Spiroplasma spp. biofilm formation is instrumental for their role in the pathogenesis of plant, insect and animal diseases

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Spiroplasma spp. are important phyto and insect pathogens, and candidate causal agent/s of transmissible spongiform encephalopathies (TSE) in man and animals. These filterable wall-less bacteria are widely distributed in nature with an unspecified environmental reservoir. In this study we showed by scanning electron microscopy that spiroplasma form biofilm on an assortment of hard surfaces including mica, nickel and stainless steel. Spiroplasma were stuck to the surfaces by fibrillar threads consistent with curli fibers (an amyloid protein found in bacterial biofilms). After a lengthy time in cultures (6 weeks), spiroplasma in biofilm bound to mica disks lost their spiral shapes and formed coccoid forms interconnected by long (>2 μm) branched membranous nanotubules, therein representing direct conjugate connections between the cells. The affinity of spiroplasma biofilms for mica and nickel, and the membrane communications suggest that soil could be a reservoir for these bacteria. The persistence of clay bound spiroplasma in soil could serve as the mechanism of lateral spread of TSEs by ingestion of soil by ruminants. Spiroplasma binding to stainless steel wire supports bacterial contamination of surgical instruments following surgery on dementia patients as a mechanism of iatrogenic transmission of TSEs, especially with resistance of spiroplasma in biofilms to drying or exposure to 50% glutaraldehyde. The discovery of biofilm formation by spiroplasma addresses questions regarding environmental persistence of these organisms in nature and suggests novel mechanisms of intercellular communication and transmission.

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Introduction

Spiroplasma are motile wall-less bacteria with the smallest genome of free-living organisms (genome = 940–2240 kb) (Tully and Whitcomb, 1992). They are primarily recognized as phytopathogens and are present in the sieve tubes of plants and on leaf surfaces. Spiroplasmas are present in the gut and hemolymph of the majority of insects, which serve in many cases as vectors for plant diseases. The two agriculturally important spiroplasma infestations are citrus stubborn disease and corn stunt disease. Spiroplasma infestation of honey bees is another agriculturally important naturally occurring spiroplasmosis (Gasparich et al., 2004). For the most part, the involvement of spiroplasma in plants or insects is of unknown significance. Conversely, spiroplasmosis may not be limited to these plant or insect hosts since spiroplasma have been found in tissues of animals and humans affected with a fatal transmissible spongiform encephalopathy (TSE) (Bastian, 1979, 1991; Bastian et al., 1981, 2004) and novel Spiroplasma spp. have been cultured from brain and eye tissues from all forms of TSE (Bastian et al., 2007, 2011). The link between spiroplasmosis and TSE is supported by reaction on Western blot of scrapie-specific hyperimmune rabbit sera with protease-treated spiroplasma proteins (Bastian et al., 1987a). Furthermore, recombinant Hsp60 protein derived from a gene identified in the genome of the suckling mouse cataract agent (SMCA) strain of Spiroplasma mirum has been shown by ELISA to identify sera from > 95% of Creutzfeldt-Jakob disease (CJD) patients (Moyer, 2004). The case for involvement of spiroplasma in the pathogenesis of TSE is supported by experimental transmission studies wherein S. mirum inoculated into rodents (Bastian et al., 1984; Tully et al., 1984) and into ruminants (Bastian et al., 2007, 2011) induces spongiform encephalopathy in a dose dependent fashion. S. mirum is neurotropic when inoculated intraperitoneally and subcutaneously into the rat (Bastian et al., 1987b). Spiroplasma spp. have also been found in crustaceans affected with neurological disease (Wang et al., 2011) and in shrimp (Nunan et al., 2005). The epidemiology of spiroplasmosis in these varied hosts has been difficult to determine due to the fastidious growth of spiroplasma, requiring special media of high osmolality (Tully and Whitcomb, 1992); therefore, spiroplasma are not readily discovered by standard culture methods, and diverse species of spiroplasma often
differ in biological growth characteristics and phenotype. Spiroplasma spp. are widely distributed in nature, and likely persist in an indeterminate reservoir with incomplete information available regarding their mode of transmission.

