Biofilm formation by *Pseudomonas fluorescens* WCS365: a role for LapD

Shannon M. Hinsa and George A. O’Toole

Department of Microbiology and Immunology, Room 505 Vail Building, Dartmouth Medical School, Hanover, NH 03755, USA

A role for the outer-membrane-associated LapA protein in early biofilm formation by *Pseudomonas fluorescens* WCS365 has previously been shown. This paper reports that lapD, a gene located adjacent to the lapA gene, also plays a role in biofilm formation. A mutation in lapD results in a conditional biofilm defect in a static assay – this biofilm phenotype is exacerbated when biofilm formation is assayed in a flow-cell system. Furthermore, a lapD mutation shows a partial defect in the transition from reversible to irreversible attachment, consistent with an early role for the lapD gene product in biofilm formation. LapD is shown to be localized to the inner membrane of *P. fluorescens*. The data show decreased LapA associated with the cell surface, but no apparent change in cytoplasmic levels of this protein or lapA transcription, in a lapD mutant. A model is proposed wherein the role of LapD in biofilm formation is modulating the secretion of the LapA adhesin.

INTRODUCTION

The ability of a bacterium to attach to a surface and form a biofilm is thought to be important for its survival in a variety of environments. Biofilm formation by pseudomonads has been shown to occur in a series of sequential events (O’Toole *et al.*, 2000). To begin the formation of a biofilm, bacteria need to interact with the substratum (Korber *et al.*, 1994; O’Toole & Kolter, 1998a). Each individual bacterium first undergoes reversible attachment, which involves contact of the pole of the cell with the surface – this interaction is relatively weak and can be easily disrupted. Eventually the cell attaches by its long axis, so-called irreversible attachment, in which the bacterium is very firmly attached to the surface, resulting in the formation of a monolayer of cells (Fletcher, 1996; Hinsa *et al.*, 2003; Jensen *et al.*, 1992; Lawrence *et al.*, 1987; Marshall, 1979; van Loosdrecht *et al.*, 1990; Zobell, 1943). Subsequent steps result in the formation of microcolonies leading to the development of a ‘mature’ biofilm (Davey & O’Toole, 2000; Reisner *et al.*, 2005; Webb *et al.*, 2003).

Previously we identified a number of transposon-generated mutations which rendered *Pseudomonas fluorescens* WCS365 unable to form a biofilm in the 96-well static assay system (O’Toole & Kolter, 1998b). Eight of the transposons had inserted into four adjacent genes (Fig. 1a) that we designated lapA, lapE, lapB and lapC (Hinsa *et al.*, 2003). All the lap mutant strains were unable to make the transition from reversible attachment to the irreversible attachment stage of biofilm maturation. Based on sequence similarity, we predicted that LapE, LapB and LapC form an ATP-binding cassette (ABC) transporter and that LapA is a large adhesion protein. We demonstrated that the LapA protein was localized to the supernatant and cell-associated fractions of the wild-type bacterium and requires the LapEBC functions for this localization. Based on these data, we proposed that the LapEBC transporter is necessary to transport LapA to the cell-associated fraction and that LapA is an adhesin necessary for *P. fluorescens* to irreversibly attach to a surface (Hinsa *et al.*, 2003).

Here we report the identification of an ORF mapping adjacent to the lapAEB locus, designated lapD, which is conditionally required for biofilm formation. Our analysis suggests that LapD, an inner-membrane protein, modulates the secretion of the LapA protein, but does not have a detectable impact on lapA transcription or LapA levels in the cytoplasm. LapD contains signature sequences of proteins that catalyse the synthesis and degradation of cyclic di-GMP. We discuss possible roles for this protein in biofilm formation.

