The *Pseudomonas fluorescens* SBW25 wrinkly spreader biofilm requires attachment factor, cellulose fibre and LPS interactions to maintain strength and integrity

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INTRODUCTION

Many bacteria are capable of forming large assemblages on plant and animal surfaces and tissues, on biological detritus, sediments, soils and other geological structures, as well as suspended flocs in water columns. Although often hard to investigate in situ, significant biological properties are attributed to these assemblages, including co-operative behaviour, competitive advantage, and defence against predators, antibiotics and immune systems, physical disturbance, etc. (for recent reviews see Costerton et al., 1995; Davey & O’Toole, 2000; Wimpenny et al., 2000; Lappin-Scott & Bass, 2001; Sutherland, 2001a; Wilson, 2001; Donlan, 2002; Dunne, 2002; Morris & Monier, 2003; Hall-Stoodley et al., 2004). These assemblages range from the largely random aggregation of bacteria growing on surfaces, in semi-solid environments or in constrained volumes, to complex structures incorporating substantial amounts of extracellular matrix material (Sutherland, 2001b; Hall-Stoodley & Stoodley, 2002; Stoodley et al., 2002; Ghigo, 2003). This latter type of assemblage represents biofilms in stricto senso, and the presence of structural matrix material provides biofilms with a cohesive physical identity that may be lacking both in colonies and in slime.

It is clear that the physical resilience of biofilms is the result of multiple interactions between matrix components (often exopolysaccharides, EPS), bacterial surface appendages (fimbriae, flagella and aggregation factors) and coatings (lipopolysaccharide, LPS) and the surface colonized by the bacteria (see references above and Dalton & March, 1998; Sutherland, 2001b; Donlan, 2002; Götz, 2002). In the case of the biofilms produced by *Salmonella typhimurium* and *Salmonella enteritidis rdar* mutants, and by the *Pseudomonas fluorescens* SBW25 wrinkly spreader, the expression of a cellulose matrix and a fimbrial-like attachment factor are the primary components contributing to biofilm strength and integrity (Römling & Rohde, 1999; Zogaj et al.,...
In an investigation of the genes required for biofilm formation by *P. fluorescens* WS (using one particular WS isolate, PR1200; Spiers et al., 2002), mini-Tn5 mutagenesis identified two major loci – the *wsp* chemosensory operon encoding the response regulator WspR, and the *wss* cellulose biosynthesis operon, which includes genes involved in the biosynthesis of cellulose. This last interaction is required during the first phase of cell interactions required for the normal development of biofilms. These findings suggest that the physical integrity of the cellulose matrix–attachment factor–bacterial cell interactions required for the normal development of *P. fluorescens* biofilms.

**METHODS**

**Bacterial strains, plasmids, culture media and growth conditions.** The *Pseudomonas fluorescens* strains used in this work are derivatives of *P. fluorescens* SBW25 (Table 1) and were grown using King’s B (KB) medium (King et al., 1954) or minimal medium containing 20 mM sucrose at 28°C. A KB microcosm consisted of a 35 ml Universal glass vial containing 6 ml KB, and was incubated with the lid held loosely in place with porous tape. *P. fluorescens* motility was assessed using 0.1% KB/0.5% (w/v) soft-agar plates. *Escherichia coli* DH5α (Gibco-BRL) and S17-1-λ<sub>S1</sub> (Simon et al., 1983) were used for DNA manipulation and conjugation. *E. coli* strains were grown using LB medium at 37°C. The plasmids pBS<sup>+</sup>-Stratagene and pGEM7f (Promega) were used for subcloning and sequencing, and the *P. fluorescens* SBW25 cosmid library in *E. coli* S17-1-λ<sub>S1</sub> was from Rainey (1999). p34S-Km<sub>3</sub> was from Dennis & Zylstra (1998). WspR and WspR19 were expressed in trans using pVS61-*wspR12-ΟTac<sup>B</sup> (wild-type WspR) and pVS61-*wspR19-ΟTac<sup>B</sup> (WspR R129C); pVS61-*ΟTac<sup>B</sup> was used as a negative control (Goyer, 2002). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml<sup>−1</sup>; chloramphenicol, 20 μg ml<sup>−1</sup>; kanamycin, 25 μg ml<sup>−1</sup>; pipercillin, 150 μg ml<sup>−1</sup>; and tetracycline, 12.5 μg ml<sup>−1</sup>. Congo red (CR) [also known as Direct Red (DR)] 28; **Table 1. *P. fluorescens* SBW25 strains used in this work**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>JB01</td>
<td>SM but NPTII promoter overexpressing <em>wss</em></td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>AS24</td>
<td>Non-chemotactic biofilm-forming strain evolved from a SBW25 cheA mutant</td>
<td>A. Spiers</td>
</tr>
<tr>
<td><strong>WS and mutants</strong></td>
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<tr>
<td>WS</td>
<td>Biofilm-forming strain (PR1200) evolved from SM</td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>WS-4</td>
<td><em>wspR</em>::mini-Tn5; isolated from a mini-transposon screen of WS</td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>WS-5</td>
<td><em>tol</em>::mini-Tn5; isolated from a mini-transposon screen of WS; mini-Tn5 is immediately upstream of ybgC, the first gene of the <em>tol</em> cluster</td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>WS-13</td>
<td><em>wssB</em>::mini-Tn5; isolated from a mini-transposon screen of WS</td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>WS-18</td>
<td><em>wssF</em>::mini-Tn5; isolated from a mini-transposon screen of WS</td>
<td>Spiers et al. (2002)</td>
</tr>
<tr>
<td>WS tolA</td>
<td><em>tolA</em>::Km&lt;sub&gt;5&lt;/sub&gt;; kanamycin-resistance cassette inserted into the <em>Sphl</em> site of <em>tolA</em></td>
<td>This work</td>
</tr>
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region of the liquid culture to the glass vials. Subsequent growth out over the A–L interface results in the characteristic WS biofilm (Spiers et al., 2003).