The prospect of resolving these issues has been facilitated by the recent discovery that Mollicutes, the simplest bacteria, form biofilm (McAuliffe et al., 2008; Simmons et al., 2007). Biofilms provide protection from stress thus is important for survival of Mollicutes and other bacteria in nature. Mycoplasma mycoides encompassed in biofilms are markedly resistant to heat shock, osmotic shock, SDS, and hydrogen peroxide compared to planktonic (free swimming) cells (McAuliffe et al., 2006). The polysaccharide matrices enclosing the bacterial populations contain extracellular DNA and proteins that facilitate communication between the bacteria with exchange of proteins and genetic elements (Nguyen et al., 2010). In this study, biofilm was shown by scanning electron microscopy (SEM) to form on a variety of surfaces including mica, stainless steel and nickel following exposure to log phase cultures of several representative Spiroplasma spp. Spiroplasma induced biofilm showed curli-like fiber attachments, which represent a functional amyloid seen in bacterial biofilms induced by both Gram positive and Gram negative bacteria, as exemplified by Escherichia coli. Curli fibers are important in attachment and entry of the bacteria into host cells (Chapman et al., 2002). The occurrence of bacterial amyloid in spiroplasma biofilms allows an understanding of the pathogenesis of spiroplasmosis that could explain the occurrence of prion amyloid in TSE tissues. Biofilms that developed on mica after lengthy exposure (6 weeks) to spiroplasma cultures showed novel nanotubular conjugate interconnections between bacteria, which, along with propensity of these bacteria to bind to mica, predict a soil habitat for these organisms.

Materials and methods

Bacterial cultures

Several species of spiroplasma representative of different clades (Gasparich et al., 2004), including GT-48 and suckling mouse cataract (SMCA) strains of S. mirum (rabbit tick spiroplasma isolates), Spiroplasma syrphidicola (tabanoid spiroplasma isolate), Spiroplasma taiwanense (mosquito spiroplasma isolate), 277F spiroplasma (flower spiroplasma isolate), Spiroplasma melliferum (honeybee spiroplasma), TAAS-1 spiroplasma (tabanoid spiroplasma isolate), and Spiroplasma citri (leafhopper/citrus spiroplasma isolate) were obtained from the Purdue Mollicute collection, under the direction of Dr. Jerry Davis. These Spiroplasma spp. were grown to log titer in M1D media in multi-well plates, each containing a sterile micaceous disk, and maintained for 3 to 6 weeks at 30 °C in a humidified incubator. SMCA cultures were also maintained on stainless steel washers in the multi-well plates (2 weeks) and in stoppered 15 ml culture tubes containing stainless steel wires (1.5 months) or nickel disks (3 weeks). Control samples were maintained in M1D media alone.

In a sequential study, stainless steel suture wires (IMEX orthopedic 22 gage 316LVM wire 2.5 cm in length) were exposed to SMCA culture in SP-4 media with individual wires separated in 8 ml capped glass vials.

Fig. 1. Spiroplasma readily formed biofilm on mica as early as 1 week exposure to broth culture. Scanning electron microscopy revealed (A) filamentous forms of SMCA strain of S. mirum partially embedded in extracellular matrix (ECM); (B) A micro colony of SMCA mostly buried in ECM; (C) SMCA biofilm forming tower structures; and (D) SMCA filaments covered with barnacle-like ECM deposits. (E) Control mica in M1D media alone revealed no ECM deposits. The extracellular matrix is outlined by short arrows and spiroplasma organisms by long arrows. Bars indicate individual magnifications.
The test wires and control wires exposed to SP-4 media alone were incubated at 30 °C over a five-week period with sampling for SEM on a weekly schedule.

Viability after drying and exposure to glutaraldehyde
Nickel wire 99.99 % Ø 0.25 mm (Advent Research Materials, Eynsham, Oxford, England), cut in 2.5 cm lengths, were exposed to either SMCA or GT-48 culture in SP-4 media, or to SP-4 media alone as a control. After 3 weeks incubation in 8 ml capped glass vials, the media were removed and the wires allowed to dry (3 to 6 weeks at 4 °C). Wires were then either 1) immersed in fresh media, or 2) plated onto SP-4 agar plates, and both incubated at 30 °C for 21 days. This latter experiment was repeated after exposure of the dried wires to a) PBS wash × 3, B) 1% glutaraldehyde for 1 min or 5 min periods, or 5% glutaraldehyde for 1 min or 50% glutaraldehyde for 1 min, followed by PBS wash × 3 and incubation on SP-4 agar plates as above.

Dark-field microscopy
Spiroplasma growth was monitored by visualization of motile spiral forms under dark-field microscopy. Culture samples were prepared for dark-field examination by placing a 5 μL drop of sample on a clean glass microscope slide, covered with a #1.5 cover slip, which was then sealed on all four sides with clear finger nail polish. Slides and cover slips were examined with a Zeiss Axio Imager A1 microscope equipped with a dark-field top lens condenser 1.2–1.4 and an EC Plan-NeoFluar 100× oil immersion objective with the 1.3 iris closed to its lowest setting (0.7).

