RESEARCH ARTICLE

Coexistence and survival of pathogenic leptospires by formation of biofilm with Azospirillum

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One sentence summary: Little is known regarding the mechanisms by which pathogenic leptospires persist in aqueous environments. This is the first report demonstrating association of pathogenic Leptospira with the environmental biofilms.

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ABSTRACT

Pathogenic Leptospira spp. represent one cause of leptospirosis worldwide and have long been regarded as solitary organisms in soil and aquatic environments. However, in the present study, Leptospira interrogans was observed to be associated with environmental biofilms with 21 bacterial isolates belonging to 10 genera. All 21 isolates were examined for their coaggregation and biofilm-forming ability with leptospires in vitro. Among these, Azospirillum brasilense RMRCBP showed maximum interspecies coaggregation with leptospiral strains (>75%, visual score of +4). Other significant coaggregating isolates belonged to the genera Sphingomonas, Micrococcus, Brevundimonas, Acinetobacter and Paracoccus. Biofilms of leptospires in combination with A. brasilense showed high resistance to penicillin G, ampicillin and tetracycline (minimum bactericidal concentration ≥ 800 μg/mL) and tolerance to UV radiation and high temperature (up to 49 °C). This study hypothesized that biofilm formation with A. brasilense protects the pathogenic Leptospira from adverse environmental conditions/stress. This coexistence of pathogenic Leptospira with other bacteria may be the key factor for its persistence and survival. However, the mechanism of biofilm formation by leptospires needs to be explored to help devise an appropriate control strategy and reduce transmission of leptospires.

Keywords: Biofilm; Microbial cooperation; Leptospira; Azospirillum

INTRODUCTION

The genus Leptospira belongs to the phylum Spirochaetes and includes both pathogenic and saprophytic species (Ko, Goarant and Picardeau 2009). Pathogenic forms of leptospires are responsible for the global re-emergence of zoonotic diseases (Levett 2001; Ko, Goarant and Picardeau 2009), causing infection in humans through contact with an infected animal’s urine or contaminated soil and water. However, there are very few details regarding the survival mechanism of pathogenic leptospires in soil and aqueous environments outside of the mammalian host (Levett 2001). As with other spirochaetes, Leptospira can alter their morphology according to environmental conditions and these changes include cell aggregation (Trueba et al. 2004), cell elongation and sphere and granule formation (Ellis et al. 1983). However, other adaptations/interactions of leptospires vital to their survival in the environment need to be explored. Bacteria are social rather than solitary organisms and rely on a range of population-level traits, such as coaggregation, biofilms, cell–cell communication and cooperative environmental resistance (Greenberg 2010; de Vargas Roditi, Boyle and
Xavier 2013). The role of coaggregation in freshwater biofilms is of particular importance because they are a major source of pathogens. Opportunistic pathogens isolated from biofilms in water systems include Mycobacterium avium, Pseudomonas aeruginosa, Klebsiella spp., Legionella spp. and Flavobacterium spp. (Rickard et al. 2004). Free-living bacteria such as Azospirillum, Sphingomonas, Micrococcus, Klebsiella and Azotobacter spp. are known for their capacity to aggregate with environmental microbes, thus affecting their dispersal and survival in soil (Burn-dman et al. 1998; Min, Zimmer and Rickard 2010).

Under the order Spirochaetales, Treponema denticola, Borre lia burgdorferi (Sapi et al. 2012), Brachyspira spp. (Wanchantheuk et al. 2010), Spirocheta spp. and Spirocoma spp. (Abed et al. 2007) are well known to form coaggregates and biofilms. A synergistic effect has been demonstrated between T. denticola and Porphyromonas gingivalis in biofilms (Mitsunori, Akihiko and Howard 2005).

Leptospira were known to be solitary organisms until they were commonly observed in multibacterial biofilms collected from water pipes of dental care units (Singh et al. 2003). In addition, leptospires have also been recently demonstrated to form biofilm in vitro (Ristow et al. 2008). However, synergism involving multiple bacteria and Leptospira in the natural environment has not been reported to our knowledge. Therefore, the present study attempted to identify the coaggregative and biofilm-forming ability of leptospires with environmental bacteria, which may enable them to act as part of a protective mechanism against adverse environmental conditions.

MATERIALS AND METHODS

Leptospiral strains

The leptospiral strains used in this study are listed in Table S1 (Supplementary data). All of the reference strains were maintained in the Leptospira repository of the National Reference Laboratory for Leptospiriosis at the Regional Medical Research Centre (ICMR), Port Blair, India. The strains were cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium supplemented with enriched medium (containing BSA and Tween 80) at 30°C for 7 days with periodic subculture.

