Type IV pili and type II secretion play a limited role in *Legionella pneumophila* biofilm colonization and retention

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Legionellae colonize biofilms in building water systems, yet little is known about their interaction with the organisms in these microbial communities. The role of *Legionella pneumophila* type IV pili and the type II secretion pre-pilin peptidase was evaluated in a model biofilm system. *L. pneumophila* strains 130b (wild-type), BS100 (a type IV pili mutant) and NU243 (a pre-pilin peptidase mutant) were assessed for attachment and retention in an established biofilm. Strains 130b and NU243 colonized the biofilm at a similar level while BS100 attached at a tenfold lower level. Over time, NU243 dropped below the level of detection while BS100 remained in the biofilm throughout the course of the experiment. The wild-type strain decreased but remained at a considerably higher level than either of the mutants. Inclusion of amoebae with BS100 allowed for attachment and retention at a level similar to 130b. NU243, which displays reduced intracellular replication, was able to establish itself and persist in the presence of amoebae. Thus, type IV pili and the pre-pilin peptidase facilitate *L. pneumophila* colonization of biofilms but are not required in the presence of a host for intracellular replication.

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INTRODUCTION

Legionella pneumophila is able to enter and replicate within the phagosome of alveolar macrophages and epithelial cells (Abu Kwaik, 1998; Gao *et al.*, 1998). *L. pneumophila* can also parasitize protozoa in a similar, but not identical, manner (Fields, 2002; Gao *et al.*, 1997). Legionellae and protozoa inhabit biofilms in building water systems, providing a reservoir of contamination that can persist for decades (Cowgill *et al.*, 2005; Fields *et al.*, 2002). The overall structure of the biofilm, in addition to providing physical protection, is a unique ecosystem in which heterogeneous species interact in a multicellular manner (Romling *et al.*, 1998; Romling, 2001; Shapiro, 1998). Colonization, micro-colony formation and maturation are distinct phases of biofilm development that require a collaborative effort from each constitutive organism, with stage-specific expression of several molecular components (Dalton & March, 1998; Le Thi *et al.*, 2001). Environmental signals may regulate which organisms can colonize a surface as well as determine their release into the planktonic milieu (Stanley & Lazazzera, 2004).

For example, the expression of type IV pili is essential to colonization and maturation of the *Pseudomonas aeruginosa* biofilm on a variety of surfaces (O'Toole & Kolter, 1998). These adhesins, expressed by numerous Gram-negative micro-organisms, are characterized by sequence conservation of the structural subunits, assembly machinery, and

mechanisms of transcriptional regulation (Strom & Lory, 1993). In *P. aeruginosa* the twitching or gliding motility associated with type IV pili are necessary for surface translocation allowing microcolony foundation (O'Toole & Kolter, 1998). These pili also participate in cell–cell communication both with related species and with diverse unrelated micro-organisms in multicellular matrices (Dorr *et al.*, 1998; Kallstrom *et al.*, 1998; Wall & Kaiser, 1999). Type II secretion, the mechanism by which most Gram-negative organisms export pilus machinery for assembly, also translocates several virulence factors that may contribute to biofilm maturation (China & Goffaux, 1999; Liles *et al.*, 1999).

In order to determine whether these molecular components are essential for biofilm formation in *L. pneumophila*, colonization and retention of pilus mutants were examined using the biofilm model described by Murga *et al.* (2001). The rotating disc reactor they designed is unique in that it seeks to effectively mimic conditions in building water systems, using a moderate water flow rate, tap water rather than rich medium, and a surface (stainless steel) in common use for potable water delivery systems. Their work demonstrated that *L. pneumophila* could colonize an established biofilm consisting of *P. aeruginosa*, *Klebsiella pneumoniae* and a *Flavobacterium* sp. In the absence of a protozoan host for intracellular replication, *L. pneumophila* was able to persist for at least 15 days, although no growth

was evident. The addition of amoebae into this system allowed for growth of *L. pneumophila*, providing a model for *L. pneumophila* persistence in building water systems.