Scanning electron microscopy (SEM)
The micaceous and nickel disks, and stainless steel and nickel wires exposed to spiroplasma cultures or media alone were rinsed with 10 mM phosphate buffered saline pH 7.5 (PBS) × 3. They were fixed by adding cold 1.25% glutaraldehyde in cacodylate buffer for 1 h and then maintained in the same buffer until processing. Each sample was mounted on a stub with adhesive and sputter coated by platinum. Specimens were examined by scanning electron microscopy (SEM) using an FEI Quanta 200 high-resolution environmental microscope.

Transmission electron microscopy (TEM)
Micaceous disks exposed to log phase cultures of SMCA and GT-48 strains of S. mirum (3 weeks) were processed as above, then embedded for sectioning by placing a conical tube over disk surface on which the biofilm was deposited. Polymerization was carried out by filling the tube with EPON. Thin sections were cut at the interface between the mica surface and the plastic EPON (to obtain a section through the biofilm) using an ultramicrotome (RMC MTXL). Thin sections were examined using a Joel JEM-1011 electron microscope.

Lectin studies
SMCA log phase cultures were incubated with 12 mm glass disks in multi-well plates for two weeks in preparation for the lectin studies. Briefly, fluorescently labeled lectins [Fluorescein Lectin Kit I-Vector Laboratories (Blastingame, CA) conjugated with fluorescein isothiocyanate (FITC) at a concentration of 2 mg/ml] were prepared by dilution of the stock solution with PBS to a lectin concentration of 10 μg/ml. Fifty microliters of this diluted lectin solution was applied to each well containing a 12 mm glass disk that had either been cultured with SMCA or M1D media alone, and then rinsed in PBS. The preparation was incubated for 30 min in the dark at room temperature, rinsed × 4 in PBS, and mounted inverted on a glass slide with Vectashield mounting media (Vector Labs, Blastingame, CA). The preparations were viewed on a Zeiss Axio Imager A1 microscope equipped with an X-cite 120 fluorescent light source and filters for green fluorescence.

Confocal microscopy
Nickel wires exposed to SMCA culture for 3 weeks (see above) were taped to a glass slide and a staining compartment created by surrounding the sample with a ridge of nail polish. Lectin staining by FITC (ConA) was carried out as above and the sample rinsed with PBS × 3, air dried at room temperature, and mounted in Cytoseal 60 (Apogent EMS Hatfield, PA) and allowed to set overnight. Specific immunofluorescence was examined using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Exton, PA) fitted with a CS APO 10× Leica objective. Emission = 520 nm; Excitation = 488 nm. Images were compiled with the Leica confocal microscopy software package.
Results

Spiroplasma exposure to mica

Observation of spiroplasma biofilm on mica by SEM and TEM

Scanning electron microscopy of micaceous disks after exposure to SMCA broth culture for 7 days growth revealed spiroplasma filaments attached to the mica surface partially embedded in a granular extracellular matrix (ECM) (Fig. 1A). Spiroplasma microcolonies were seen either embedded in the ECM (Fig. 1B) or incorporated into Tower-like structures, as described in other Mollicute biofilms (Simmons et al., 2007) (Fig. 1C). The granular ECM was deposited on the spiroplasma filaments like barnacles, as shown in Fig. 1D. Control disk exposed to media alone is shown in Fig. 1E. Fluorescent studies of glass disks exposed to SMCA broth cultures for 2 weeks revealed binding of Concanavalin A and Wheat Germ Agglutinin to the ECM of the biofilm, while glass disks in M1D media were negative, as shown in Fig. 2. The lectin binding studies confirmed the polysaccharide nature of the ECM.

![Image](https://example.com/image.png)

Fig. 3. All Spiroplasma spp. tested formed biofilm on mica after 3 weeks, but with some variability. Scanning electron microscopy of (A) GT-48 strain of S. mirum; (B) S. citri; (C) S. taiwanense; and (D) S. syrphidicola showed formation of biofilm with spiroplasma filaments buried in ECM. (E) GT-48; and (F) Spiroplasma melliferum showed attachment of large ball-like collections of organisms to the mica surface. The extracellular matrix is identified by short arrows and spiroplasma by long arrows. Bars indicate magnification.
in the SMCA biofilm (Johnsen et al., 2000; Strathmann et al., 2002), which is characteristic of Mollicute biofilm (McAuliffe et al., 2008; Simmons et al., 2007). Additional SEM studies showed that all of the Spiroplasma spp. tested produced biofilms after exposure of the mica to spiroplasma broth cultures for 3 weeks duration, as shown in Fig. 3. The amounts of biofilm formed by different spiroplasma species on mica were variable. By comparison, TAAS-1 spiroplasma formed sparse biofilm on mica, while SMCA and GT-48 strains of S. mirum formed abundant ECM deposits. S. melliferum biofilms on mica showed minimal ECM, but numerous large medusa-like balls of spiral bacteria attached to the mica surface, as shown in Fig. 3F. Fig. 3 shows that certain spiroplasma species (S. taiwanense; 277F spiroplasma and S. syrphidae), formed abundant ECM on the mica surface, but with few spiroplasma filaments visible.