METHODS

**Bacterial strains, plasmids and culture conditions.** *Pseudomonas fluorescens* WCS365 was grown in lysogeny broth (LB) or in minimal media, as specified, at 30 °C. The minimal salts medium used was M63 (Pardee *et al.*, 1959) supplemented with 1 mM MgSO4 and either 0.2% glucose +0.5% Casamino acids (CAA) or 0.4%...
sodium citrate. For flow cells, M63 medium with 1 mM MgSO4 and 1 mM citrate or 1 mM glucose was used. Escherichia coli was grown in LB at 37°C. Antibiotics were added at the following concentrations: (i) E. coli: tetracycline (Tc), 15 μg ml⁻¹; gentamicin (Gm), 10 μg ml⁻¹; (ii) P. fluorescens: Tc, 45 μg ml⁻¹; nalidixic acid (Nal), 20 μg ml⁻¹; Gm, 35 μg ml⁻¹. Arabinose at a final concentration of 0.2% was added to induce gene expression from the pMQ72 vectors. To test for cellulose production, 0.02% calcium was added to LB plates supplemented with 5 μM FeCl₃, as described by Leigh et al. (1985). Congo red (40 μg ml⁻¹) and Coomassie blue (20 μl ml⁻¹) were added to LB agar plates to assess polysaccharide production (Friedman & Kolter, 2004). Alternatively, Congo red (40 μg ml⁻¹) and Coomassie blue (20 μl ml⁻¹) were added to M63 minimal medium plus glucose or citrate and supplemented with agar (at 0.8%, 1% and 1.5% agar). After inoculation, these plates were incubated at either 30°C or room temperature for up to several days. Sudan black staining for EPSII (galactoglucomannan) was performed according to Liu et al. (1998). Swimming was tested on LB solidified with 0.3% agar, or M63 minimal medium plus citrate or glucose, solidified with 0.3% agar. Swimming assay plates were incubated at either 30°C or room temperature.

**Molecular techniques.** Plasmids were constructed using standard molecular methods (Ausubel et al., 1990) or in Saccharomyces cerevisiae by in vivo recombination (Longine et al., 1998). Plasmid constructs and vectors were transformed into chemically competent E. coli Top10 cells (Invitrogen) and then transferred into P. fluorescens by electroporation.

The sequence of the DNA flanking the transposon insertion was determined by cloning the lapD19 transposon insertion and determining the DNA sequence flanking the element, as described by O’Toole & Kolter (1998b). Further sequence surrounding the lapD19 insertion was obtained by sequence walking with primers designed from the vector oriented towards the transposon insertion (primers P2 and P3; see Table S1, available as supplementary data with the online version of this paper). This additional P. fluorescens sequence was used to design PCR primers (P268 and P269 within the lapD ORF and P265 within the lapA gene) to confirm the gene order inferred from sequence analysis. The resulting PCR product, obtained from primers P265-P269, was sequenced to verify the gene orientation. The lapD sequence was compared to two sequenced strains, P. fluorescens Pf0-1 (http://genome.jgi-psf.org/finished_microbes/pfl/pfl.home.html) and P. putida KT2440 (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3. spl?database=gpp). PSORT analysis was carried out on the available internet site (http://www.psort.org/).

**Construction of the lapD knockout mutant.** The primer pair P781-P782 was used to amplify an ~900 bp segment of chromosomal DNA from 630 bp upstream of the lapD gene to 270 bp downstream of the predicted translation start of the lapD gene. The primer pair P783-P784 was used to amplify an ~670 bp segment of chromosomal DNA from 520 bp upstream of the lapD gene stop codon to 150 bp downstream of the stop codon. Primer P782 contained sequence that overlapped with the P783 primer, and vice versa for P783. The P781-P782 PCR product (1 μl) and the P783-P784 PCR product (1 μl) were added to another PCR reaction to