Many Gram-negative organisms have an absolute requirement for type IV pili and the type II secretion system in order to establish a biofilm and allow for maturation (Bechet & Blondeau, 2003; Entcheva-Dimitrov & Spormann, 2004; Giltner *et al.*, 2006; Moorthy & Watnick, 2004). However, *L. pneumophila* is a facultative intracellular parasite of a broad range of free-living protozoa, and thus may have evolved different mechanisms for the biofilm niche compared to free-living Gram-negative bacteria. This study investigated the ability of *L. pneumophila* to colonize and persist in the Murga model when altered in the expression of type IV pili or the type II secretion system. Strain NU243 contains a kanamycin resistance (Km^R) cassette inserted in *pilD* that abolishes expression of the pre-pilin peptidase, PilD. PilD cleaves the leader sequence of proteins, such as the major type IV pilus subunit, PilE, which are exported via the type II secretion mechanism. Thus, this strain fails to express both type IV pili and type II secretion products (Liles *et al.*, 1999). The second mutant, BS100, contains a Km^R insertion in *pilE* that eliminates expression of the major pilin subunit, leaving type II secretion unaffected (Stone & Abu Kwaik, 1998).

METHODS

Bacterial strains, protozoa and media. *L. pneumophila* serogroup 1 strain 130b (ATCC BAA-74) and its derivatives NU243 (*pilD::Km^R*) and BS100 (*pilE::Km^R*) have been described previously (Engleberg *et al.*, 1984; Liles *et al.*, 1999; Stone & Abu Kwaik, 1998). The heterotrophic Gram-negative bacteria used to form the biofilm matrix were *Pseudomonas aeruginosa* (ATCC 2770), *Klebsiella pneumoniae* (DMS#92-08-28a) and a *Flavobacterium* strain ('*F. xanthomonas*' CDC-65). The protozoan component was the amoeba *Hartmannella vermiformis* (ATCC 50237) (Fields *et al.*, 1990). All bacteria were grown from frozen stocks of not more than three passages on artificial media within the laboratory.

The medium for *L. pneumophila* was buffered charcoal yeast extract (BCYE) with antibiotic supplement. The formulation is, per litre: 18.3 g BCYE agar base (BBL), 2 g glycine, 10 g L-cysteine, 80 mg cycloheximide, 13.22 mg polymyxin B and 5 mg vancomycin. *L. pneumophila* was incubated for 5–7 days at 37 °C with 5% (v/v) CO₂. The heterotrophs were grown on R2A agar (Difco) at 25 °C for 3 days. The amoebae were grown in axenic medium at 37 °C and subcultured at 3–4 day intervals to prevent encystment, according to standard protocols (Fields *et al.*, 1990). The formulation for axenic medium is, per litre: 10 g peptone and yeast extract, 1 g yeast DNA, 15 mg folic acid, 1 mg haemin, 500 mg dibasic anhydrous sodium phosphate, 362 mg monobasic anhydrous potassium phosphate and 10% (v/v) fetal bovine serum.

Biofilm reactor and sampling methods. The biofilm reactor has been described elsewhere (Murga *et al.*, 2001). Briefly, 1×10^8 c.f.u. of each base organism were added to the reactor and allowed to form a biofilm on a disc rotator containing 316L stainless steel coupons (Biosurface Technologies) in a 1:10 dilution of R2A broth for 3 days at 30 °C. R2A broth consisted of, per litre: 500 mg each of yeast extract, proteose peptone #3, Casamino acids and glucose; 300 mg each of sodium pyruvate and dibasic potassium phosphate; and 50 mg magnesium sulfate. After the initial incubation period,

R2A broth diluted 1:100 was connected for constant flow of 1 ml min⁻¹ for 24 h followed by de-chlorinated tap water for the remainder of the experiment. Retention time under constant flow was 8.3 h.

One week after beginning the flow of tap water 1×10^8 c.f.u. of *L. pneumophila* and 4×10^6 cells of *H. vermiformis*, where applicable, were added to a reactor (day 0). Samples from both the bulk liquid and stainless steel coupons were taken on days 1, 4, 7, 11 and 16, then collected in polystyrene tubes to inhibit adherence by the amoebae. Samples of the bulk liquid were taken by pipette through a portal in the lid of each reactor. Coupons were aseptically removed from the biofilm rotator, rinsed three times in Butterfield buffer (425 mg l⁻¹ monobasic potassium phosphate, pH 7.2) then deposited in 10 ml 10 mM PBS pH 7.2. The coupons were alternately vortexed for 30 s then sonicated for 30 s for three repetitions (setting #18 on a 60 Sonic Dismembrator, Fisher Scientific) to remove the biofilm from the surface. The collection liquid was then homogenized for 30 s (PRO2000 homogenizer, PRO Scientific) to yield a suspension of single cells.