Scanning electron microscopy of all spiroplasma biofilms revealed fibrillar attachments of the spiroplasma filaments and coccolid forms to the mica, as shown in Fig. 4. These fibrillar structures closely resemble the curli-like fibers seen in other bacterial biofilms (Barnhart and Chapman, 2006) that represent bacterial amyloid. Transmission electron microscopy of SMCA and GT-48 biofilms on mica documented the amyloid-like fibrillar nature of these attachments, as shown in Fig. 4E.

Nanotubular connections in spiroplasma biofilms

Scanning electron microscopy of spiroplasma biofilms after 6 weeks incubation with mica revealed mostly coccolid shaped organisms 200 to 400 nm in diameter (Fig. 5). Many of the spiroplasma were interconnected by straight nanotubular structures measuring over two microns in length and 15 nanometers in width (Fig. 5). Several of these nanotubules fragmented during voltage change on the scanning electron microscope indicating that they were fragile membranous structures (Fig. 5B). Nanotubular connections have been previously reported between other bacteria (Dubey and Ben-Yehuda, 2011), showing bulbous swellings as observed in the spiroplasma biofilms (Figs. 5A,D). Many of the nanotubular structures showed branching interconnecting several spiroplasma coccolid forms (Figs. 5B,D). A tower structure seen in a GT-48 spiroplasma biofilm on mica at 3 weeks revealed numerous nanotubular connections between spiroplasma coccolid forms over its surface (Fig. 5C).

Spiroplasma exposure to stainless steel

Scanning electron microscopy of stainless steel washers exposed to SMCA broth cultures for 2 weeks revealed biofilm comparable to that seen on mica (data not shown). Scanning electron microscopy of stainless steel wires after 6 weeks exposure to SMCA broth cultures showed the presence of stacked deposits of ECM (Fig. 6), especially concentrated to defects in the wire surface (Fig. 6C). Rare spiroplasma filaments were identified showing amyloid-like fibrillar attachments. Scanning electron microscopy identified matrices of fibrils suggestive of patches of amyloid deposits on the stainless steel surface (Fig. 6D). The recognition of these deposits as amyloid is supported by comparison to the ‘fragmented wire-like appearance’ of amyloid deposits on the outer portions of photoreceptors observed by SEM in aging mouse eyes (Hoh Kam et al., 2010). Scanning electron microscopy of wire in M1D media showed no ECM deposits (Fig. 6A). A sequential SEM study of SMCA exposure to stainless steel wire revealed progression of the biofilm formation over a five week period (Fig. 7) despite planktonic organisms have been shown to reach log phase peak at 4 to 8 days with substantial decline in numbers after day 14 (Bastian et al., 1988). During this time period there was increased complexity.

![Fig. 4](image-url) All Spiroplasma spp. induced biofilm showed ultrastructural evidence of functional bacterial amyloid. Scanning electron microscopy of biofilms generated by (A) GT-48; (B) S. citri; (C) S. syrphidae; and (D) SMCA showed numerous curli-like fibrillar attachments to the mica surface (long arrows), short white arrows showed extracellular matrix. Transmission electron microscopy of (E) GT-48 biofilm revealed collections of fibrils consistent with amyloid. All biofilm studies were prepared by 3 weeks exposure of the spiroplasma cultures to the micaceous disks. Bars indicate magnification.
of the biofilm with coccoid forms and thin nanotubular interconnections. The coccoid forms may represent response to stress, likely related to the increased acidity of the culture media (Fig. 7).

**Spiroplasma exposure to nickel**

Since bacterial biofilm formation is enhanced by the presence of nickel in the media (Perrin et al., 2009), we undertook to determine if spiroplasma showed enhanced biofilm formation on this metal surface. Scanning electron microscopy of nickel disks exposed to SMCA broth cultures for 3 weeks revealed abundant biofilm formation (Fig. 8). Both filamentous and coccoid forms of spiroplasma were seen on the surface of the nickel. Matrices of fibrillar structures with the ‘fragmented wire-like appearance’ characteristic of SEM studies amyloid fibrils (Hoh Kam et al., 2010) were abundant on the nickel surface (Fig. 8E). The nickel surface of disks exposed to the acidity of the log phase spiroplasma culture was pitted likely due to leaching of nickel into the acid media (Fig. 8). The surfaces of the control nickel disks in M1D media were intact and showed no ECM deposits. Confocal microscopy of FITC-labeled ConA lectin binding to SMCA biofilm formed on nickel wire revealed incomplete coverage as supported by the SEM findings (Fig. 9).