Screening for environmental biofilm-forming bacteria

Five sterile glass rods (12′′) were buried up to 3′′ deep in 10 diferent waterlogged paddy field soils (28 ± 1°C, pH 6.5 ± 0.4) and allowed to rest for 48 h. Biofilm formation at the air–liquid and soil–water interfaces was observed. The rods were brought to the laboratory in aseptic condition and biofilms were scraped out. These were pooled in 1 mL of normal saline (0.8% NaCl) and vortexed for 15 min. Three aliquots (40 μL each) of biofilm suspension were streaked onto nutrient agar (NA; Hi-Media, India) medium and incubated at 28°C for 48 h. Individual colonies were picked and replicated onto NA medium to obtain pure colonies.

Screening and isolation of Leptospira from biofilm

To isolate the Leptospira, each biofilm suspension was filtered through a 0.22-μm filter (Millex-GV). The filtrate was inoculated onto semi-solid EMJH medium supplemented with enrichment medium (containing BSA + Tween 80) and 1.5% fetal calf serum and incubated at 28°C for 1 month, with its growth being monitored periodically. Once appropriate growth was achieved, leptospiral isolates were subcultured onto fresh EMJH liquid medium and maintained for further studies. The remaining biofilm suspension was subjected to DNA extraction using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s protocol. PCR assay was carried out using primers targeting the lipL32 gene (Ahmed et al. 2006) for Leptospira spp.

Identification of isolates

Environmental bacterial isolates and leptospiral isolates were cultured in nutrient medium for 12 h and EMJH medium for 6–7 days at 28°C. Genomic DNA was extracted following the procedure described by Ausubel et al. (1995). PCR was performed using 16S rRNA primers (Table S2, Supplementary data) following standard procedures (Lane et al. 1985). The amplified products were sequenced using DNA Analyzer 3730 (Applied Biosystems, CA, USA). Partial 16S rRNA gene sequences and sequences of closely related strains in the NCBI (National Center for Biotechnology Information) database were retrieved and assembled using MEGAS software (Tamura et al. 2011) and unknown sequences were identified using the ClustalW alignment tool of MEGAS.

Coaggregation of Leptospira with environmental isolates

A modified quantitative spectrophotometric assay described by Ledder et al. (2008) was used to determine the coaggregation activity between leptospires and 21 test bacterial isolates (environmental biofilm isolates). Briefly, bacteria were harvested by centrifugation at 5000 g for 10 min and resuspended in coaggregation buffer (0.1 mM CaCl2, 0.1 mM MgCl2, 0.15 M NaCl and 3.1 mM NaN3 in 1 mM Tris buffer, pH 7.0) (Cisar, Kolenbrander and McIntire 1979). Strains and isolates were washed three times separately in coaggregation buffer and later resuspended to give an OD600 of 1.0. Equal volumes of each suspension were mixed in sterile cuvettes and the optical densities were recorded. Autoaggregation was determined using an identical method by combining two equal volumes of the same bacterial suspension. The mixtures were kept for 1 h at room temperature (25 ± 1°C) to allow coaggregation to occur and the OD value was recorded. The percentage of coaggregation was calculated using the following equation:

\[
\text{Coaggregation} = \frac{([\text{Pre-incubation value OD}_{600}]}{([\text{Pre-incubation value OD}_{600}]} - \text{Test value OD}_{600}) \times 100
\]

To characterize the mechanisms of coaggregation among leptospires and environmental isolates, a strongly coaggregating environmental isolate (RMRCBP) was selected and resuspended at an OD600 of 1.0 in coaggregation buffer as described above. Similarly, leptosporial strains at a concentration of ~2 × 108 cells/mL were prepared as described above. Equal volumes of each suspension (500 μL) were thoroughly mixed and incubated at room temperature (25 ± 1°C) for 2 min.

Visual aggregation assays

Coaggregation was scored by visual assay as described by Cisar, Kolenbrander and McIntire (1979). The assay was performed three times, by assigning consistent scores. Coaggregation between isolates was scored from 0 to +4 as follows: 0, no visible coaggregation; +1, small uniform coaggregates; +2 large coaggregates with turbid suspension; +3, large coaggregates that
settled rapidly, leaving a turbid supernatant; and +4, large co-aggregates that settled rapidly, leaving a clear supernatant. Cell suspensions containing single isolates were scored similarly to determine autoaggregation (self-aggregation) according to the method of Vornhagen et al. (2013).

