



Twitching motility among pathogenic *Xylella fastidiosa* isolates and the influence of bovine serum albumin on twitching-dependent colony fringe morphology

Cheryl D. Galvani, Yaxin Li, Thomas J. Burr & Harvey C. Hoch

Department of Plant Pathology, Cornell University-New York State Agricultural Experiment Station, Geneva, NY, USA

Correspondence: Harvey C. Hoch,
Department of Plant Pathology, Cornell
University-New York State Agricultural
Experiment Station, Geneva, NY 14456 USA.
Tel.: +1 315 787 2332; fax: +1 315 787
2389; e-mail: hch1@cornell.edu

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Abstract

Fourteen *Xylella fastidiosa* isolates from grapevines exhibiting Pierce's disease symptoms in California, Texas, and South Carolina were examined for type IV pilus-mediated twitching motility, a phenotype previously observed in a Temecula isolate from California. All isolates except one from South Carolina (SC 19A97) exhibited colonies with a peripheral fringe on PW agar, a feature indicative of twitching motility; however, when individual cells of SC 19A97 were examined at higher magnifications twitching motility was observed. The presence and width of colony peripheral fringes were related to the amount of bovine serum albumin (BSA) present in the medium; no or low levels of BSA (0–1.8 g L⁻¹) permitted development of the widest fringe, whereas higher levels (3.5–6.0 g L⁻¹) severely limited, and in many instances prevented, peripheral fringe development. The growth rate of the wild-type Temecula isolate in PW broth with different concentrations of BSA was similar for all tested concentrations of BSA; however, growth was significantly reduced in medium without BSA.

Introduction

Xylella fastidiosa is a gram-negative, nonflagellated bacterium that causes economically important diseases of grape, citrus, peach, almond, coffee, and many other crops (Purcell & Hopkins, 1996; Simpson *et al.*, 2000; Van Sluys *et al.*, 2003). The bacteria are limited to colonizing the water-conducting xylem vessels of host plants. Such colonization frequently results in obstruction of water and nutrient transport, resulting in disease symptoms and often in plant death. Because *X. fastidiosa* lack flagella, the mechanism by which the bacteria are disseminated through the xylem vascular system has not been well characterized. Recently, it was demonstrated that a Temecula isolate of *X. fastidiosa* that causes Pierce's disease of grapevine developed a 'peripheral fringe' around colonies (Meng *et al.*, 2005) grown on periwinkle wilt (PW) agar (Davis *et al.*, 1981), a trait associated with type IV pilus-mediated twitching motility in other bacterial species (Bradley, 1980; Glessner *et al.*, 1999; Semmler *et al.*, 1999; Liu *et al.*, 2001; Huang *et al.*, 2003). It was further shown that the Temecula isolate could migrate upstream in a fabricated microfluidic chamber against rapidly flowing medium via twitching motility, and that similar migration occurred *in planta* against the flow of

xylem sap (Meng *et al.*, 2005). The observation that the Temecula isolate of *X. fastidiosa* exhibited colony morphologies consistent with twitching motility and that it was capable of migrating against a transpiration stream in xylem vessels led us to ponder why such colony morphologies had not been readily observed in previous reports. The *X. fastidiosa* genome sequence indicates that the bacterium contains at least thirty genes responsible for pilus assembly or function (Simpson *et al.*, 2000; Van Sluys *et al.*, 2003), and earlier descriptions of the genus indicated that some cells had 'fimbria-like fibrils' at the cell poles (Wells *et al.*, 1987). Because the pathogenic Temecula isolate exhibited twitching motility (Meng *et al.*, 2005), it was sought to determine whether this isolate was representative of other wild-type pathogenic *X. fastidiosa* isolates, or whether it was an anomaly, in which case the previous description of twitching motility (Meng *et al.*, 2005) would not be broadly applicable for a role of type IV pili in pathogenesis by this bacterium. Therefore peripheral fringe development in a range of pathogenic *X. fastidiosa* isolates obtained from various grape-growing regions in the United States was assessed. During the course of the survey of the pathogenic isolates, it was discovered that the concentration of bovine serum albumin (BSA) in the medium dramatically

influenced whether or not a peripheral fringe was associated with the colonies. Thus, the influence of BSA on peripheral fringe development in *X. fastidiosa* is also reported here.