**Viability of spiroplasma biofilm on nickel after drying and exposure to glutaraldehyde**

The formation of spiroplasma biofilm on stainless steel and nickel surfaces raised the issue whether the organisms would survive drying conditions or harsh astringents used to clean surgical instruments. In a preliminary study to test resistance of spiroplasma biofilms, we showed that SMCA and GT-48 spiroplasma biofilms on nickel wire survived drying (3 to 6 weeks) (Fig. 10). The growth on the SP-4 agar plates was most prominent around wires that had sunk into the agar indicating the microaerophilic nature of the organisms (Konai et al., 1996). The GT-48 biofilms dried on nickel wire survived exposure to 1%, 5%, and 50% concentrations of glutaraldehyde with only slightly delayed growth on the agar (Fig. 10). These studies suggest the need for a more comprehensive survivability study for spiroplasma comparing the susceptibility of planktonic and biofilm bound organisms, and different *Spiroplasma* spp. to environmentally harsh physical and chemical conditions.

**Discussion**

*Spiroplasma* spp. induced biofilm-enhanced survivability and reservoir of infection

Our discovery that *Spiroplasma* spp. produced biofilm on a wide assortment of surfaces represents a major advance in understanding the biological nature of these unique wall-less organisms. First of all, spiroplasma are known to survive on leaf surfaces in spite of exposure to adverse environmental conditions including desiccation (Tully and Whitcomb, 1992). The presence of spiroplasma biofilm on the undersurface of the leaf would protect the organism in the confines of a stable humid environment of a biofilm (Eberl et al., 2007). Secondly, the strong binding of spiroplasma biofilm to mica...
(Fig. 1) indicates a penchant for clay, and therein suggests a possible soil reservoir for these organisms (Lundsford et al., 2000). The formation of a biofilm by bacteria lends to essential communication between organisms, which would allow their survival in an unfavorable microenvironment (Annous et al., 2009; Walker et al., 2004). The unique nanotubular connections shown between spiroplasma organisms in biofilms (Fig. 5) have been described in biofilms formed by other bacteria (Annous et al., 2009; Dubey and Ben-Yehuda, 2011). A soil reservoir for spiroplasma would lead to direct involvement of the bacteria in the rhizosphere biology that is closely tied to plant health (Danhorn and Fuqua, 2007; Walker et al., 2004).

Since spiroplasma have been implicated in the pathogenesis of chronic wasting disease (CWD) (Bastian et al., 2004), a TSE in deer, it is a possibility that a soil reservoir for these bacteria could account for lateral transmission of CWD in ruminants (Mathiason et al., 2009). Soil contamination in deer pens is suspected to come from excrements shed by infected deer or in the pasture from decomposing carcasses. TSE infectivity has been found in feces (Safar et al., 2008) and in urine (Seeger et al., 2005). It is interesting that scrapie is prevalent during birthing (Touzeau et al., 2006); placental tissues from scrapie-affected sheep are infectious (Onodera et al., 1993) and scrapie has been experimentally transmitted orally by feeding of fetal tissues from pregnant scrapie-affected animals (Pattison et al., 1974). The causal agent/s of TSE are known to survive in clay and humus soils (Cooke et al., 2007; Johnson et al., 2006; Mathiason et al., 2009), and oral transmission of TSE is well established in ruminants (Hamir et al., 2009) and in rodents (Kruger et al., 2009). In naturally occurring scrapie, oral passage may be engendered by lesions commonly seen on the oral mucosa of sheep (Watson et al., 2006). Other ruminants become infected by their propensity to ingest large amounts of soil (Fries et al., 1982; Mayland et al., 1977; Thornton and Abrahams, 1983).

The confusion arises because ‘prion amyloid’ is used interchangeably with TSE infectivity, and the measure of TSE infectivity of soil samples in those studies has been determined by a mouse model rather than by demonstration of a concentration of prion amyloid in the soil samples. This discrepancy is emphasized by recent studies suggesting that prion amyloid is not a surrogate marker of TSE infectivity (McLeod et al., 2004; Scherbel et al., 2007; Solassol et al., 2004; Yao et al., 2005) and that other factors are involved in the pathogenesis of TSE (Telling, 2000). In this scenario, we hypothesize that spiroplasma is shed by CWD affected ruminants, then forms biofilm in the soil by binding to clay particles, and migrates out of the biofilm into the new host following ingestion.