Influence of heat on coaggregation

The effect of heat on coaggregative interaction was determined by heating RMRCBP at 85°C for 30 min (Kolenbrander 1995). A suspension containing an equal volume of leptospiral strains and RMRCBP was made and the aggregation pattern was visualized and scored as described above.

Biofilm formation assay

A dual system of biofilm formation was studied between leptospiral strains and RMRCBP, as described by O’Toole (2011). Briefly, 25-μl aliquots of (a) Leptospira alone (L. interrogans Pomona, 2 × 10⁷ cells/mL) and (b) co-culture (L. interrogans Pomona with RMRCBP at equal concentrations of 2 × 10⁷ cells/mL) were prepared. These cultures were added into wells of a flexible polyvinyl chloride (PVC) 96-well plate (Corning, Rochester, NY, USA) containing 175 μL of growth medium (EMJH). The cells were allowed to develop biofilm at 30°C (without shaking) for 48 h, replenishing with fresh growth medium every 12 h. The fresh EMJH medium in the well served as a control and the experiment was performed in triplicate. Each well was washed three times with phosphate-buffered saline (PBS) under aseptic conditions to remove unbound bacteria. After washing, the wells were screened for biofilm formation by using crystal violet as described elsewhere (O’Toole 2011). To quantify the attached Leptospira and Azospirillum spp., cells were scraped and DNA was extracted from the sediment using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s protocol. Quantitative real-time PCR (SYBR Green method) was performed to target the lpl32 and mnfH genes (Table S2, Supplementary data) for Leptospira spp. and Azospirillum spp., respectively (Ganoza et al. 2006). Biofilm formation was determined using an identical method by combining two equal volumes of the same leptospiral suspension. The experiment was repeated for 25 strains (Table S1, Supplementary data) of Leptospira and the ability of these strains to form biofilm with RMRCBP was tested as described above.

Microscopic analysis

Leptospira interrogans Pomona and RMRCBP were grown in EMJH medium, washed twice with PBS and centrifuged at 2800 g. The culture pellet was suspended in sterile paddy field water (pH 6.8; filter sterilized with a 0.22-μm filter, Millex-GV) at an optimum cell density of 2 × 10⁷ cells/mL. Glass slides (76 × 26 mm, Hi-Media, India) were incubated by submerging half of the slide into a bacterial suspension for different time intervals (1, 6, 16, 24, 48, 64, 72, 160 and 190 h) at 28°C. After incubation, slides were gently rinsed three times in PBS (pH 7.4) and observed under a dark-field microscope (Zeiss AXIO SCOPE A.1, Germany). Similarly, for scanning electron microscopy (SEM), L. interrogans and A. brasilense biofilms were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer and kept overnight at 4°C. These samples were rinsed three times in 0.1 M cacodylate buffer and washed with water. Samples were gradually dehydrated in 10–100% ethanol baths, desiccated and carbon evaporated (Ristow et al. 2008). Samples were observed with a secondary electron lens detector using SEM. All of these experiments were carried out in triplicate.

Viability and recovery

The viability and recovery of Leptospira was studied using biofilms of Leptospira and isolated RMRCBP in 96-well PVC plates. Alamar blue dye (Pettit et al. 2005) was used to evaluate viability. Five microliters of Alamar blue solution (Hi-Media, India) were added to each well and the plate was incubated for 6 h. The viability was noted by observing the color intensity and the plate was read under 570 and 600 nm using a spectrophotometer.

Leptospira interrogans Pomona and isolated RMRCBP were transferred to sterile PBS (pH 7.4; filter sterilized with a 0.22-μm filter, Millex-GV) at an optimum cell density of 2 × 10⁷ cells/mL. The tubes were incubated in static conditions at 28°C to allow for the formation of biofilm. The duration of viability and recovery of live leptospires was checked after 1 year of incubation, as described by Bjerkan, Witoso and Bergh (2009). Briefly, the content was thoroughly scraped and centrifuged at 2800 g. The pellet was mixed with 1 mL of EMJH liquid medium and sonicated at 40 Hz for 10 sec five times with a 30-sec gap per cycle (Labsonic, Sartrius Stedim Biotech, Germany). The disintegrated bacterial suspension was filtered through a 0.22-μm filter (Millipore) and the filtrate was collected. Recovery of the leptospires was determined by inoculating 0.5 mL of the mixture into fresh EMJH medium and incubating at 30°C for 1 month, with regular monitoring of the growth.