One of the previously identified WS mini-Tn5 mutants, WS-5, was found not to be associated with either of the *wsp* or *wss* operons (Spiers et al., 2002). WS-5 cells form an unusual colony morphology that is intermediate between that of WS and the non-biofilm-forming SM strain that produces smooth colonies on agar plates. Colonies of WS-5 bind Congo red, indicating that it expresses cellulose, and this strain is able to form weak biofilms when incubated in liquid microcosms. In this work, we reveal the genomic location of the transposon insertion responsible for the observed defects in WS-5 and show how defects in LPS expression affect A–L biofilm strength by significantly altering the cellulose matrix–attachment factor–bacterial cell interactions required for the normal development of WS biofilms.


**General molecular biology methods.** Standard molecular biology techniques were used according to current protocols or manufacturer’s instructions. Optical densities were determined using 4 mm-pathway plastic cuvettes and a Spectronic 20 Genesys (Spectronic Instruments) spectrophotometer. Conjugation and electroporation were used to transfer DNA between or into *E. coli* and *P. fluorescens*. Ligation mixtures were dialysed against deionized water for 30 min using 20 μm HA MF-membrane filters (Millipore) before electroporation. Plasmid and cosmids DNA was isolated from *E. coli* using Qiagen mini-spin kits. DNA was analysed by TBE-agarose gel electrophoresis and ethidium bromide-staining. The following subclones of the cosmids pAS256 were made for sequencing purposes: pAS257-260 and 263 were HindIII fragments in PBS; pAS261 and 262 were *Sal* fragments in PBS; pAS265, 266, 268 and 269 were partial HindIII deletions of pAS260; and pAS267, 271 and 272 were *Sphl* fragments in pGEM7 (further mapping data are available on request). Nucleotide sequence was obtained by automated sequencing using standard vector and sequence-specific primers.

**Construction of the WS tolA mutant.** This mutant was produced by the insertion of a kanamycin-resistance (Km) cassette into tolA. This was achieved by cloning Km from p34S-Km3 into the *Sphl* site of pAS258, a PBS* clone containing the three HindIII fragments covering the *ybgC-tolB* region (Fig. 1), to give pAS298. This plasmid was electroporated into WS, and Km* integrants were isolated. These were grown in non-selective medium then plated onto KB plus kanamycin. Individual colonies were tested for piperacillin sensitivity (bla from pAS298 confers resistance to piperacillin, Pp) indicating vector excision, and an appropriate Km* Pp* isolate chosen as WS tolA.

**LPS analysis.** LPS samples were obtained from cultures using EDTA extraction. Overnight KB cultures were first diluted to an OD560 of ~0.5 to equalize cell numbers. Cells from 2 ml of culture were resuspended in 100 μl deionized water; 400 μl 250 mM EDTA (pH 8.0) was added and the suspension vortexed vigorously for 5 s. The suspension was incubated at 37°C for 30 min and vortexed every 10 min. The supernatant was recovered for analysis after centrifugation at 10,000 g for 5 min. Aliquots were examined using 18% deoxycholic acid polyacrylamide gel electrophoresis (DOC-PAGE) and silver staining (Bio-Rad) according to Reubs et al. (1998).

The monoclonal antibody (mAb) BC12-CA4 (Meyer & Dewey, 2000) was used to detect LPS by ELISA assay. This mAb recognizes an unidentified antigen from *B. cinerea* and binding is inhibited by rhamnose (Rha), suggesting that the target is a Rha-glycosylated protein. It was tested as a possible mAb against pseudomonads as these bacteria contain substantial Rha polymers as part of the conserved LPS A-band O-polysaccharide component (Rocchetta et al., 1998). KB-grown cells were resuspended in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 8.0), adjusted to an OD560 of ~0.5 to equalize cell numbers, and used to produce a 10^–10^ dilution series in PBS. Aliquots of 100 μl were adsorbed to a MaxiSorp ELISA Plate (Nunc) overnight at 4°C. After washing with PBS/0.05 % (v/v) Tween-20, mAb was added and incubated for 1 h at 37°C. Bound mAb was detected using anti-mouse polyclonal immunoglobulin peroxidase conjugate (Sigma) and TMB substrate (Adgen) and absorbance measured at 450 nm. mAb binding was tested as AA50 OD560, having adjusted for cell numbers. Measurements were performed in duplicate.