The formation of biofilm by spiroplasma also explains the unconventional resistance of these organisms to adverse physical and chemical treatments (Bastian, 1991). In our laboratory, SMCA and GT-48 strains of S. mirum have been shown to survive near boiling temperatures and levels of ionizing radiation incompatible with survival of E. coli, which was used as a control (unpublished data). In preliminary experiments, we have shown these wall-less bacteria to survive exposure to glutaraldehyde at concentrations up to 50% (Fig. 10) (1 to 2% glutaraldehyde is routinely used for tissue fixation). Other bacteria protected by a polysaccharide matrix in biofilm have shown remarkable survivorship (1000 times) versus planktonic organisms (McAuliffe et al., 2006). Biofilm formation by Spiroplasma spp. offers an explanation why a wall-less spiroplasma pathogen can survive environmental insults comparable to the biologic properties attributed to the transmissible agent/s of

Fig. 6. Spiroplasma formed biofilm on stainless steel wires. Scanning electron microscopy of (A) stainless steel wire in M1D media showed no surface deposits. SMCA strain of S. mirum formed biofilm on the stainless steel wire manifested by deposits of extracellular matrix (B), concentrated to defects on the wire surface (arrows) (C). (D) Matted collections of short fibrils in the SMCA biofilm on the stainless-steel surface were consistent with amyloid deposits (Hoh Kam et al., 2010). Bars indicate magnification.
TSE (Bastian, 1991). Future studies of spiroplasma resistance to environmental extremes will have to take into consideration planktonic versus biofilm bound organisms.

**Nanotubular conjugate connections in spiroplasma biofilm**

The nanotubular structures observed by SEM connecting spiroplasma organisms (Fig. 5) have only recently been described in bacterial cultures grown on a solid surface (Dubey and Ben-Yehuda, 2011). Similar structures have been described in eukaryotic cell preparations, especially seen interconnecting dendritic cells (Hurtig et al., 2010). These structures are larger in eukaryotes but otherwise identical to those described in bacterial preparations (Dubey and Ben-Yehuda, 2011) and those seen in spiroplasma biofilm preparations in this study. The membranous nanotubular connections between spiroplasma were long measuring over 2 μm in length, and showed complex branching. These interconnections were seen between spiroplasma coccoid forms, which are representative of the multiformity of the spiroplasma organism (Bastian et al., 1984). The fragile membranous nanotubules in spiroplasma biofilms measured 15 nm in diameter with bulbous

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**Fig. 7.** Spiroplasma biofilm formed on stainless steel wire with a gradual buildup over five weeks exposure to SMCA broth culture. The sequential study showed increasing acidity over this time frame, but the culture fluids remained clear (insets). After one week exposure to SMCA culture, SEM revealed rare patches of biofilm (A)/(B). (C) Wire exposed to media alone revealed no deposits, even at sites of irregularities on the metal surface (D). After two weeks exposure to SMCA culture, SEM showed increased amounts of biofilm deposits (E)/(F) with lengthy microtubular communications (G). After three weeks exposure to SMCA culture, SEM showed increased and more complex deposits on the wire surface (H)/(I)/(J) with bleb-like and coccoid forms projecting from the biofilm deposits. After four weeks exposure to SMCA culture, SEM revealed (K)/(L) more widespread coverage of the metal surface and biofilm with less distinct micro-colony formation. One large tower structure (M) was seen with (N) numerous coccoid forms attached by thin nanotubular connections to the parent tower deposit. After five weeks exposure to SMCA culture, there was (O)/(P) widespread but incomplete biofilm coverage. (Q) Coccoid forms were evident on the surface of many of the biofilm deposits, and (R) granular deposits were seen in defects on the metal surface. No apparent damage to the metal surface by the biofilm was seen. Bars indicate magnification.
swellings measuring 30 nm in diameter, as described in other bacterial preparations (Dubey and Ben-Yehuda, 2011). In this study, these conjugate connections were only observed in SEM studies of spiroplasma biofilms after lengthy (6 weeks) incubation of the micaceous disks in the bacterial cultures. These truly remarkable structures suggest a mechanism for direct sharing of cellular contents dispelling the idea that bacteria are solely dependent upon extracellular molecules in the environment for communication (Dubey and Ben-Yehuda, 2011). This phenomenon seen in a wall-less bacterium with a small genome suggests that this spiroplasma model may be ideal for investigating this form of direct communication between bacteria.

