetersenii (Bulach et al., 2006).

**Quantification of biofilm formation**

Biofilm formation can be quantified by crystal violet staining of the cells attached to the surface. In the present

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**Fig. 4.** Scanning electron microscopy of L. interrogans biofilms on a glass surface. At 8 days, cells were strongly attached to the glass surface at the liquid–air interface (A–D). The pathogenic strain forms biofilms consisting of large mound-shaped microcolonies (a). Micrographs show organisms embedded in a matrix (B–D). (D) Higher magnification of the boxed area in (B).
study, we showed that 12-well polystyrene plates yielded the largest amount of surface-attached biomass for *L. biflexa* (Fig. 1B). In light of these results, we assayed biofilm formation of *L. biflexa* in polystyrene plates at 30 °C under static conditions. In this assay, biomass was formed at the bottom of the wells and at the liquid–air interface (data not shown), and crystal violet staining reached a maximal $A_{600}$ of approximately 2 after 2 days. Afterwards, the crystal violet staining became lower and $A_{600}$ values were low as soon as 72 h incubation (Fig. 6). The increase in crystal violet staining and biofilm production was correlated with bacterial growth in the medium and ceased in the stationary growth phase.

To study the roles of the flagella and motility in the biofilm formation, we tested the *L. biflexa flaB* mutant (Picardeau *et al.*, 2001), which is defective in the synthesis of the endoflagella and non-motile, in polystyrene plate assays. The *flaB* mutant was able to form biofilms, showing that the flagella of leptospires, which are located in the periplasm, are not essential for biofilm formation. However, the *flaB* mutant exhibited a delay in biofilm formation (Fig. 6). To determine the effect of alginate on biofilm formation by *L. biflexa*, we generated mutants in two of the genes, *alg1* (LEPBlA2006) and *alg2* (LEPBlA2008), that could be involved in alginate biosynthesis. LEPBlA2006 and LEPBlA2008 exhibit 41 and 66% similarity with the putative alginate biosynthesis protein AlgJ of *Pseudomonas syringae* and a putative alginate O-acetyltransferase of *Bacillus anthracis*, respectively. The average level of maximum biomass for the mutant strains was similar to the parent strain. However, this level was reached with a 24 h lag for the *alg1* mutant compared with the parent strain. The lack of an obvious phenotype in these mutants could be due to functional redundancy with other alginate biosynthetic genes.

We recently identified transposon mutants of *L. biflexa* that were not able to use some iron sources for survival (Louvel *et al.*, 2005, 2006). Using transposon mutagenesis, we could...
also screen a library of random mutants in a polystyrene plate assay to identify biofilm-defective mutants.

Role of biofilm formation in *Leptospira* spp.

Bacterial biofilms have a structurally complex and dynamic architecture and can develop on many surfaces. Both saprophytic and pathogenic *Leptospira* strains were found to form surface-associated biofilms in standing cultures. The progression of biofilm formation by *Leptospira* spp. mimics other biofilms described in the literature, beginning with individual bacteria adhering to the abiotic surface, expansion into colonies, and formation of a three-dimensional structure (Hall-Stoodley et al., 2004).

Our data, based on SEM and TEM, show differences in biofilm architecture between a strain of the pathogen *L. interrogans* serovar Lai and a strain of the saprophyte *L. biflexa* serovar Patoc in the tested conditions. This may be correlated with phylogenetic and lifestyle differences between pathogenic and saprophytic strains. *L. interrogans* is an important animal and human pathogen, but it was not previously considered as a biofilm-building species. Our study revealed that the majority of pathogenic strains produced detectable biofilms on abiotic surfaces in *vitro*. Bacteria in biofilms exhibit properties distinct from those of planktonic cells, such as increased resistance to biocides and antimicrobial agents (Hall-Stoodley et al., 2004). Multi-species biofilms may also contribute to gene transfer between micro-organisms (Wang et al., 2002). It was recently shown that severe leptospirosis was associated with exposure to a high concentration of leptospires in environmental water samples (Ganoza et al., 2006). Biofilm formation may contribute to long-term survival in environmental water (Trueba et al., 2004). In our study, natural mineral water was also used as the sole source of nutrient supply to allow the development of *L. biflexa* biofilms. The ability of pathogenic *Leptospira* to survive in aquatic ecosystems in biofilms could be one of the main factors controlling environmental survival and disease transmission. Since the long-term colonization of proximal renal tubules of mammalian maintenance host species by pathogenic leptospires is believed to proceed via the formation of cell aggregates (A. I. Ko, unpublished data), biofilm formation may also play an important role in maintaining chronic carriage of the pathogen *L. interrogans* in animal reservoirs. The determination of the structure and the mechanism of colonization of kidneys in the animal reservoir will no doubt be of fundamental importance.

Further studies are required to study the biofilms of these ubiquitous organisms in the context of their environmental habitats such as fresh water or renal tubules.

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