**Microscopy and FACS.** Propidium iodide (PI) was used to assess the leaky membrane phenotype of mutants after Gaspar et al. (2000). PI (20 μM) was added to KB cultures and incubated at 28°C in the dark for 30 min before examination with an Olympus BX50 epifluorescence microscope. Calcofluor (Fluorescent Whitener 28, Sigma) was used to assess the presence of cellulose in biofilms or colony material. Calcofluor (10 μM) was added to samples resuspended in KB, then incubated at 28°C for 2 h before washing with fresh KB and subsequent examination. A fluorescence-activated cell sorter (FACS) was used to determine the percentage of cells stained with PI. Overnight KB cultures were diluted to an OD560 of 0–100–0–150 in fresh KB and incubated with PI for 30 min. FITC–(orange) fluorescence and scattering were measured for 100,000 events, and the percentage above a threshold determined by preliminary comparison between WS and WS-5 was recorded for each culture.

**Biofilm and cellulose assays.** Bacterial attachment to the glass of KB microcosms in the meniscus region was determined quantitatively using crystal violet as previously described (Spiers et al., 2003) and presented as the relative attachment with respect to the WS biofilm [A570 OD560 WS*]. The absolute strength of KB-grown biofilms was determined by placing glass balls in the centre of each biofilm until it broke, sank or was ripped from the sides of the microcosm vial, to determine the maximum deformation mass (MDM) (grams) (Spiers et al., 2003). In the case of the complementation and chemical interference experiments, biofilms were incubated on the bench at 20–22°C to minimize physical disturbance; as a result, MDM values were lower than those obtained at 28°C. In the complementation experiments, overnight cultures of strains to be tested were diluted to an OD560 of 1.00. KB microcosms were then inoculated with 100 μl aliquots of single strains or a mixture of...
two strains (in a total volume of 100 µl) in such a manner that each test used the same total number of cells. In the interference experiments, 120 µl 500 mM EDTA or water was added to mature KB-grown biofilms at the meniscus in order to avoid disruption of the biofilm. The EDTA was allowed to mix by diffusion over a period of 2 h before MDM were determined. A quantitative measure of cellulose experience by various strains in KB microcosms, whereas morphology typical of wrinkly spreaders (Spiers et al., 2002). As the first step in this work, we characterized the WS-5 was isolated from a mini-Tn mutagenesis of the WS strain, and is defective in expression of the wrinkled colony function) than the WS-5 mini-Tn insertion (in which tolA gene was sufficient to explain the WS-5 phenotype, we made a WS tolA mutant in which tolA was disrupted and downstream tolB:ybgF expression compromised (Fig. 1). WS tolA− showed a WS-5-like colony morphology, expressed cellulose and produced weak biofilms in KB microcosms, suggesting that it was the disruption of the functionally known tol genes, rather than the functionally uncharacterized ybgC gene, that was responsible for the phenotype of WS-5. In contrast to WS-5 (WS tol:: mini-Tn5), WS tolA in shaking cultures produced more floccular material, suggesting that the tolA mutation generated a more severe phenotype (through the inactivation of TolA and loss of Tol-Pal function) than the WS-5 mini-Tn5 insertion (in which tolQRB-pal-ybgF expression was reduced, allowing some Tol-Pal function to be preserved). For this reason, the rest of our work focused on the comparative analysis of WS-5 alone.

**Identification and analysis of the mini-Tn5 insertion site in WS-5**

In order to determine the location and genetic identity of the mini-Tn5 insertion site in WS-5, we screened a P. fluorescens SBW25 cosm library for clones that complemented WS-5 in trans and restored the WS phenotype on KB agar plates. One cosmID was isolated (pAS256) and restriction analysis indicated that it contained a ~20 kb fragment, which was then randomly subcloned and end-sequences obtained. This sequence-sampling allowed identification of two well-conserved gene clusters at either end of the cosmid insert: the gsv glycine cleavage system (Okamura-Ikeda et al., 1993), and tol-pal (also referred to as tol-oprL) (Sturgis, 2001). When located on the unfinished SBW25 genome, the sequences identified a single contig covering the entire cosmid insert region. Using a mini-Tn5-specific primer and nested-PCR sequencing, the insertion site of mini-Tn5 in WS-5 was determined immediately upstream of ybgC, the first gene in the tol-pal cluster (i.e. WS-5 was WS tol:: mini-Tn5) (Fig. 1).

Tol-Pal proteins are involved in the normal interaction of the inner and outer membranes and are found throughout the eubacteria (Sturgis, 2001; Lazzaroni et al., 1999; Llubes et al., 2001). Tol-Pal system mutants typically show impaired control of membrane channels (resulting in problems with uptake or export, leakage of proteins from the cytoplasm, sensitivity to pH and osmotic stress) and the disruption of outer-membrane or cell-surface components, including a reduction in or loss of LPS expression (Gaspar et al., 2000).