Materials and methods

Maintenance and storage of bacterial isolates

Wild-type *X. fastidiosa* Temecula (ATCC 700964) and other wild-type isolates were maintained at 28 °C on PW agar (Davis *et al.*, 1981) without phenol red and with 3.5 g L⁻¹ instead of 6.0 g L⁻¹ BSA (Cat. No. A-8918, Sigma, St Louis, MO). It should be noted that following this study, Sigma discontinued production of A-8918 and replaced it with A-7979, which is inferior in supporting the growth of *X. fastidiosa*. A survey of BSA products by various manufacturers revealed that a Gibco product (Cat. No. 15260, Invitrogen, Carlsbad, CA) was equivalent to the original Sigma product for supporting growth. The transposon-generated mutants 6E11 (*fimA*⁻) and 1A2 (*pilB*⁻) (Meng *et al.*, 2005) were similarly maintained in culture as the Temecula parent, but with 50 µg mL⁻¹ kanamycin added to the PW agar. Wild-type isolates of *X. fastidiosa*, recently isolated from diseased grapevines and with few transfers in *in vitro* culture, were obtained from California, Texas, and South Carolina (Table 1). Isolates and mutants were stored frozen at -80 °C in PW broth containing 7% final concentration DMSO (Sigma). Owing to the reported loss of virulence during successive transfers in axenic culture (Hopkins, 1985; de Souza *et al.*, 2003), all wild-type isolates and mutants were handled with fewer than ten serial transfers following retrieval from freezer storage.

Assessment of colony morphology

Using rounded toothpicks, cells from 5–7-day-old *X. fastidiosa* cultures were spotted aseptically onto PW agar containing four different BSA concentrations: 0, 1.8, 3.5, or 6.0 g L⁻¹. Each isolate or mutant was spotted at ten sites on the agar surface of each plate. Cells were grown at 28 °C. Colony morphologies were assessed after 4–7 days with an Olympus SZX12 dissecting microscope using off-center lighting. The experiment was repeated at least three times.

Confirmation of twitching motility

As above, cells were spotted onto PW agar containing the four BSA concentrations. After 2–3 days of growth, colony morphologies were assessed with an Olympus IMT-2 inverted microscope. For examination of colony twitching, time-lapse digital images of selected individual colony peripheries were captured using METAMORPH image software as previously reported (Meng *et al.*, 2005). Alternatively, for isolates that did not readily exhibit a peripheral fringe around colonies, *X. fastidiosa* cells were spread sparsely onto either the agar surface or onto a sterilized sheet of cellophane overlaid on the agar surface and examined for twitching motility after 1–2 days of growth. The experiment was repeated at least two times.

Growth rate analysis

Cells from 5–7-day-old PW plates were used to make a cell suspension in PW medium containing four different BSA concentrations: 0, 1.8, 3.5, or 6.0 g L⁻¹. The initial OD for each cell suspension was adjusted to an absorbance of 0.1 at 600 nm. The cultures were incubated at 28 °C with

Table 1. Wild-type isolates and mutants of *Xylella fastidiosa*

Isolate	Description	Source/(reference, if any)
SC 17A54	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
SC 17A97	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
SC 18A1	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
SC 18A4	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
SC 19A11	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
SC 19A13	Pathogenic wild-type from grapevine	C.J. Chang; South Carolina
Texas 1.3	Pathogenic wild-type from grapevine	D. Appel; Texas
Texas 3.3	Pathogenic wild-type from grapevine	D. Appel; Texas
Conn Creek	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
Bakersfield	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
Medieros	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
STL	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
Temecula	Pathogenic wild-type from grapevine	S. Lindow; California (Hendson <i>et al.</i> , 2001)
Traver	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
UCLA	Pathogenic wild-type from grapevine	A. Purcell; California (Hendson <i>et al.</i> , 2001)
Mutants		
1A2	<i>pilB</i> mutant of Temecula; type I pili only; twitch-negative; non-motile	H.C. Hoch, T.J. Burr (Meng <i>et al.</i> , 2005)
6E11	<i>fimA</i> mutant of Temecula; type IV pili only; twitch-positive; motile	H.C. Hoch, T.J. Burr (Meng <i>et al.</i> , 2005)

continuous agitation at 195 r.p.m. Cell density was determined at days 0, 1, 2, 3, and 4 by measuring OD at $A_{600\text{ nm}}$. The experiment was repeated twice.