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**Fig. 1.** Biofilm formation in a static system. (a) Organization of the lap chromosomal region in P. fluorescens WCS365 (not to scale). Approximate size of genes: lapD, 1-9 kb; lapA, 26-0 kb; lapE, 1-4 kb; lapB, 2-2 kb; lapC, 1-4 kb. (b) Biofilm formation in the 96-well plate assay. This figure illustrates biofilm formation following an 8 h incubation in M63 medium supplemented with glucose + CAA. The surface-attached bacteria were stained with crystal violet to aid visualization. (c) Complementation analysis of the ΔlapD mutant. Biofilm formation in the 96-well plate assay by wild-type and ΔlapD strains grown in M63 medium supplemented with glucose + CAA was measured at 8 h. Biofilm formation was also measured for both strains carrying either the vector control (pMQ72) or the lapD-containing plasmid (pMQ72-lapD). (d) Measuring LapD levels. A Western blot was developed with LapD antibodies on membrane fractions collected after 16 h growth on minimal medium supplemented with citrate. The ΔlapD strain carrying the lapD complementing plasmid (pMQ72-lapD) was grown in the presence of the inducer arabinose (lane 3) and in the absence of arabinose (lane 4).
serve as the template. Primers P781 and P784 were used to amplify the 1530 bp pair product by using PCR. The resulting product generates a deletion of 1670 nt internal to lapD. The PCR product was cloned into pGEM-T vector according to the manufacturer’s instructions (Promega). Both the P781 and P784 primers contained an EcoRI site, which was not found in the knockout fragment. The knockout fragment was digested from the pGEM-T vector, using EcoRI, and ligated into pEX18-Tc, which had previously been digested by EcoRI and treated with bacterial alkaline phosphatase. The resulting plasmid, pEX18-DKO, was used to build a deletion of the lapD gene as previously described (Hoang et al., 1998) with the following modifications: the conjugation was performed at room temperature and the selection was performed at 30 °C. The presence of the deletion of the lapD gene was confirmed by PCR using the DKOrev and P265 primer pair.

**Construction of the lapD-carrying plasmid.** The lapD gene was amplified with primers P744 and P745, which flank the gene and also contain 40 bp tails homologous to the MCS of plasmid pMQ72 (R. Shanks & G. O’Toole, unpublished data). This amplicon was recombined into linearized pMQ72 via yeast homologous recombination in S. cerevisiae (Longtine et al., 1998). In the pMQ72-based vectors the lapD gene is under the control of the arabinose-inducible promoter pBAD and induced with 0.2% arabinose.

**Biofilm assays.** Biofilm formation was measured using the microtitre dish assay system, performed as described previously (O’Toole & Kolter, 1998a, b; O’Toole et al., 1999), using minimal M63 medium supplemented with 0.2% glucose and 0.5% CAA or 0.4% citrate as the growth substrate. We have reported previously that using either glucose and CAA or citrate as the growth substrate promotes biofilm formation that is dependent upon LapA (Hinsa et al., 2003). However, growth on citrate does promote a more robust biofilm than growth on glucose + CAA (Hinsa et al., 2003). To quantify biofilm formation in the 96-well format the crystal violet stain was solubilized in 150 μl 30% glacial acetic acid and the absorbance determined at 550 nm; the absorbance from at least five wells was averaged for each assay and the standard deviation is represented by error bars.

Measurement of reversible and irreversibly attached cells was performed as reported by Hinsa et al. (2003). Briefly, the bacterial strains were grown overnight in M63 supplemented with either glucose + CAA or 0.4% citrate and subcultured into this same minimal medium, grown to mid-exponential phase, diluted 1:5 in the same medium, then 500 μl of this dilution was placed in a 24-well microtitre plate for microscopy. Movies were collected immediately at one frame per second for 60 s for analysis of reversible and irreversible attachment. Bacteria that were attached by a single pole of the cell and showed movement during the course of the 60 s movie were defined as ‘reversibly attached’ to the surface, as were bacteria that attached to the surface or detached from the surface during the course of the movie. Cells firmly attached to the surface by the long axis of the cell and which did not move during the 60 s interval in which the movie images were captured were defined as ‘irreversibly attached’. Three movies of 60 s each were captured for each strain from three independent wells.