**RESULTS**

**Initial phenotypic characterization of WS-5**

WS-5 was isolated from a mini-Tn5 mutagenesis of the WS strain and is defective in expression of the wrinkled colony morphology typical of wrinkly spreaders (Spiers et al., 2002). As the first step in this work, we characterized the phenotype of WS-5 on agar plates and in liquid microcosms. WS-5 colonies on both KB and LB agar were smooth-like and did not show the normal wrinkled colony morphology of the WS. After 1 day, the colonies typically were smaller and more waxy-looking than those produced by the SM strain, and over a period of 2–3 days became less SM-like but never fully WS. WS-5 colonies stained orange with CR on agar plates, and WS-5 produced very weak biofilms in KB microcosms. Examination of Calcofluor-stained colony and biofilm material by fluorescent microscopy confirmed that WS-5 expressed cellulose.
Confirmation of the leaky-membrane phenotype

In order to determine whether WS-5 showed the leaky-membrane phenotype typical of Tol-Pal mutants, we assessed membrane integrity using the fluorescent DNA-binding dye propidium iodide (PI). This hydrophilic dye cannot pass through the bacterial membrane, and can only bind DNA if the membrane has been damaged. Fluorescent microscopy of exponential-phase WS-5 cells grown in KB with PI showed that a significant number of cells stained with the dye (and cells were misshapen), indicating that WS-5 shows the expected Tol-Pal leaky-membrane phenotype. In contrast, most WS cells did not stain with PI and showed no evidence of misshapen cell morphologies. We used FACS analysis to quantify the relative differences in PI uptake, and found that WS-5 (WS tol::mini-Tn5) staining was 3.35-fold greater than the mean uptake for SM, WS, WS-4 (WS wspR::mini-Tn5) and WS-18 (WS wssF::mini-Tn5) cells.

Some Tol-Pal system mutants are able to utilize small molecular mass molecules as sole carbon sources that diffuse across the damaged membrane, which otherwise could not cross into the cytoplasm, where they are metabolized (Llamas et al., 2003). In a test of this particular phenotype, we found that WS-5 was able to grow on minimal agar supplemented with sucrose, whereas neither the SM nor WS strains could use the disaccharide as the sole carbon source. These findings are all consistent with the leaky-membrane phenotype expected from the mini-Tn5 insertion site in WS-5.

WS-5 is insensitive to WspR reactivation of the WS phenotype

In order to determine how a disruption of the Tol-Pal system might result in weak biofilm formation by WS-5, we first examined whether the WS phenotype in WS-5 could be recovered by WspR expressed in trans. Previous work has identified WspR as a regulator of both cellulose and attachment-factor expression (Speirs et al., 2002, 2003). When expressed in trans in SM, both wild-type WspR (WspR12) and the constitutively active mutant WspR19 produce WS-like colony morphologies (Goymer, 2002). We determined the colony phenotypes of SM and WS-5 (WS tol::mini-Tn5) carrying pVSP61-ΩTCR, pVSP61-wspR12-ΩTCR and pVSP61-wspR19-ΩTCR on both KB and LB agar plates. The control plasmid pVSP61-ΩTCR did not alter either SM or WS-5 colony morphologies, and both pVSP61-wspR12-ΩTCR and pVSP61-wspR19-ΩTCR produced WS-like colonies in SM. In contrast, neither pVSP61-wspR12-ΩTCR nor pVSP61-wspR19-ΩTCR altered the colony morphology of WS-5.

These findings indicate that WS-5 is not a mutant in which the WS phenotype has been turned off, or in which the signal that activates the WS phenotype has been interrupted. It therefore seemed most likely that a third component required for normal WS biofilm formation was no longer available. Of all of the phenotypes associated with Tol-Pal system mutants, we considered that the loss of LPS expression was most likely to have an impact on biofilm formation. We therefore directly tested this hypothesis by examining whether LPS expression was reduced in WS-5, whether WS-5 cells showed altered hydrophobicity, and whether WS biofilm strength could be changed by chemical interference targeted at LPS–cellulose fibre–attachment factor interactions.

Expression of LPS

LPS expression is strongly reduced in Tol-Pal mutants (Gaspar et al., 2000). In order to determine whether WS-5 showed a similar reduction in LPS expression, we prepared LPS EDTA-extracts from overnight KB cultures in which cell densities had been first equalized. These extracts were electrophoresed using DOC-PA gels that were then silver-stained to reveal the major LPS bands (Fig. 2). WS-5 (WS tol::mini-Tn5) expressed insignificant amounts of LPS when compared with either WS, WS-4 (WS wspR::mini-Tn5) or WS-18 (WS wssF::mini-Tn5). LPS levels in WS and WS-5 were also investigated using the mAb BC12-CA4. Although the binding of mAb BC12-CA4 to P. fluorescens was weak, ELISA assays clearly showed a significantly greater (5.7 ×) mAb binding to WS than WS-5 cells (P=0.0384) (ΔA450 OD600-1 ±SE: WS, 0.554 ±0.022; WS-5, 0.097 ±0.011), further supporting our DOC-PAGE observations that WS-5 does not express detectable amounts of LPS.