Susceptibility to antibiotics

Biofilm containing L. interrogans Pomona and isolated RMRCBP were grown in 96-well PVC plates as described above. Antibiotics (penicillin G, ampicillin and tetracycline) were prepared in concentrations ranging from 0.1 to 800.0 μg/mL (U/mL for penicillin G) (Murray and Hospenthal 2004). To determine the antibiotic exposure, 200 μL of the pre-diluted antibiotic solution (penicillin G, ampicillin and tetracycline) was mixed with EMJH medium and added to wells. Control wells contained 200 μL of fresh culture at a concentration of 2 × 10⁷ cells/mL, which was maintained at 30°C (Murray and Hospenthal 2004). Antibiotic solutions were discarded and the wells were filled with 100 μL of PBS. After exposure to antibiotics, the viability and recovery of leptospires were estimated as described above. The experiment was performed in triplicate.

Resistance to UV

Biofilm of L. interrogans Pomona with isolated RMRCBP were allowed to form on 96-well plates as described above. Approximately 200 μL of paddy field surface water (filter sterilized with a 0.22-μm filter, Millex-GV) was added to each well and the same amount of planktonic leptospiral cells (2 × 10⁷ cells/mL) in paddy field surface water (pH 6.8) were added to empty wells as a control. Experimentation with UV exposure was carried out as described by Tang, Dziallas and Grossart (2011). Lids of all 96-well plates were opened and the plates exposed to continuous UV light for 2 h on a sterile bench. UV light was produced by 15 Watt bulbs (Philips 15W/G15T8) placed ~15 cm above the plates. The plates were cooled with ice placed underneath to avoid overheating by UV radiation. After each exposure, plates were completely wrapped in aluminum foil to prevent further exposure to light.
UV dosage ($D; \mu W/cm^2$) was calculated as follows:

$$D = I \times T$$

where $I$ is UV intensity and $T$ is exposure time (600, 1200, 2400, 3000, 3600, 4200, 4800, 5400, 6000, 6600 and 7200 sec). Average UV intensity as per the manufacturer was 2304 $\mu W/cm^2$ at the level of the plates, which yielded respective UV dosages of $1.3 \times 10^6$, $2.7 \times 10^6$, $4.1 \times 10^6$, $5.5 \times 10^6$, $6.9 \times 10^6$, $8.2 \times 10^6$, $1.1 \times 10^7$, $1.2 \times 10^7$, $1.4 \times 10^7$, $1.5 \times 10^7$ and $1.6 \times 10^7$ $\mu W/cm^2$.

Susceptibility to high temperature

Aliquots (4 mL) of $L.\ interrogans$ Pomonca alone and co-culture mixture ($L.\ interrogans$ with isolated RMRCPB at $2 \times 10^7$ cells/mL) in paddy field water (pH 6.8; filtered sterilized with a 0.22-$\mu$m filter, Millex-GV) were added to glass tubes with a screw cap. Sterile paddy field water in triplicate served as a control. The tubes were incubated at 28°C for biofilm formation and after visible biofilm was formed, tubes were exposed to various temperature ranges (30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 49, 50, 51, 52, 54, 56, 58 and 60°C) by placing the tubes in a water bath for different time intervals (20, 40 and 60 min). After exposure, the biofilm was scraped and sonicated as described previously (Joe et al. 2009) and then inoculated into fresh EMJH medium and incubated at 30°C. The growth was monitored periodically using dark-field microscopy.

Soil adsorption test

The adsorption of $L.\ interrogans$–RMRCPB coaggregates in paddy field soil (pH 6.8) was tested using a modified method described previously (Smith and Turner 1961). Paddy field soil (20 g) was autoclaved in a glass tube. Sterile PBS was poured onto the soil and its level was maintained at 2 cm above the soil. One milliliter of $L.\ interrogans$ Pomonca and isolated RMRCPB ($2 \times 10^7$ cells/mL) mixture was added to the tube and allowed to stand in the incubator at 28 ± 2°C for 24 h. Three milliliters of surface liquid content were aspirated and 3 g of surface soil were collected for genomic DNA extraction as described above. The concentration of leptospiral genomic DNA was quantified in surface water as well as in the soil by using quantitative real-time PCR (Ganoza et al. 2006). The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Screening for environmental biofilm-forming bacteria