For the flow-cell studies, the once-flow-through continuous culture flow-cell system was assembled as described by Christensen et al. (1999) and modified M63, as described above, was utilized as the growth medium.

**Imaging.** Epifluorescent and phase-contrast microscopy were performed with a model DM IRBE microscope (Leica Microsystems) equipped with an Orca model C4742-5 CCD camera (Hamamatsu). Images were acquired and processed on a Macintosh G4 loaded with OpenLab software (Improvision).

### LapD and P. fluorescens biofilm formation

**Protein localization and Western analysis.** Inner- and outer-membrane protein samples were prepared according to Nunn & Lory (1993), with modifications. Twenty millilitre cultures grown in minimal M63 medium supplemented with citrate and MgSO₄ were grown shaking at 30 °C for ~16 h. The cultures were centrifuged for 10 min at 6000 g and the bacterial pellet was resuspended in 1·5 ml 200 mM Tris/HCl buffer (pH 7·5) and 20 mM EDTA. A Thermo Spectronic French press mini-cell was used to lyse the cells by processing twice at 20 000 p.s.i (1·38 MPa). Next, the samples were centrifuged at 12 200 g to pellet unbroken cells. The supernatant was removed and centrifuged at 100 000 g for 60 min at 4 °C. The pellet was resuspended in 500 μl Tris/HCl buffer (20 mM, pH 7·6) and then mixed with 500 μl 0.2% Sarkosyl in 20 mM Tris/HCl (pH 7·6). The samples were placed on a shaker at room temperature for 20 min. The samples were then centrifuged at 100 000 g for 60 min at 4 °C. The supernatant fraction containing the inner membrane was removed and the pellet (outer membrane) was resuspended in 1 ml 20 mM Tris/HCl (pH 7·6). The combined inner- and outer-membrane fraction was prepared as described above with the modification that after centrifugation at 100 000 g for 60 min at 4 °C, the pellet was resuspended in 300 μl PBS and regarded as the total membrane fraction.

Cell-associated and cytoplasmic fractions were prepared as previously described (Hinsa et al., 2003). Briefly, the cell-associated fraction was generated by pelleting a 20 ml culture of bacterial cells, then resuspending the pellet in 400 μl PBS. The cell pellet was vortexed for 5 s to dislodge proteins weakly associated with the bacterial outer membrane, the bacteria were recentrifuged and the LapA-containing supernatant fraction was assayed by Western blotting. LapA was not detected in the inner- or outer-membrane fraction (Hinsa et al., 2003).

SDS loading buffer was mixed with each sample, followed by heat denaturation at 95 °C for 10 min. The protein samples for the SecY protein analysis were placed at 37 °C for 10 min instead of 95 °C (Duong & Wickner, 1999). The samples were resolved on a gradient polyacrylamide gel (4–15%) at 25 mA. The protein was transferred to a nitrocellulose membrane in transblot buffer as described by Towbin et al. (1979). Western blots were developed using antibodies prepared to LapA, LapD, LapE and SecY. The LapA and LapE antibodies have been described previously (Hinsa et al., 2003). A LapD peptide (CSEKLRVESQDNLTLGAN) was synthesized and used to generate antibodies against the LapD protein (Bio Synthesis Incorporated). A SecY peptide (CERGQRHAVHYAKRQGKVFVA), conserved in P. fluorescens and Pseudomonas aeruginosa, was synthesized and used to generate antibodies against the SecY protein (Bio Synthesis Incorporated). Western blots were developed with ECL Western detection reagents (Amersham).