Relative hydrophobicity of WS strains

LPS expression is known to affect the surface charge and/or relative hydrophobicity (Hr) of bacterial cells (Rocchetta

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\text{Fig. 2. LPS expression in WS-5 is strongly reduced in comparison with the wrinkly spreader and other mutants. From left to right: LPS samples from WS, WS-4 (WS wspR::mini-Tn5), WS-5 (WS tol::mini-Tn5) and WS-18 (WS wssF::mini-Tn5). The major LPS bands are indicated by triangles. LPS samples were prepared from overnight KB cultures adjusted to give the same OD_{600} cell density. Samples were extracted using EDTA, electrophoresed in an 18% DOC-polyacrylamide gel and then silver-stained to detect LPS.}\n\]
Comparison between WS and WS-5 biofilms

In order to quantify the differences in the physical characteristics between the WS-5 biofilm and those produced by WS and other mutants, we determined the relative attachment and maximum deformation mass (MDM, strength) of 3-day-old KB-grown biofilms. Although the ability of WS-5 (WS tol::mini-Tn5) to attach to the surface of the glass microcosms was not significantly different from that of WS or WS-18 (WS wssF::mini-Tn5) (P=0.0547) (Fig. 3a), the absolute strength of the WS-5 biofilm was substantially less than that of WS or WS-18 (P<0.0001) (Fig. 3b). In order to determine whether the reduced strength of WS-5 biofilms was due to a decreased rate of growth, we measured growth in KB over 24 h for WS, WS-5 (WS tol::mini-Tn5) and WS-18 (WS wssF::mini-Tn5) (ΔOD₆₀₀ h⁻¹±SE: WS, 0.058±0.001; WS-5, 0.071±0.001; WS-18, 0.068±0.001). The three growth rates were not significantly different (P=0.4254), indicating that the reduced WS-5 biofilm strength could not be due to a decreased rate of growth. Finally, we also measured the relative amounts of cellulose produced by each strain using a CR-binding assay. WS and WS-5 (WS tol::mini-Tn5) bound similar amounts of CR (P=0.0965) and slightly less (P=0.04) than WS-18 (WS wssF::mini-Tn5) (P=0.024) (ΔOD₆₀₀ OD₆₀₀ WS⁻¹±SE: WS, 0.317±0.019; WS-5, 0.368±0.019; WS-18, 0.464±0.033). CR binds components other than cellulose, and in the case of WS and WS mutants, CR is bound by cellulose and attachment factor (Spiers et al., 2003). Having demonstrated that WS, WS-5 and WS-18 show the same degree of attachment and similar levels of CR-binding, we therefore conclude that the three strains express similar levels of cellulose. This finding indicates that the reduced strength of WS-5 biofilms is not due to a reduced level of cellulose expression.

Recruitment to the A–L Interface

The differences in relative hydrophobicity (H₄) between WS, WS-5 and WS-18 cells might affect A–L biofilm strength by enhancing the recruitment of cells to the meniscus region where initial attachment to the glass surface of microcosms occurs, and by maintaining cells within the developing biofilm during growth. We examined differences in recruitment by monitoring OD₆₀₀ at the bottom of the liquid column using standard spectrophotometer cuvettes. We reasoned that bacterial cells may adhere to the surface of the cuvette, or remain a homogeneous suspension of cells, and thus show no change in OD₆₀₀. Alternatively, the cells may be recruited to the surface (by chemotaxis or random motion) and maintained through attachment to the walls or aggregation with other bacteria at the surface. First, however, we determined that WS, WS-4 (WS wspR::mini-Tn5), WS-5 (WS tol::mini-Tn5), WS-13 (WS wspR::mini-Tn5) and WS-18 (WS wssF::mini-Tn5) cells were motile by direct microscopic examination, and significantly different from the non-chemotactic AS24 (an evolved SM cheA) (P<0.0001) (migration through soft agar, mm h⁻¹: WS, 0.681±0.019; WS-5, 0.394±0.019; WS-18, 0.594±0.019; cf. Other strains)