BSA influence on biofilm development

A 20 μL cell suspension of wild-type *X. fastidiosa* Temecula cells was added to 30 mL PD2 medium with either 0, 1.8, 3.5, or 6.0 g L^{-1} BSA in a 125-mL flask. The experiment was carried out in triplicate for each BSA concentration. Flasks were incubated at 28 °C with shaking on an orbital shaker (ThermoForma, Marietta, OH) at 100 r.p.m. Following eight and 20 days of growth, the appearance and relative amount of biofilm that formed on the side of the flask was noted. The experiment was repeated twice.

Results and discussion

Wild-type pathogenic isolates of *X. fastidiosa* from California, South Carolina, and Texas (Table 1) were examined on PW agar for the presence of a peripheral colony fringe, which is indicative of type IV pilus-mediated twitching motility. Eight of the 15 isolates examined are depicted in Fig. 1. All isolates, except SC 17A97 from South Carolina, exhibited a fringe around the periphery of the colony. However, subsequent time-lapse imaging of SC 17A97 cells spread over the agar surface or over cellophane overlaid on agar revealed that they too exhibited twitching motility similar to all the other isolates (not shown). Fringe width varied with the isolate (and with the concentration of BSA as noted below), and was in general consistent with replicated colonies of the same isolate within the same plate as well as in subsequent tests. Several isolates, e.g. Texas 1.3, SC 18A1, SC 17A54, and the 'standard' Temecula as well as the *fimA* mutant 6E11 had consistently wider fringes compared with other isolates such as Conn Creek and UCLA (Figs 1 and 2). Higher magnification of the fringe revealed the presence of

twitching, motile *X. fastidiosa* cells, either singly or in aggregates (Fig. 3). Expansion of similar fringe regions and movement of individual bacterial cells has been documented for a number of bacterial species (Bradley, 1980; Glessner *et al.*, 1999; Semmler *et al.*, 1999; Liu *et al.*, 2001; Huang *et al.*, 2003), including *X. fastidiosa* (Meng *et al.*, 2005; also see <http://www.nysaes.cornell.edu/pp/faculty/hoch/movies>). For comparison purposes, the *pilB* mutant 1A2, which does not have type IV pili and therefore does not exhibit twitching motility, had a smooth colony margin (Fig. 2).

As noted above, fringe width among the various isolates varied greatly. The reason for this was not discerned, but could be due to any number of factors such as differences in number of type IV pili, length of these pili, extension and retraction rates of the pili, colony expansion rate vs. twitching and fringe expansion rate, etc. In addition, the presence and number of type I pili on these isolates may also influence the twitching motility rate and hence fringe width. Already, it is known that the wild-type Temecula isolate has both types of pili, and the *fimA* mutant 6E11 has only type IV pili (Meng *et al.*, 2005). Under identical conditions, the *fimA* mutant (lacking type I pili) has a wider fringe than the Temecula parent (Li *et al.*, 2007). When present, attachment to the substratum by type I pili may be acting as a motility 'brake' holding the cells back from more rapid motility, resulting in a narrower fringe. Attachment to various surfaces by cells with only type I pili results in more pronounced biofilm formation (Meng *et al.*, 2005).

For any given isolate, the presence and width of the peripheral fringe was affected by the amount of BSA present in the PW medium. For the isolates that exhibited a peripheral fringe, higher BSA concentrations generally reduced or eliminated the appearance of the peripheral fringe (Figs 2 and 3). For some isolates, as well as the *fimA* mutant 6E11, a barely perceivable fringe was frequently observed at the highest BSA concentration, while medium with no BSA