**Quantitative RT-PCR (qRT-PCR) analysis.** The wild-type and the ΔlapD mutant strains were subcultured from overnight planktonic cultures grown in M63 minimal medium supplemented with MgSO₄ and sodium citrate, subcultured and grown to late-exponential phase in this same medium. RNA was obtained using the Qiagen RNA kit as instructed with the following modification that lysozyme digestion was performed for 5 min. Following elution, a second DNase treatment was performed with 50 U DNase I (Roche) for 1 h at room temperature. The RNA was then purified with the Qiagen kit following the manufacturer’s instructions. The RNA concentrations were determined and normalized before cDNA was generated. First-strand cDNA synthesis was carried out with 3 μg RNA using the First-Strand cDNA synthesis kit according to the manufacturer’s instructions (Amersham Biosciences). For a positive normalization control, primers were designed to the constitutively expressed rplU gene (P780 and P779). The primer set (P771 and P772) was used to quantify lapA gene expression in the wild-type and ΔlapD mutant strains. The SYBR Green PCR kit from Applied Biosystems was used.
for qRT-PCR reactions, according to the manufacturer’s instructions, and the reactions were run in an ABI Prism 7700 Sequencing Detection System. Statistical analysis was performed using a Student’s t-test in Excel.

RESULTS

Initial molecular characterization of the lapD mutant

A mutant strain carrying the sad-19 transposon mutation was identified based on its biofilm defect in a 96-well plate assay when glucose and CAA were provided as growth substrates (O’Toole & Kolter, 1998b). We identified the gene disrupted by the transposon by sequencing the region adjacent to the cloned transposon fragment. The DNA sequence obtained was then compared with the P. fluorescens Pf0-1 and P. putida KT2440 genome sequences. The transposon was found to map to an ORF we designated lapD; therefore the mutant allele was designated lapD19. The transposon had inserted just past the midpoint of the lapD ORF. The lapD gene is 88% identical at the nucleotide level to ORF93 (contig 483) of P. fluorescens Pf0-1 and 71% identical to ORF PP0165 of P. putida KT2440.

Based on the P. fluorescens Pf0-1 genome we predicted that the lapD gene would map close to the previously identified lap region (Fig. 1a) (Hinsa et al., 2003). To determine the chromosomal location of lapD, sequence walking was performed from the cloned lapD19::Tn5 transposon and PCR was performed to verify the orientation of this gene relative to that of lapA (data not shown). Based on these data we determined that lapD is adjacent to and transcribed in the opposite direction from lapA (Fig. 1a). The organization of the lap genes in P. fluorescens WC365 is identical to that found in the sequenced strain P. fluorescens Pf0-1.

The mass of LapD is predicted to be ~71 kDa, and based on PSORT analysis, is an inner-membrane protein containing two transmembrane domains. Based on the sequence of the lapD gene we predict that this ORF would encode a protein containing HAMP, GGDEF and EAL domains. A HAMP domain is commonly found in the cytoplasmic portion of a membrane-bound protein and is hypothesized to play a role in sending a signal from the periplasmic N-terminus to the cytoplasmic C-terminus of a protein (Aravind & Ponting, 1999). LapD also has a predicted GGDEF domain that has 36% identity and 52% similarity to the consensus sequence across 152 amino acids. Some GGDEF-domain-containing proteins have variable motifs (Spiers et al., 2003), including LapD, which contains a shortened GGEF sequence instead of the typical GGDEF residues. This same unusual sequence is found in the predicted LapD proteins encoded by P. fluorescens Pf0-1, P. putida KT4220 and P. aeruginosa PAO1 (PA1433). LapD also has a predicted EAL domain, but although LapD has 37% identity and 51% similarity across 233 amino acids of the EAL domain sequence (Galperin et al., 2001), LapD has a KVL sequence instead of the hallmark EAL sequence. Proteins containing the GGDEF and EAL domains are predicted to possess diguanylate cyclase and phosphodiesterase activities, respectively, thus controlling the intracellular levels of the nucleotide cyclic di-GMP (Galperin et al., 2001; Simm et al., 2004).