Curli-like amyloid in spiroplasma biofilms suggest a role in neurodegenerative diseases

The amyloid fibrillar connections seen in spiroplasma biofilms (Fig. 4) represent surface adhesin protein/s first described in Gram negative bacterial biofilms (Olsen et al., 1989; Otzen and Nielsen, 2008). These adhesin proteins referred to as curli fibers have also been demonstrated in biofilms of Gram positive bacteria (Jordal et al., 2009), from which spiroplasma are derived by genomic reduction or degenerative evolution. The process of curli formation in E. coli biofilms involves the polymerization of a bacterial secreted protein (CsgA) by the controlled expression of a surface membrane protein (CsgB); CsgB forms the nucleus for extension of repeat units of CsgA into a beta-sheet amyloid structure (Hammer et al., 2007; Wang and Chapman, 2008). The repeat beta-sheets are stacked perpendicular to the fiber axis (Chapman et al., 2002) and this process can show cross-species formation of amyloid with the CsgB of one species interacting with the CsgA of another species. These curli fibers are important virulence factors for bacteria and allow attachment of the organism to both eukaryotic cells and inert surfaces such as clay. Curli fibers show specific binding to fibronectin, laminin and plasminogen, allowing the bacteria to bind to and invade host tissues (Larsen et al., 2007). Biofilm-associated bacteria differ from their planktonic counterparts with respect to the genes that are transcribed (Donlan, 2002). In E. coli and other enterobacteria, curli amyloid is highly conserved (Romling et al., 1998), especially the protein sequences (Barnhart and Chapman, 2006), but generally there appears to be a wide variation in genetic production of bacterial amyloids suggesting independent evolution of this capability (Jordal et al., 2009). Virulence related adhesion proteins of S. citri, which closely resemble curli fibers by SEM, are not part of the spiroplasma genome, but rather are produced by a plasmid (Berg et al., 2001). No genomic basis for biofilm formation has been found in our completed genome study of SMCA (data not published), or in the recently reported genome of S. melifera (Alexeev et al., 2011). However, in both studies most of the ORFs discovered are of unknown function (hypothetical proteins). The role of functional amyloid formation in spiroplasma biofilms needs to be clarified in the pathogenesis of spiroplasma-related diseases.

Bacterial amyloid can initiate formation of pathogenic or misfolded amyloid from interaction with diverse host proteins (Otzen and Nielsen, 2008). This template-directed process brings up a possible relationship between bacterial amyloids and neurodegenerative diseases, which are characterized by uncontrolled formation of misfolded amyloid proteins (Otzen and Nielsen, 2008; Wang and Chapman, 2008). Preformed amyloid can also stimulate the formation of misfolded amyloids from totally different host proteins; the possibility is that numerous proteins can be induced to amyloid formation by extension and
self-assembly (Takahasi and Mihara, 2004). Bacterial amyloid injected into susceptible mice has led to the development of amyloidosis in these animals (Lundmark et al., 2005). Other amyloids such as A-beta from brains affected with Alzheimer’s disease have been shown to initiate conversion of normal proteins to amyloid isoforms (Xing et al., 2001). Placing normal prion (PrP\(^\text{c}\)) isoform in contact with misfolded prion amyloid (PrP\(^\text{res}\)) and submitting the mixture to repeated sonication cycles lead to self-assembly and propagation of the abnormal prion amyloid proteins, but without a corresponding increase in TSE infectivity (Klingeborn et al., 2011; Soto and Castilla, 2004). Prion protein assembles into amyloid by a fibrillation process initiated from a seed precursor state (Stöhr et al., 2008). Occasionally normal PrP\(^{c}\) controls appear to convert spontaneously to misfolded prion forms (Murayama et al., 2006).

These data suggest that prion amyloid formation is independent from infectivity, thereby supporting our hypothesis that another factor is involved in the causality of TSEs. We propose that spiroplasma functional bacterial amyloid from spiroplasma biofilms will form a nucleus for conversion of PrP\(^c\) to the prion amyloid without contact with TSE-affected tissues; a project confirming this scenario is underway in our laboratory. Bacterial amyloid conversion of normal host proteins into an amyloid isoform would explain the role of spiroplasma or other bacteria in the pathogenesis of neurodegenerative diseases including prion diseases (Fowler et al., 2007).

**Bacterial amyloid and biofilm is likely important in iatrogenic transmission of CJD**

Spiroplasma-induced biofilm on stainless steel relates to an important issue in clinical medicine; namely what is the mechanism for iatrogenic passage of Creutzfeldt-Jakob disease infectivity via surgical instruments (Bastian, 1991) or wire electrodes (Bemoulli et al., 1977) after application of standard sterilization procedures? Similar inadvertent transmission of CJD has occurred from contamination of surgical instruments used in eye surgery, especially involving the retina and posterior globe (S-Juan et al., 2004); this phenomenon is likely enhanced by predilection of TSE infection to the eye as suggested by experimental spiroplasmosis in ruminants (Bastian et al., 2011). Thiazole dyes have detected amyloidogenic fibrils on the stainless-steel surface of surgical instruments that have been exposed to TSE brain homogenate (Lemmer et al., 2008; Lipscomb et al., 2007). TSE infectivity has been definitively documented to bind to stainless steel by experimental transmission of TSE in mice through implantation of stainless-steel wire that had been incubated with TSE brain homogenates (Flechsig et al., 2001; Yan et al., 2004; Zibeley et al., 1999). However, prion amyloid, an infection-specific protease-resistant protein that is deposited in tissues of TSE-affected animals and humans, has not been definitively identified on the wire surfaces (Lemmer et al., 2008).