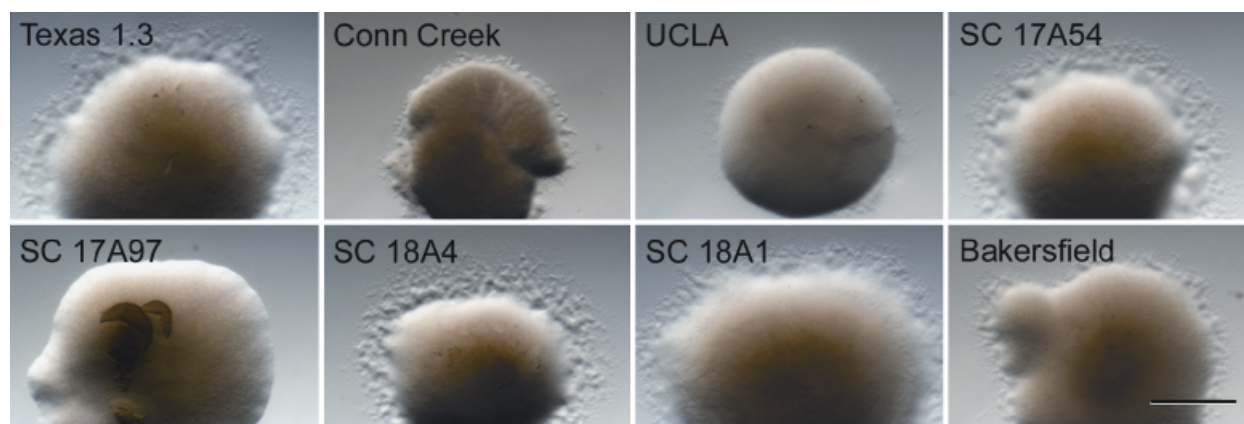


Fig. 1. Colony characteristics of eight wild-type *Xylella fastidiosa* isolates grown on PW agar without BSA. All isolates, except SC 17A97, exhibited a peripheral fringe, which is indicative of type IV pilus-mediated twitching motility by the bacteria. Magnification bar, 0.5 mm.

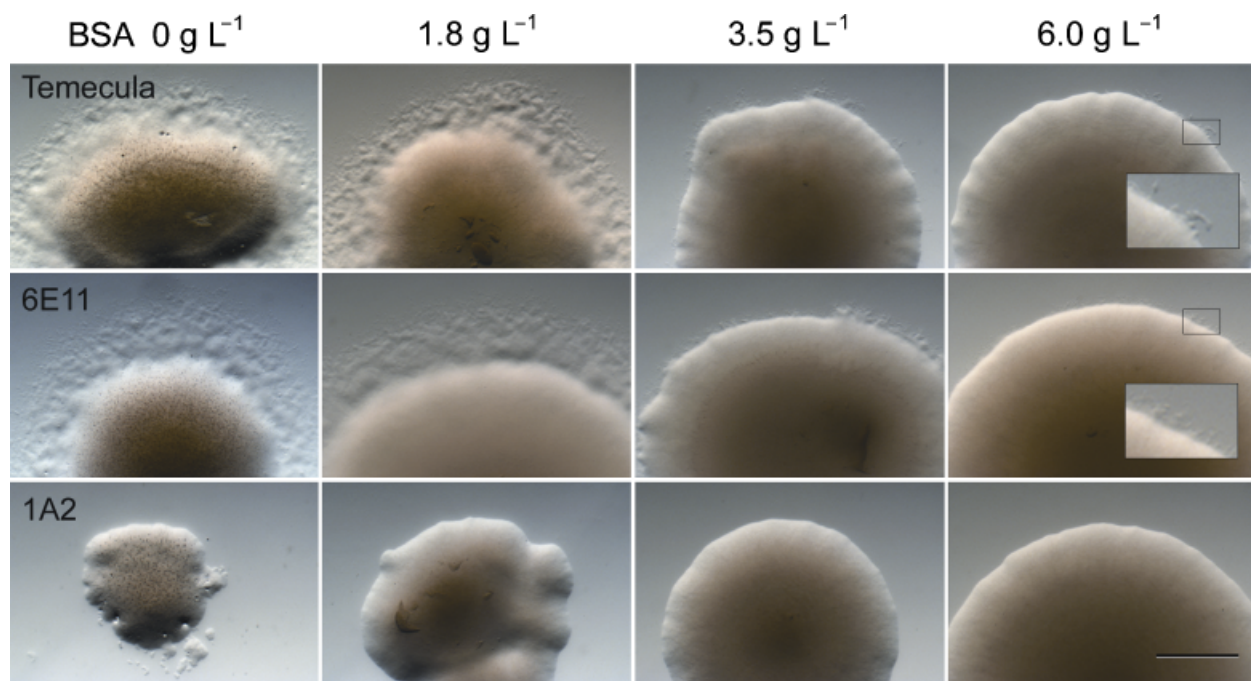


Fig. 2. Colony margin characteristics of the Temecula wild-type *Xylella fastidiosa* isolate and mutants 6E11 and 1A2 grown on PW agar with different concentrations of BSA. Inset of noted regions of Temecula and 6E11 (6.0 g L^{-1} BSA) depicting higher magnification of colony margin. Magnification bar, 0.5 mm.