Fig. 3. Relative attachment and strength of WS-5 A–L biofilms. (a) Relative attachment for SM, WS, WS-4 (WS wspR::mini-Tn5), WS-5 (WS tol::mini-Tn5), WS-13 (WS wspR::mini-Tn5) and WS-18 (WS wssF::mini-Tn5) (OD₆₀₀ OD₆₀₀ WS⁻¹); (b) Maximum deformation mass (MDM) for WS, WS-5, and WS-18 (SM, WS-4 and WS-13 do not produce biofilms). Microcosms were incubated at 28 °C for 3 days before assay. Means±SE are shown.
WS-4, which is not impeded by attachment factor, 0.836 ± 0.022; and AS24, 0.125 ± 0.019). The relative OD<sub>600</sub> at the bottom of KB liquid columns was found to decrease for WS, WS-5 and WS-18 as cells migrated towards the surface over a period of 1 h (Fig. 4). WS cells showed a significantly greater (3–5×) level of recruitment to the surface than either WS-5 (WS<sub>tol·</sub>mini-Tn5) or WS-18 (WS<sub>wssF·</sub>mini-Tn5) (P = 0.006) (relative OD<sub>600</sub> ± SE: WS, 0.8877 ± 0.0013; WS-5, 0.9224 ± 0.0066; WS-18, 0.9299 ± 0.0011). In contrast, WS-4 (WS<sub>wpkR·</sub>mini-Tn5) cells, unable to express cellulose or attachment factor, showed a substantially different behaviour in which cells initially adhered to the sides of the cuvette before slowly migrating towards the surface after 20 min. We also measured the ability of WS, WS-5 and WS-18 biofilms to maintain cells within the developing biofilm during growth. After 3 days, the proportion of cells found in the liquid column under the biofilm was significantly greater (3–4×) for WS-5 (WS<sub>tol·</sub>mini-Tn5) and WS-18 (WS<sub>wssF·</sub>mini-Tn5) than for WS (P = 0.0001) (though the total OD<sub>600</sub> achieved by WS, WS-5 and WS-18 was the same, P = 0.5022). From these findings it is clear that the weaker WS-5 and WS-18 biofilms are unable to recruit and maintain cells within the biofilm as efficiently as the WS.

Chemical interference of interactions amongst biofilm components

Previously, we have shown that the presence of the dye CR resulted in a significant decrease in the strength of WS biofilms by interfering between the normal cellulose fibre and/or attachment factor interactions (Spiers et al., 2003). In order to further elucidate interactions between biofilm components, we tested WS, WS-5 and WS-18 biofilm strengths in the presence of Ca<sup>2+</sup>, Fe<sup>3+</sup>, EDTA and various diazo dyes structurally related to CR. The metal anions are expected to bind EPS/LPS and alter the normal cell-surface charge distribution; EDTA chelates Mg<sup>2+</sup>, which is known to have a major role in LPS charge-neutralization (Groisman et al., 1997; Rocchetta et al., 1999). In contrast, the diazo dyes bind cellulose differentially due to slight structural variations (Kai & Mondal, 1997), and are expected to interact similarly with the WS attachment factor.

In initial tests, we found that both Ca<sup>2+</sup> and Fe<sup>3+</sup> severely affected growth in KB microcosms, whereas the addition of more Mg<sup>2+</sup> (KB contains ~6 mM Mg<sup>2+</sup>) had no effect on MDM, and therefore these were not tested further. However, at low levels of EDTA (2–10 mM), a significant decrease in WS and WS-18 (WS<sub>wssF·</sub>mini-Tn5) MDM was observed, whereas WS-5 (WS<sub>tol·</sub>mini-Tn5) MDM was not significantly affected even at 10 mM EDTA (P = 0.1333) (Fig. 5a). (5 mM EDTA had no effect on maximum growth rate, but 10 mM EDTA resulted in a 0.2× reduction in growth rate.) EDTA might act to prevent irreversible interactions that occur during biofilm development, or it might act to destabilize reversible interactions that maintain biofilm strength. To determine which of these possibilities was more likely, we tested the MDM of mature WS biofilms 2 h after EDTA had been added to a final concentration of 10 mM. There was no significant difference in MDM between the EDTA-treated biofilms and water-treated negative controls (P = 0.6708), indicating that EDTA affects the establishment of irreversible interactions that form during biofilm development, rather than destabilizing reversible interactions (i.e. the constant association and dissociation of cellulose fibres, attachment factor and LPS) that might occur in the mature biofilm.

We also tested CR and related diazo dyes (DR 2, DB 1, 14, 15 and 53) (Fig. 5b), having first determined that no dye showed a toxic effect on growth at the concentration tested. None of the dyes increased the relative MDM of WS-5 (WS<sub>tol·</sub>mini-Tn5) (P = 0.9804). However, DB 1 and DB 53 differentiated WS-5, DB14 differentiated WS-18 (WS<sub>wssF·</sub>mini-Tn5), and DR 2 differentiated WS from the other two strains. These findings confirmed our expectations that EDTA and the diazo dyes would differentiate between WS, WS-5 and WS-18 biofilms through differential interference of interactions between biofilm components. This strongly suggests that biofilm strength is the result of multiple interactions between biofilm components, and that WS-5 biofilms lack some component found in both WS and WS-18 biofilms.

Complementation of WS-5 biofilms with WS-4

If biofilm development and final strength are the result of multiple cellulose fibre–attachment factor–LPS interactions,
we reasoned that the weak WS-5 biofilm may be complemented by a second strain capable of expressing LPS, but which cannot express cellulose or attachment factor (e.g. WS-4). In order to test this expectation, we determined the MDM of KB-grown WS-4 (WS wspR::mini-Tn5)/WS-5 (WS tol::mini-Tn5) mixed biofilms (Fig. 6).