Out of 10 biofilm samples screened from different paddy fields, the lipL32 gene was detected in six samples, one of which yielded an isolate (M 10) of $L.\ interrogans$ (GenBank accession number KF170283). Along with $L.\ interrogans$ strain MT 10, 21 isolates of diverse bacterial genera, namely Azospirillum, Sphingomonas, Exiguobacterium, Bacillus, Aeromonas, Lysinibacillus, Acinetobacter, Paracoccus, Brevundimonas and Micrococcus (Fig. 1, Table S3), were identified in the biofilm samples. These genera are ubiquitous and are a common bacterial genera found in biofilms formed in fresh water worldwide (Burdman et al. 1998; Rickard et al. 2004; Vornhagen et al. 2013). Singh et al. (2003) also observed some of the genera (e.g. Sphingomonas and Bacillus) as well as Escherichia, Geobacter and others along with saprophytic Leptospira spp. in biofilms of dental water unit systems.

Coaggregation assay of Leptospira with environmental isolates

Among 21 bacterial isolates in the coaggregation assay, 8 isolates showed coaggregation (~40%) and a positive visual coaggregation score of >1 (Table S4). Of these, $A.\ brasilense$ RMRCPB (GenBank accession number KF150630) showed the highest intensity of interspecies coaggregation (~75%) with leptospiral strains in vitro (Figs 2 and 3D). A previous report showed that bacteria are interlinked with only a few specific coaggregating partners and together all form a network of multispecies biofilm (Vornhagen et al. 2013). In this study, $A.\ brasilense$ RMRCPB combined with each Leptospira in the study set exhibited rapid and complete settling of large coaggregates, leaving a clear supernatant with a visual score of +4 (Table 1). Coaggregation scores for leptospiral strains ranged from +1 to +2 (data not shown). The coaggregation result was in accordance with previous reports where Azospirillum spp. coaggregated (~60%) with Pseudomonas, Bacillus, Azotobacter and Azorhizobium spp. (Joe et al. 2009). A similar high level of coaggregation was also observed among oral bacteria (Ledder et al. 2008). The other significant coaggregating genera in this study were Sphingomonas, Micrococcus, Paracoccus, Acinetobacter, Brevundimonas and Exiguobacterium (Table S4), which were the commonly found coaggregating genera in aqueous biofilms (Rickard et al. 2003; Rickard et al. 2004; Vornhagen et al. 2013). Among these, Azospirillum, Sphingomonas and Micrococcus were shown to function as bridging organisms in biofilm formation (Burdman et al. 1998; Rickard et al. 2002).

Azospirillum is a major inhabitant of soil worldwide and is an important nitrogen fixer that colonizes the soil rhizosphere by aggregating with other environmental bacteria (Greer-Phillips, Stephens and Alexandre 2004; Lerner et al. 2009). Though leptospires were detected in agricultural fields, there are no such reports of their coaggregation or biofilm formation with other bacteria. However, Leptospira were shown to form coaggregates with each other in viscous medium, which is believed to be one of the survival strategies in medium lacking nutrients (Trueba et al. 2004). Heating (up to 65°C) of $A.\ brasilense$ RMRCPB inhibited coaggregation with all of the leptospiral strains tested. A similar observation was obtained by Joe et al. (2009), in which heat treatment of $A.\ brasilense$ MTCC-125 was found to considerably inhibit coaggregation. This indicates that $A.\ brasilense$ might carry proteinaceous adhesins that mediate coaggregation, which have been described in other bacteria such as Streptococcus sanguis, Fusobacterium nucleatum and Bifidobacterium adolescentis (Kolenbrander 1995; Burdman et al. 1999; Ledder et al. 2008).