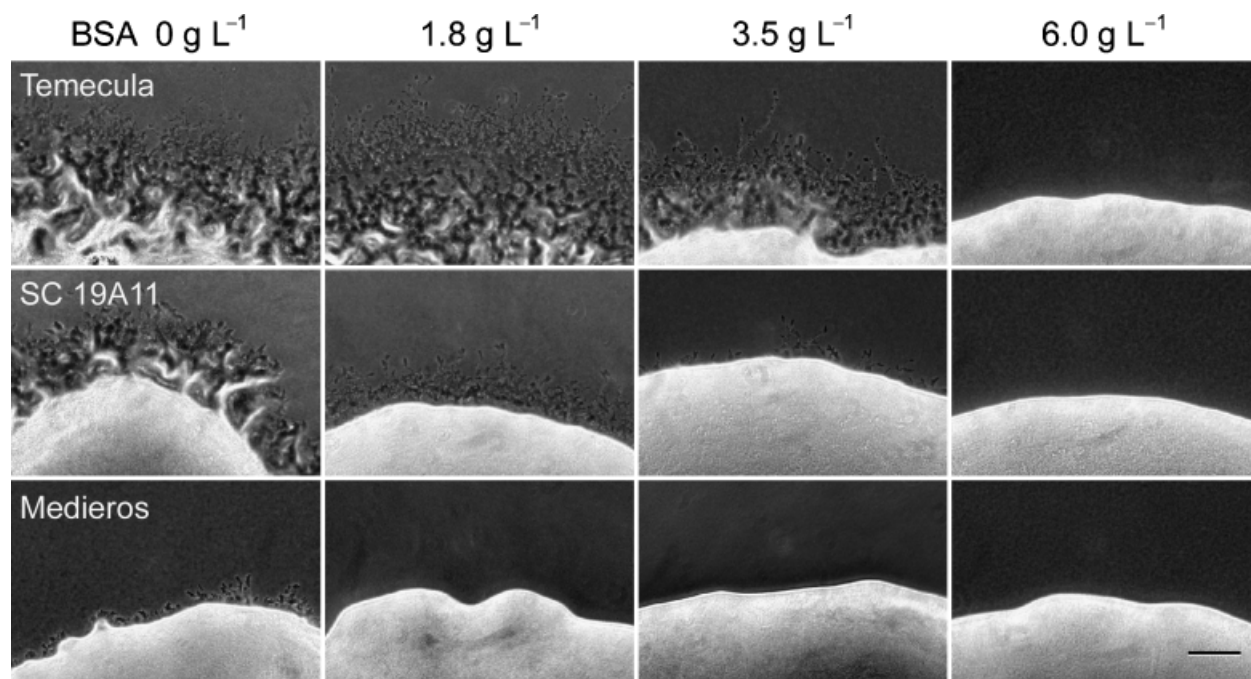


Fig. 3. Higher magnification of colony margin characteristics of the Temecula, SC 19A11, and Medieros wild-type *Xylella fastidiosa* isolates grown on PW agar with different concentrations of BSA. Magnification bar, 0.1 mm.

produced the widest and most pronounced fringe (Fig. 2). At the same time, the higher BSA concentrations nearly always produced larger colonies (Fig. 2) which, in part, substantiated the original report describing the need for BSA

in PW medium for *X. fastidiosa* (Davis *et al.*, 1980). Furthermore, the growth of *X. fastidiosa* in PW broth containing BSA was significantly enhanced compared with PW broth without BSA (Fig. 4).

Development of biofilm on culture vessel walls is frequently noted as a measure of bacterial adhesion to these surfaces (Marques *et al.*, 2002). Previously, biofilm development in the wild-type Temecula isolate of *X. fastidiosa* and several mutants grown in PD2 medium without BSA had been described (Meng *et al.*, 2005). Considering that BSA influenced peripheral fringe development in *X. fastidiosa* colonies on PW agar, the influence of BSA on biofilm formation in PD2 medium augmented with various concentrations of BSA was further examined. Following 8 days of growth in shaken flasks, *X. fastidiosa* developed rings of biofilm at the air–medium interface. There appeared to be little difference in the amount of biofilm among the four BSA concentrations, although the cells grown without BSA displayed slightly more biofilm. More notable was the clarity of the medium following growth; medium lacking BSA was clear, whereas medium containing 1.8, 3.5, or 6.0 g L⁻¹ BSA was turbid. It is likely that all cells in the medium lacking BSA were either attached to the flask wall or to each other, whereas in the turbid medium containing BSA many cells were planktonic. In support of this observation, Leite *et al.* (2004) reported that growth in PW medium (which contains 6.0 g L⁻¹ BSA) stimulated *X. fastidiosa* cells to be planktonic. An additional explanation for the lack of turbidity in cultures grown without BSA is that albumin