Biofilms produced from WS-5 alone or 1:9 WS-4/WS-5 were significantly weaker than 1:4 WS-4/WS-5-mixed biofilms ($P=0.0465, 0.0486$), but none of the mixed biofilms reached the strength of WS biofilms. Nevertheless, this result demonstrates that WS-4 can partially complement WS-5 biofilm strength. Furthermore, similar partial complementation of JB01 (SM NPTII::wss) biofilms with WS-13 (WS wssB::mini-Tn5) was also seen (JB01 is an SM derivative overexpressing cellulose but not expressing attachment factor, which produces a particularly weak biofilm: Spiers et al., 2002). When JB01 was complemented with WS-13 expressing attachment factor, the MDM of the 1:1 WS-13/JB01 biofilm was significantly greater than that of a biofilm of JB01 alone ($P<0.0001$). In both the WS-4/WS-5 and WS-13/JB01 tests, the increased MDM of the mixed biofilms is not the result of differences in growth rates, as in both cases, significant complementation was only seen with higher initial ratios of the strain that could not produce a biofilm alone (i.e. the significant comparisons are between 1:9 and 1:4 WS-4/WS-5, and between 1:9 and 1:1 WS-13/JB01). These findings strongly suggest that the strength of the WS biofilm is due to the combination of cellulose, attachment factor and LPS, and that partial complementation can be achieved by expressing all three components by two strains in different combinations.

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**Fig. 5.** EDTA and diazo dyes interfere with normal interactions between biofilm components and result in altered biofilm strengths. (a) The addition of EDTA to standard KB microcosms had a significant effect on the relative maximum deformation mass (MDM) of both WS (●) and WS-18 (WS wssF::mini-Tn5) (○) biofilms, but no significant effect on the very weak WS-5 (WS tol::mini-Tn5) (△) biofilm except at high concentrations of EDTA. (b) Diazo dyes significantly altered the relative MDM of WS (dark bars) and WS-18 (white bars) biofilms, but had little impact on WS-5 (light grey bars) biofilms. Ctrl, no dye added. CR is also known as DR 28. Microcosms were incubated at 28 °C for 3 days before assay. Mean±SE of relative MDM shown for both assays. Note the log scale in (b).

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**Fig. 6.** WS mutant A–L biofilms can be partially complemented and show significant increases in strength. The WS-5 (WS tol::mini-Tn5) biofilm can be complemented by WS-4 (WS wspR::mini-Tn5), leading to a $1.7 \times$ increase in maximum deformation mass (MDM) (white bars). WS-5 expresses attachment factor and cellulose, but not LPS; WS-4 expresses LPS, but not cellulose or attachment factor. Similarly, the JB01 (SM NPTII::wss) biofilm is complemented by WS-13 (WS wssB::mini-Tn5), leading to a $3.9 \times$ increase in MDM (grey bars). JB01 expresses cellulose and LPS, but not attachment factor; WS-13 expresses LPS and attachment factor, but not cellulose. Neither WS-4 nor WS-13 produces biofilms. The MDM of WS in this experiment was 0.151±0.93 g (dark bar off-scale). Microcosms were inoculated with single strains, or with mixtures of strains, such that each test used the same total number of cells. Microcosms were incubated at 20–22 °C for 3 days before assay. Means±SE are shown.
DISCUSSION

In previous analyses of factors required for development of the WS A–L biofilm, we demonstrated that partially acetylated cellulose and a fimbrial-like attachment factor were significant determinants of the strength and structural integrity of the WS biofilm (Spiers et al., 2003). In this work, we have extended our analysis to the wrinkly spreader-defective mini-Tn5 mutant WS-5, which produces weak biofilms despite the production of partially acetylated cellulose and fimbrial attachment factor.

The site of the mini-Tn5 insertion in WS-5 was shown to be immediately upstream of ybgC, the first gene in the highly conserved tol-pal gene cluster, and it exerts polar effects on downstream gene expression. The Tol-Pal system proteins are involved in maintaining the correct functional relationship between the inner and outer membranes, and mutants often show a variety of pleiotropic effects, including the loss of LPS expression (Gaspar et al., 2000). We confirmed that this was the case in WS-5 by DOC-PAGE and ELISA assays.

LPS plays a major role in determining the surface-charge or relative hydrophobicity (Hr) of the cell (Rocchetta et al., 1999), and is involved in bacterial attachment to surfaces and biofilm formation; it differentiates between biofilm and planktonic cells, as well as affecting colony morphology in a number of bacteria (Giwercman et al., 1992; Genevax et al., 1999; Mireles et al., 2001; Nesper et al., 2001; Landini & Zehnder, 2002; de Lima Pimenta et al., 2003; Rashid et al., 2003). WS-5 cells showed a significantly different Hr from WS cells, and maintenance of WS-5 cells in the biofilm was less efficient than that of WS. In addition, the strength of the WS biofilm was more sensitive to chemical interference than that of the WS-5 biofilm. Each of these findings, along with the demonstration that the WS-5 biofilm can be partially complemented by an LPS-expressing strain, strongly suggests that LPS-dependent and charge-sensitive interactions are important in WS biofilm development, and that complex interactions between cellulose fibres, attachment factor and LPS determine the final strength of WS biofilms.