Biofilm formation with Leptospira

The dual-species model was selected for the study of biofilm formation between leptospiral strains and $A.\ brasilense$ RMRCPB. This model is useful for studying different types of microbial interactions (Simões, Simões and Vieira 2007; Anderson et al. 2008). Although $A.\ brasilense$ RMRCPB aggregated to form biofilms with all of the leptospiral strains tested, the intensity of its aggregation with different leptospiral strains varied over a period of 48 h (Fig. 3B and C). As none of the leptospiral strains formed an auto biofilm over 48 h, there duration of forming auto biofilm ranged between 4 to 21 days. Three leptospiral strains (Hebdomadis, L-14 and 507) did not form any auto biofilm even after 21 days of incubation (data not shown). These results are consistent with an earlier study in which biofilm formation depended on the leptospiral strain and number of passages (Ristow et al. 2008). The present study clearly indicated that
Figure 1. The inferred phylogenetic tree showing the diversity of biofilm bacteria, including 22 paddy field biofilm isolates and 32 reference strains. Evolutionary distances were determined with pairwise dissimilarities of the 16S rRNA gene sequences and the dendrogram was generated using the neighbor-joining algorithm (MEGA5). Closed triangles indicate MT 10 and RMRCBF used for biofilm characterization in vitro and closed circles indicate other biofilm isolates.
Azospirillum spp. play a major role in influencing Leptospira to form biofilms. This phenomenon was previously described in other spirochaetes such as Treponema spp., a distinct relative of Leptospira, wherein P. gingivalis 381 provides a platform for T. denticola to form biofilm (Chen, Palmer and Kuramitsu 2002; Vesey and Kuramitsu 2004; Yamada, Ikekami and Kuramitsu 2005). Quantitative PCR analysis of leptospiral strains and A. brasilense indicated that freshly isolated L. interrogans MT 10 and a few leptospiral strains that showed greater tendency to form auto-biofilm had a higher affinity towards A. brasilense RMRCPB, i.e. Paidjan, But-6, Poi, Sari, Salinem, Patoc-1 and M84, whereas some strains, namely Bankinan 1, Hund utrc-IV and Moskva V, showed a lower affinity to form biofilm with A. brasilense RMRCPB. However, quantitative PCR showed that A. brasilense is required in specific concentrations (ranging between $1.7 \times 10^7$ and $1.8 \times 10^7$ cells/mL), irrespective of the leptospiral strains used for co-culturing (Fig. 3A). This is because coaggregation promotes biofilm integration by facilitating attachment to partner species. This contributes to the extended coaggregation of the second population in dual-species biofilms through competitive interaction (Min and Rickard 2009).

**Microscopic analysis**

Leptospira interrogans–A. brasilense cells formed a dense layer on the glass slides, increasing in a time-dependent manner and reaching a maximum level at 48 h. Initially, small aggregates were formed between L. interrogans and A. brasilense cells (Fig. 4A1 and A2). These aggregates adhered to the glass surface and, with time, formed a mature biofilm. Biofilms were denser at the air–liquid interface, as both leptospires and Azospirillum are aerobic in nature and actively motile. SEM analysis revealed intertwined networks of attached cells of L. interrogans and A. brasilense that served as a scaffold for further biofilm development over time. At higher magnification, tightly packed structures were observed, linked together by a complex network of leptospires and Azospirillum and surrounded by matrix (Fig. 4B1 and B2). It is unclear whether the matrix was synthesized by leptospires or Azospirillum, even though both are known to produce extracellular matrix. The extracellular matrix of Azospirillum spp. has been reported to consist of poly-β-hydroxybutyrate, lipopolysaccharides and proteins (Burdman et al. 1998). However, the composition of the leptospiral biofilm matrix is yet to be explored.

**Viability and recovery of leptospires**

In the co-culture, leptospires remained viable for up to 18 months. A similar observation was made in L. interrogans, Leptospira biflexa and Leptospira meyeri by Barragan et al. (2011), in which Sphingomonas spp. consortium was found to support leptospiral persistence for up to 1 year. Often, dominant
Figure 3. Biofilm-forming ability of different leptospiral strains with A. brasilense RMCPB. (A) Quantitative real-time PCR assay for concentration of attached Leptospira spp. and A. brasilense RMCPB per well in dual-species biofilm. Among the strains used, the number of attached Leptospira ranged between $8.1 \times 10^6$ and $1.6 \times 10^7$ cells/mL. (B) Biofilm quantification by crystal violet staining. Biofilm formed by various strains of Leptospira as visualized with crystal violet staining on a PVC surface (crystal violet staining of wells that contained only plain medium is depicted as control). The amount of biofilm was quantitatively measured at OD$_{570}$. Error bars are standard errors derived from three replicate experiments. (C) The intensity of biofilm formed by various Leptospira strains with A. brasilense RMCPB at the air–liquid interface as visualized by crystal violet staining. (D) Leptospira strains and A. brasilense RMCPB coaggregation. The intensity of coaggregation is shown after 1 h of incubation.