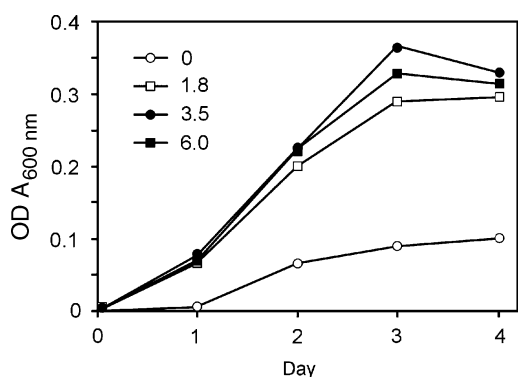


Fig. 4. Growth of *Xylella fastidiosa* Temecula wild-type isolate in PW medium with different concentrations of BSA. Cell density was determined at days 0, 1, 2, 3, and 4 by measuring OD at A_{600 nm}.

supports growth of *X. fastidiosa*. By 20 days of growth, the amount of biofilm in flasks without BSA was distinctly more than in medium with BSA (Fig. 5). It is likely that BSA coats the surface of the flask and prevents firm cell adherence, which is the initial step in biofilm formation. Cooksey *et al.* (2004) similarly observed decreased biofilm formation with increasing levels of BSA in PD3 medium. There is evidence in a number of other bacterial species and at least one mammalian cell line that the presence of BSA either reduced or completely eliminated cell attachment to various substrates (Burchard, 1986; Fuquay *et al.*, 1986; Tamada & Ikada, 1993; Al-Makhlafi *et al.*, 1994; Cunliffe *et al.*, 1999). BSA acts as a surfactant, thereby reducing surface tension and impeding cell attachment to a substrate (Burchard, 1986; Tamada & Ikada, 1993; Al-Makhlafi *et al.*, 1994; Cunliffe *et al.*, 1999).

Until recently, there had not been evidence that *X. fastidiosa* translocated via twitching motility *in vitro* or *in planta*. When the genome sequence of *X. fastidiosa* was published, ORFs similar to type IV pilus genes in other bacteria were reported, and it was suggested that the pili might have a role in *X. fastidiosa* adhesion and translocation (Simpson *et al.*, 2000; Van Sluys *et al.*, 2003). Recently, it was shown for the first time that *X. fastidiosa* possesses two distinct types of polar pili: long, type IV pili and short, type I pili (Meng *et al.*, 2005). Analysis of various pilus-defective mutants further demonstrated that the long, type IV pili are involved in twitching motility on an agar surface, in a microfabricated flow chamber, and *in planta* (Meng *et al.*, 2005). Type IV pilus-defective mutants, e.g. 1A2 (*pilB*⁻) did not develop a colony fringe. Mutant 6E11 is defective for the shorter type I pili (*fimA*⁻), and continued to exhibit a fringe; in fact, 6E11 usually had a wider fringe than the wild-type Temecula parent isolate. Colony morphologies of the Pierce's disease isolate of *X. fastidiosa* were first described by Wells *et al.* (1987) as 'smooth opalescent' and 'rough opalescent'. Subsequently, Davis *et al.* (1981) described colony morphologies of xylem-limited bacteria (now known to be isolates of *X. fastidiosa*) that cause phony disease of peach and plum leaf scald to be '... smooth, whereas the surface of the lesser elevated portion of the colonies was