We have previously noted that the P. fluorescens WS A–L biofilm and colony morphology are similar to those produced by Escherichia coli and Salmonella sp. (Spiers et al., 2002), and in each case, cellulose fibres and curli/Tafi fimbriae are required for both biofilm strength and the rdar colony morphology (Römling & Rohde, 1999; Zogaj et al., 2001; Solano et al., 2002). In S. enterica biofilms, Tafi fibres appear as a tangled amorphous matrix when cellulose is present, but when it is not, the fibres adopt a more normal, slightly curved linear structure (White et al., 2003). Further analysis of E. coli and S. enterica biofilms has also revealed the presence of a third matrix component, an anionic extracellular polysaccharide, which requires cellulose in order to maintain a close association with cells (White et al., 2003). These findings, along with our observations regarding the WS biofilm, indicate that the structure and physical properties of bacterial biofilms are the result of multiple interactions between various matrix components – the main EPS matrix fibres, proteinaceous attachment fibres (fimbriae and flagella), LPS and additional polysaccharides. In parallel research, we are undertaking a comprehensive screen for new WS mutants, in which ISphoA/hah disruptions of the cellulose acetylation genes, putative LPS biosynthesis and membrane-associated genes have been identified (S. Gehrig, A. Spiers & P. Rainey, work in progress), further underlining the importance of these interactions in the WS phenotype.

LPS is generally anchored to the bacterial outer membrane via lipid A (Rocchetta et al., 1999). However, LPS is also known to be released from cells during normal growth, and cell-free LPS accumulates in cultures after cell lysis (Cadieux et al., 1983; Ishiguro et al., 1986; Al-Tahhan et al., 2000). This suggests that some of the cellulose fibre–attachment factor–LPS interactions important to WS biofilm strength may be cell-independent, insofar that once LPS has been produced and released at one location, bacterial cells may not be required to remain in place to maintain biofilm strength. Indeed, the WS biofilm might result from the aggregation of locally expressed and largely cell-free cellulose, attachment factor and released LPS, with the bacterial cells free to move within the biofilm as it develops and as environmental conditions change. This possibility is supported by WS biofilm microscopy, in which few bacteria were found to be closely associated with the cellulose matrix, and most found to be mobile within the spaces of the biofilm (Spiers et al., 2003).

Biofilm matrices are known to be chemically complex, with 85–98% of the total organic carbon present as excreted polymers and products from cell lysis, and the balance in intact cells (Sutherland, 2001a). It is becoming increasingly apparent that the physical structure of many biofilms is not primarily the result of the expression of one matrix component, but of several interacting elements. Furthermore, although the main component might be specifically expressed during biofilm development, the expression of other components may not be restricted to the biofilm. The added complexity of matrix components and expression patterns has an obvious impact on the study of biofilm development. While the formation of biofilms may confer a growth advantage at surfaces, biofilm matrix compounds need not be biofilm-specific; for example, flagella and pili play a role in motility as well as initial attachment, and EPS glycocalyx may play a protective role as well as contribute to formation of the biofilm matrix.

The finding that the structural aspect of biofilm matrices is more complex than originally thought has implications for the ‘architectural’ nature of biofilm development (Wimpenny et al., 2000; Ghigo, 2003). If biofilm development is a genetically programmed growth mode, then far more biosynthetic pathways would need to be controlled than currently recognized. On the other hand, if biofilm growth is a consequence of bacterial attachment, the resulting community structure may be better defined by a small
number of specific biosynthetic pathways, plus a number of less-specific systems that contribute to the overall physical-chemical structure of the biofilm matrix. If this is so, then we predict that biofilm growth will show a variable requirement for secondary matrix components and a degree of redundancy, as these will not be uniquely required and may be complemented by other components.

In the case of natural biofilms, ranging from those growing on dental surfaces or other human tissues to true water-, soil- or plant-associated environmental biofilms (Davey & O’Toole, 2000; Wilson, 2001; Morris & Monier, 2003), different members of the biofilm may contribute to different parts of the biofilm matrix. Given the continuously changing composition of biofilms during establishment, growth and maturity, a dynamic and complex physical-chemical matrix can only be expected. Many of the phenotypes associated with biofilms, such as increased resistance to stress and antimicrobial agents, may be the consequence of the microenvironment heterogeneity within biofilms (Ghigo, 2003). The complexity of both the biofilm matrix and community underlies the ecological success of this type of assemblage, and may also explain the value of such structures to both pathogenic and opportunistic bacteria.

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REFERENCES


Involvement of LPS in the wrinkly spreader biofilm