Table 1. Coaggregation scores of RMCPB with 26 leptospiral strains used in this study.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Genomospecies/strain</th>
<th>Coaggregation score, % (±SD)</th>
<th>Visual assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L. interrogans/Ballico</td>
<td>75.26 (±1.14)</td>
<td>+4</td>
</tr>
<tr>
<td>2</td>
<td>L. interrogans/Bankinang 1</td>
<td>77.37 (±3.37)</td>
<td>+4</td>
</tr>
<tr>
<td>3</td>
<td>L. interrogans/Paidjan</td>
<td>75.08 (±2.72)</td>
<td>+4</td>
</tr>
<tr>
<td>4</td>
<td>L. interrogans/Hond urech-IV</td>
<td>77.46 (±0.58)</td>
<td>+4</td>
</tr>
<tr>
<td>5</td>
<td>Leptospira krischneri/3522 C</td>
<td>76.03 (±1.70)</td>
<td>+4</td>
</tr>
<tr>
<td>6</td>
<td>L. interrogans/Djasiman</td>
<td>75.42 (±2.32)</td>
<td>+4</td>
</tr>
<tr>
<td>7</td>
<td>L. interrogans/Moskva V</td>
<td>75.42 (±1.23)</td>
<td>+4</td>
</tr>
<tr>
<td>8</td>
<td>L. interrogans/CH-31</td>
<td>75.95 (±2.82)</td>
<td>+4</td>
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<td>L. interrogans/Hebdomadis</td>
<td>75.22 (±6.17)</td>
<td>+4</td>
</tr>
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<td>10</td>
<td>Leptospira fanei/But-6</td>
<td>78.87 (±1.42)</td>
<td>+4</td>
</tr>
<tr>
<td>11</td>
<td>L. borgpetersenii/Poi</td>
<td>77.27 (±2.13)</td>
<td>+4</td>
</tr>
<tr>
<td>12</td>
<td>L. borgpetersenii/Sari</td>
<td>76.71 (±0.69)</td>
<td>+4</td>
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<td>13</td>
<td>Leptospira noguchii/CZ-214-K</td>
<td>76.21 (±1.37)</td>
<td>+4</td>
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<td>14</td>
<td>L. interrogans/Pomona</td>
<td>75.25 (±1.24)</td>
<td>+4</td>
</tr>
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<td>+4</td>
</tr>
<tr>
<td>16</td>
<td>L. interrogans/Salinem</td>
<td>78.00 (±0.60)</td>
<td>+4</td>
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<td>+4</td>
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<td>18</td>
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<td>19</td>
<td>Leptospira santarosai/1343-K</td>
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<tr>
<td>20</td>
<td>L. borgpetersenii/ Peripelician</td>
<td>76.43 (±1.39)</td>
<td>+4</td>
</tr>
<tr>
<td>21</td>
<td>L. borgpetersenii/M84</td>
<td>75.34 (±1.90)</td>
<td>+4</td>
</tr>
<tr>
<td>22</td>
<td>L. krischneri/Nicalaeva</td>
<td>76.17 (±2.34)</td>
<td>+4</td>
</tr>
<tr>
<td>23</td>
<td>L. meyeri/Peluodo</td>
<td>75.25 (±5.23)</td>
<td>+4</td>
</tr>
<tr>
<td>24</td>
<td>Leptospira inadai/L-14</td>
<td>77.39 (±1.21)</td>
<td>+4</td>
</tr>
<tr>
<td>25</td>
<td>L. noguchii/507</td>
<td>77.20 (±4.69)</td>
<td>+4</td>
</tr>
<tr>
<td>26</td>
<td>L. interrogans/MT 10</td>
<td>80.98 (±1.61)</td>
<td>+4</td>
</tr>
</tbody>
</table>

Percentage of coaggregation as measured by OD$_{600}$ change over 1 h (see the Materials and methods section). Data are means from three separate experiments (±SD). Coaggregation scores were assessed by a visual assay. All test combinations had rapid and complete settling of large coaggregates leaving a clear supernatant (visual score +4).
coaggregating bacteria compete and suffocate coexisting bacteria under the matrix to a non-viable stage. However, in association with A. brasilense RMRCPB, leptospires remained viable for longer. Metabolic pathways for the persistence of leptospires in these co-cultures remain unclear. However, it is indicative that they depend on these environmental bacteria for nutrients. Several studies argue that leptospires acquire iron from different sources, including siderophores of other bacteria. However, this aspect has not been investigated thoroughly (Louvel et al. 2006). Genome sequencing has revealed that L. interrogans possesses several genes to facilitate better survival in the environment before encountering a mammalian host. However, Leptospira borgpetersenii was found to have lost all vital survival genes related to environmental sensing, metabolite transport and utilization (Bulach et al. 2006).