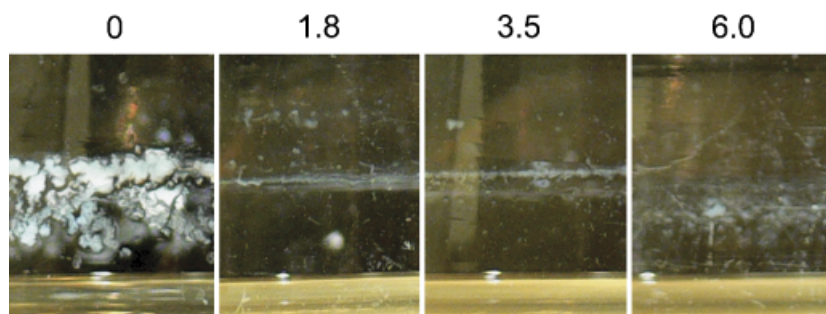


Fig. 5. Biofilm development of *Xylella fastidiosa* Temecula wild-type isolate following 20 days of growth in PD2 medium with different concentrations of BSA (g L⁻¹).

rough'. The description of 'rough' colony morphologies was interpreted as being equivalent to the peripheral fringe observed in many isolates of pathogenic *X. fastidiosa*, and indicative of twitching motility.

Why has the peripheral fringe not been observed more widely in X. fastidiosa cultures? The observations suggest that it is related to the BSA content of the medium, at least as far as PW medium is concerned. The original recipe for PW agar (Davis *et al.*, 1981) included the equivalent of 6 g L⁻¹ of BSA, the highest concentration used in the current study. Studies suggest that twitching motility is not the only type of bacterial motility inhibited by the presence of BSA (Dworkin *et al.*, 1983; Burchard, 1986). It was demonstrated that the surfactants Tween 80 and BSA inhibited swarming and gliding motility in *Cytophaga* U67 (Burchard, 1986). It was suggested that inhibition of motility occurred because surfactants prevented adhesion of the bacteria to a surface. Addition of BSA to growth medium completely inhibited gliding motility of *Myxococcus xanthus* (Dworkin *et al.*, 1983). In the case of twitching motility, type IV pili must attach to a surface in order for a cell to translocate via pilus retraction (Mattick, 2002). Thus, in the presence of BSA or other surfactants, type IV pilus-mediated motility will not occur. Interestingly, there is evidence that BSA stimulates twitching in *Pseudomonas aeruginosa* (Huang *et al.*, 2003), and if that were true for *X. fastidiosa* as well, the peripheral fringe would have been expected to be enhanced with increasing BSA concentrations, which was not observed.

In bacteria of the genera *Rochalimaea* (formerly *Rickettsia*) (Myers *et al.*, 1969), *Neisseria* (Glass & Kennett, 1939; Mueller & Hinton, 1941), and *Bordetella* (Mishulow *et al.*, 1953; Wilson, 1963), it was reported that substances such as activated charcoal or starch can be substituted for serum; therefore, BSA is likely not serving a specific nutritive role in bacterial growth. The fact that charcoal or starch can replace serum suggests that the role of BSA may be nonspecific; serum may 'detoxify' growth media by sequestering growth-inhibiting substances. BSA is not essential for growth in *X. fastidiosa*; however, growth is slower in its absence (Fig. 2) (Davis *et al.*, 1980).

In *X. fastidiosa*, biofilm formation is inhibited by the presence of BSA in the medium, and there is a direct relationship between increasing BSA concentration and a reduction in biofilm formation (Cooksey *et al.*, 2004). It is likely that the surfactant quality of BSA imparts a coating on surfaces that impedes bacterial cell or pilus attachment, and therefore biofilm formation. Biofilm development begins with the attachment of a single layer of cells to a substrate, which is followed by the attachment of additional layers of cells and the production of exopolysaccharide. If the initial step of cell attachment is prevented by BSA, then biofilm will not be produced.

Importantly, it was demonstrated that the Pierce's disease Temecula isolate of *X. fastidiosa* is not unique with respect to twitching motility and colony fringe development. All pathogenic isolates from California, South Carolina, and Texas, except SC 17A97, exhibited these properties on PW agar. When examined in greater detail, isolate SC 17A97 cells also exhibited twitching motility; thus, it is likely that most other *X. fastidiosa* isolates will have this phenotype. The data show that the concentration of BSA in PW agar dramatically influenced the appearance of the peripheral fringe. At the concentration of BSA used in the original PW recipe (Davis *et al.*, 1981), the peripheral fringe of the bacterial colonies is either greatly reduced or absent. Although BSA is not a natural component of xylem fluid, the results suggest the crucial role that grape xylem wall surface chemistry likely plays in the initial attachment of *X. fastidiosa* cells to the xylem, and the subsequent progression of Pierce's disease. An examination of xylem fluid composition in resistant vs. susceptible plants may help us to better predict strategies for controlling this pathogen.

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