Viable counts of heterotrophic bacteria were determined by plating serial dilutions on appropriate artificial media. *L. pneumophila* colonies were enumerated by first treating the sample with an acidic solution of potassium chloride for 15 min to reduce the viability of heterotrophs and amoebae (DeLuca *et al.*, 1999). The legionellae were then deposited onto a 0.2 µm filter (Millipore Isopore Membrane GTTP) that was transferred to a tube containing sterile distilled, de-ionized water. Serial dilutions were plated from this suspension after vortexing for 1 min. Viable counts of amoebae were determined as previously described (Bowman *et al.*, 1996) by placing 100 µl of serial dilutions from the original bulk or coupon sample on non-nutrient agar plates spread with *Escherichia coli* DH5α followed by incubation at 37 °C for 5 days. Microscopy was used to follow trophic spread, thereby determining the limiting dilution. This method provides only the order of magnitude of trophic amoebae present in the system and does not directly measure trophs or cysts. All data presented herein represent the means of at least four independent experiments. Statistical analysis of points compared was done with paired Student's *t* tests.

RESULTS

Biofilms in the absence of protozoa

No significant variation in the initial attachment to the established biofilm was observed between the wild-type, 130b, and the *pilD* mutant, NU243 (Fig. 1a). In contrast, the *pilE* mutant strain, BS100, attached at a significantly lower level ($P < 0.01$). Retention of 130b and NU243 declined over time, with NU243 undetectable on day 11. BS100 and 130b were recovered throughout the the experiment at levels similar to their respective attachment. There was a slight but statistically insignificant ($P > 0.1$) increase in 130b recovery on day 16.

The number of legionellae in the bulk water of each reactor was determined at the same time intervals (Fig. 1b). There was no significant shedding of legionellae from the biofilm. On day 16 there was a slight increase in detection of 130b coincidental with the increased viable counts recovered from the coupons (Fig. 1a). However, the increase, when compared to the two mutant strains, is statistically insignificant ($P > 0.1$) due to the high standard deviation.

Fig. 1. Legionellae recovered from biofilm without amoebae: *L. pneumophila* strains 130b, BS100 or NU243 recovered as sessile forms from the coupons (a) and as planktonic forms in the bulk liquid (b) of a model biofilm reactor. Note that the y-axis is logarithmic in (a) and linear in (b). These data represent the mean $\pm$ SD of six independent experiments.

Viable counts of the bacteria that formed the base biofilm were also determined beginning 1 day before the addition of the *L. pneumophila* and continuing throughout the experiment. The ratio of *P. aeruginosa* to *K. pneumoniae* to '*F. xanthomonas*' was approximately 3:3:1 both on the coupons and in the bulk water. The base biofilm bacteria were unaffected by the addition of *L. pneumophila*, remaining at a constant level of approximately 10⁸ c.f.u. per coupon and 10⁵ c.f.u. ml⁻¹ in the bulk water regardless of the strain of *L. pneumophila* added (data not shown).

Biofilms in the presence of amoebae

L. pneumophila and the amoeba, *H. vermiformis*, were added to the reactor without prior mixing after the bacteria of the base biofilm had been allowed to establish a biofilm for 10 days. Strains 130b and BS100 colonized the established biofilm in a similar manner (Fig. 2a). Both strains displayed a steady increase of approximately 2 logs over 16 days and did not appear to have reached a plateau.

NU243 is impaired in its ability to replicate within a protozoan or mammalian host (Liles *et al.*, 1999) and was

Fig. 2. Legionellae recovered from biofilm that included amoebae. *L. pneumophila* 130b, BS100 or NU243 recovered as sessile forms from the coupons (a) and as planktonic forms from the bulk liquid (b) of a model biofilm reactor containing amoebae are shown in a semi-logarithmic format. These data represent the mean $\pm$ SD of four independent experiments.

thus expected to disappear from the biofilm in a similar way to what was observed in the absence of amoebae (Fig. 1a). No NU243 were recovered on day 1 but over 10² c.f.u. per coupon were detected on day 4, identical to the wild-type and simple pilin mutant (Fig. 2a). However, NU243 did not increase after day 4, but declined slightly to a steady state of roughly 5 $\times$ 10² c.f.u. per coupon on day 16.