Susceptibility to antibiotics

Resident bacteria in biofilms can be more resistant to antibacterial compounds (such as disinfectants, antibiotics and surfactants) than planktonic cells (Davey and O’Toole 2000; Stewart 2002). Leptospires are susceptible to drugs, namely penicillin G (25–100 U/mL), ampicillin (12.5–50 μg/mL) and tetracycline (50–100 μg/mL), though the minimum inhibitory concentration and minimum bactericidal concentration (MBC) varied among strains (Murray and Hospenthal 2004). The MBC for L. interrogans Pomona in biofilm with A. brasilense was significantly higher at >800 U/mL, 800 μg/mL and >800 for penicillin G, ampicillin and tetracycline, respectively. This is more than 4-fold higher than required in the planktonic state, for which the MBCs of penicillin G, ampicillin and tetracycline were 50 U/mL, 12.5 μg/mL and 50 μg/mL, respectively. The MBCs of penicillin and ampicillin for planktonic A. brasilense RMRCPB were higher than for planktonic L. interrogans, but this occurred to a lesser extent with tetracycline (Fig. 5A). This was similar to previous reports in which Azospirillum spp. was resistant to antibiotics such as penicillin (\(\geq 125\) U/mL) and ampicillin (\(\geq 50\) μg/mL) (Alonso, Giraudo and Calzolari 1987; Boggio and Roveri 2003). In the present study, the reason for high antibiotic resistance of Leptospira-Azospirillium biofilm is unclear. However, recent evidence suggests that the EPS (extracellular polymeric substance) matrix surrounding the attached cells provides an effective barrier, restricting the ability of biocides to penetrate into the biofilm (Drenkard andAusubel 2002; Stewart 2002).

Resistance to UV and high temperature

Both free-living and pathogenic leptospires survive in soil and water for several months and are naturally exposed to solar UV radiation (Stamm and Charon 1988). In the UV radiation study, the planktonic form of leptospires survived only for 10 min, whereas in the presence of A. brasilense RMRCPB, survival was extended up to 20 min (t-test, P value \(\geq 0.005\)) (Fig. 5C). This was similar to previous reports on planktonic leptospires and other microorganisms (Smith and Turner 1961; Wong-ekkabut et al. 2009; Chadsuthi et al. 2010). Leptospires in biofilm were able to withstand high intensities of UV (\(4.1 \times 10^6\) mWs/cm²), which is nearly two times higher than the dosage required to eliminate pathogens in drinking water (\(2.2 \times 10^6\) mWs/cm²), US Environmental Protection Agency, 2006). The optimum temperature for the growth of Leptospira was reported to be 25–30°C and it does not grow beyond 42°C (Noguchi 1918; Johnson and Harris 1967). However, in biofilms with A. brasilense RMRCPB, leptospire viability was enhanced at higher temperatures. Planktonic leptospires were killed at 42°C within 6 h, whereas in biofilms with A. brasilense RMRCPB they survived for >6 h at 49°C (Fig. 5D). By residing inside an aggregate or biofilm, leptospires were able to shield themselves from external stress. However,
the mechanism of the interaction was not clear. It could be suggested that other competent environmental organisms may protect pathogens (such as *Leptospira*) against harmful UV radiation and high temperatures.

### Soil adsorption

*Leptospiras* can persist in the environment irrespective of the season (Barragan et al. 2011) and their adsorption to soil may favor this persistence. In the present study, there was a significant reduction in the concentration of *leptospiras* in water when *L. interrogans* Pomona was co-inoculated with *A. brasilense* RMRCPB in waterlogged soil in vitro (t-test, P value $\geq 0.005$). However, in surface soil the adsorption rate of *Leptospira* was 48.6–52.0% and this increased to 84.6–91.2% for the co-inoculated mixture (Fig. 5B). This inverse effect has been attributed to coaggregation, which leads to biofilm formation and might facilitate easy adsorption to the soil.

The phenomenon of microorganisms participating in the mechanism of social behavior is well known. Microorganisms cooperate among themselves to exploit resources and counter environmental stress. *Leptospiras* are highly susceptible to adverse environmental stress. This selection pressure, combined with anthropogenic activity, leads to unique microbial interactions. The present study demonstrates the ability of *leptospiras* to interact with other microbes to form biofilms, facilitating their ability to overcome harmful effects of the environment and enabling them to survive for longer. The mutualism between the two bacteria remains to be explored. However, it is evident that *Leptospira* benefits from this positive interaction. This study indicates that *Leptospira* is not solitary but a social organism, depending on a range of population-level traits, such as biofilms, cell–cell communication and cooperative stress resistance. Observations from this study could lay the foundation for further research to provide a better understanding of the interaction between divergent bacteria.

### SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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Conflict of interest. None declared.

REFERENCES