Prion amyloid cannot be differentiated from other amyloid proteins such as Alzheimer’s disease-related Amyloid-β or bacterial amyloids since all have the properties of protein amyloids (Shewmaker et al., 2009). It cannot be assumed that infectivity on the wire surface represents prion amyloid since several studies have shown that loss of immunoreactivity for prion does not correlate with biological inactivation (McLeod et al., 2004; Scherbel et al., 2007; Solassol et al., 2004; Yao et al., 2005). Both TSE infectivity and bacterial amyloids have a predilection for nickel (Luhr et al., 2009; Perrin et al., 2009), a component of stainless steel. Although infection related proteins such as prion amyloid may become adherent to the instruments, we suggest that adherence of Spiroplasma spp. to the stainless steel surface provides an alternative explanation for persistence of TSE infectivity on surgical instruments that cannot be removed by standard sterilization techniques. If spiroplasma bound on surgical instruments is responsible for iatrogenic transmission of TSE from one patient to another by reuse of TSE contaminated neurosurgical instruments or wire electrodes (Bastian, 1991; Bemoulli et al., 1977), then proper cleaning using ultra-sonication and hydrogen peroxide that is effective for removal of other bacterial biofilms could markedly reduce the danger of transmission (Presterl et al., 2007). This approach is supported by a long history of removing bacterial biofilms from metal surfaces, which may involve application of other
chemicals including EDTA that is effective in dissolving bacterial biofilms (Banin et al., 2006). It is interesting that TSE infectivity is susceptible to these treatments (Fichet et al., 2007; Kuczius et al., 2007; Orem et al., 2006).

Conclusion

Our observation of Spiroplasma spp. generated biofilms with unique features including bacterial amyloid and nanotubular intercelular conjugate connections goes far in establishing a foundation for understanding the biologic properties of spiroplasmosis. Future goals attainable through this approach may be the discovery of reservoirs of pathogenic spiroplasma in nature, possibly in soil. These data may provide critical information regarding the pathogenic mechanisms involved in spiroplasmosis. The discovery of a functional amyloid in spiroplasma biofilms will stimulate a search for identification of genes related to adhesin protein/s that may serve as an important virulence factor for these pathogenic bacteria. In regard to TSEs, those factors responsible for disease progression may be due to toxicity of prion peptides (Forloni et al., 1999) with the spiroplasma initiating the process or providing the trigger. There are data supporting normal PrP as a receptor protein for a bacterium (Watarai et al., 2003) and recovery of Spiroplasma spp. from TSE-affected brain tissues via passage in embryonated eggs (Bastian et al., 2007) or by direct inoculation of eye contents from scrapie-affected sheep into M1D media (Bastian et al., 2011) favors that TSE pathology is due to the direct action of the bacterial replication.

Conflict of interest statement

The authors have no conflict of interest.

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Fig. 10. Spiroplasma embedded in biofilm on the surface of nickel wire remained viable after extensive drying and treatment with glutaraldehyde. The experiment involved exposure of nickel wire to S. mirum (GT-48 strain) culture in Sp-4 media for three weeks, followed by drying (three to six weeks), then explanting onto Sp-4 agar plates and incubating for 21 days at 30 °C. (A) Control wire revealed no growth after exposure to Sp-4 media alone. (B)(C) Numerous colonies were seen in the agar plates migrating from the surface of wires exposed to the GT-48 cultures (in duplicate). Wires buried in the agar showed the most growth indicating the microaerophilic nature of the organism. (D) Spiroplasma growth was seen by inverted microscopy arising all along the wire surface and migrating into the agar (Original magnification ×100). (E) Spiroplasma growth from the wire surface into the agar was evident after washing ×3 with PBS. (F) Control wire exposed to media alone revealed no growth. A second experiment involved exposure of the wires after drying (three to six weeks) to varying concentrations of glutaraldehyde, washing ×3 in PBS, then explanting onto Sp-4 agar plates and incubating for 21 days at 30 °C. Inverted microscopy of (G) wire treated with 1% glutaraldehyde for one minute revealed abundant growth along the wire surface. Spiroplasma growth was seen from wire surfaces into the agar after treatments with (H) 5% glutaraldehyde for one minute, 1% glutaraldehyde for 5 min (data not shown), and (I) 50% glutaraldehyde for one minute. (E)(F)(G)(H)(I) Original magnification ×200.
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References