In the bulk water, strains 130b and BS100 were present from day 1 at roughly 10³ c.f.u. ml⁻¹. Viable counts for both strains peaked on day 4 at roughly 5 $\times$ 10³ c.f.u. ml⁻¹ then declined, to a final concentration of 10² c.f.u. ml⁻¹ on day 16 (Fig. 2b). Note that the planktonic counts of both 130b and BS100 declined as sessile counts increased (compare Fig. 2a and 2b). NU243 was undetectable on day 1 but increased to almost 10² c.f.u. ml⁻¹ on day 4, remaining at approximately this level until day 16.

The ratio of *P. aeruginosa* to *K. pneumoniae* to '*F. xanthomonas*' remained approximately 3:3:1 for both sessile and planktonic fractions. Addition of amoebae reduced the base biofilm total counts from 10⁸ c.f.u. per coupon to 10⁵ c.f.u. per coupon and 10⁶ c.f.u. ml⁻¹ in the planktonic to 10⁴ c.f.u. ml⁻¹ (data not shown). Amoebae

from the biofilm increased by one order of magnitude between days 1 and 4 to plateau at approximately 10^2 cells per coupon regardless of the strain of *L. pneumophila* introduced. All reactors contained roughly 10^2 cells ml^{-1} in the planktonic fraction on days 1–16 (data not shown).

DISCUSSION

Data from experiments performed in the absence of amoebae indicate a role for both type IV pili and the pre-pilin peptidase component of the type II secretion system in colonizing an established biofilm. *L. pneumophila* strain BS100 (Pile[−]) was unable to establish itself in a pre-formed biofilm at levels similar to its isogenic wild-type, signifying a role for type IV pili in deposition on a solid surface. This impairment in colonization was not evident in experiments using NU243 (PilD[−]), a strain lacking not only type IV pili but the entire type II secretion system downstream of leader sequence cleavage. Though almost ubiquitous in Gram-negative bacteria, type IV pili are not the only means used to attach to other cells or abiotic surfaces (Dalton & March, 1998). *L. pneumophila* has several uncharacterized pili as well as other cell surface adhesins (Stone & Abu Kwaik, 1998, 1999). Thus, it is possible that the severe mutation in NU243 which destroys several tiers of protein expression may trigger overexpression of a PilD-independent adhesin. Further study of the cell surface adhesion molecules of legionellae is needed to clarify these results.

The action of a putative secondary mechanism of attachment did not correlate with retention of *L. pneumophila*. NU243 could establish itself in the biofilm at a level similar to wild-type but did not persist past day 9. This suggests that colonization and retention are distinct phases requiring expression of different cell surface components or extracellular proteins. The PilD pre-pilin peptidase is essential for the secretion of numerous degradative enzymes required for pathogenesis (Aragon *et al.*, 2000; Cianciotto, 2001). These data imply a role for PilD-dependent secretory products in the retention of *L. pneumophila* in a biofilm, as well.

The presence of a protozoan host is more critical to colonization and retention than the presence of type IV pili or type II secretion. The wild-type and the Pile[−] mutant displayed statistically identical colonization and retention profiles in the presence of amoebae. This is in direct contrast to the greatly reduced attachment of the Pile[−] mutant in the absence of amoebae. Inclusion of amoebae was also able to partially compensate for the decreased retention of the PilD[−] mutant strain. The increase of NU243 from undetectable levels on day 1 to 10^2 on day 4 indicates that these observations are most likely due to intracellular replication. Furthermore, it would seem that undefined factors in the biofilm are able to partially restore the PilD[−] mutant's ability to replicate intracellularly.

Unlike other Gram-negative organisms commonly studied in biofilm models *L. pneumophila* does not have an absolute requirement for type IV pili or type II secretion. Its unique

lifestyle as a facultative intracellular parasite may diminish its need for primary attachment and retention mechanisms. In the absence of protozoa there are obviously mechanisms other than pilin expression functioning to allow colonization and keep the legionellae within the biofilm. Characterization of the methods utilized by this organism for attachment and persistence in the biofilm will require further investigation.

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