The lapD gene is conditionally required for biofilm formation

To verify that lapD is important for biofilm formation we disrupted this gene in P. fluorescens WC365, resulting in an unmarked knockout mutation (ΔlapD). The disruption of the gene was confirmed by PCR analysis (data not shown). The ΔlapD mutant, like the lapD19 transposon mutation, was defective for biofilm formation in the static 96-well biofilm plate assay, with an approximately 43% reduction in crystal violet staining compared to the wild-type (Fig. 1b). We used the ΔlapD mutant in all subsequent studies. There was no difference in growth rate between the wild-type and the ΔlapD mutant in minimal medium supplemented with glucose + CAA or citrate (data not shown).

We carried out complementation analysis to confirm that the lapD gene was responsible for the biofilm-defective phenotype of the ΔlapD strain. The lapD gene was amplified from P. fluorescens DNA and cloned into pMQ72 via homologous recombination in S. cerevisiae (Longtine et al., 1998), resulting in plasmid pMQ72-lapD. In the pMQ72-lapD construct the lapD gene is under the control of the arabinose-inducible promoter derived from the pBAD plasmid (Guzman et al., 1995). Both pMQ72 and pMQ72-lapD were introduced into the wild-type strain and ΔlapD mutant and these strains were analysed for biofilm formation (Fig. 1c).

Biofilm formation by the wild-type strain is not altered when carrying either pMQ72 or pMQ72-lapD and grown in the presence of arabinose (first three bars, Fig. 1c). These data suggest that expression of the lapD gene from this plasmid does not lead to changes in biofilm formation by the wild-type strain. Biofilm formation by the ΔlapD strain carrying the pMQ72 vector is not affected when growing under inducing conditions; however, biofilm formation by the ΔlapD strain is restored to wild-type levels when the pMQ72-lapD construct is grown in the presence of arabinose (last three bars in Fig. 1c). Western blots show that the level of LapD in the ΔlapD/pMQ72-lapD is slightly higher than the wild-type, and LapD production is induced in the presence of arabinose (Fig. 1d). These data confirm that the lapD gene plays a role in biofilm formation by P. fluorescens.

We showed previously that mutations in lapA rendered cells defective in the transition from reversible, polar attachment to irreversible attachment wherein cells are firmly adhered to the substratum by the long axis of the cell (Hinsa et al., 2003). We performed similar studies with the ΔlapD mutant strain grown on glucose + CAA (Fig. 2a). The wild-type strain shows ~90% irreversibly attached bacteria versus the lapA mutant, wherein only ~5% of the bacteria are irreversibly attached. These findings correspond to the
phenotype reported previously (Hinsa et al., 2003). The \( \Delta \text{lapD} \) mutant shows an intermediate phenotype, with \( \sim 40\% \) of the cells irreversibly attached. The partial defect in irreversible attachment of the \( \Delta \text{lapD} \) mutant is consistent with the partial biofilm defect observed for this strain in the 96-well plate assay (Fig. 1b).

To characterize the kinetics of biofilm formation in the 96-well assay system we performed a time-course study in glucose- and citrate-based media. The extent of biofilm formation was determined by measuring crystal-violet-stained biomass accumulating on the walls of the microtitre dish over 24 h (O’Toole et al., 1999). In glucose+CAA medium, the wild-type strain forms a significantly better biofilm compared to the \( \Delta \text{lapD} \) strain over the first 8 h, with approximately 45\% less crystal violet staining observed for the \( \Delta \text{lapD} \) wells. By 24 h, the \( \Delta \text{lapD} \) strain is able to form a biofilm comparable to the wild-type strain (Fig. 2b). These data suggest that the \( \Delta \text{lapD} \) mutant is delayed in biofilm formation in glucose + CAA medium.

Surprisingly, in citrate medium there is no significant difference between the wild-type and the mutant over the 24 h period (Fig. 2c). Biofilm formation by the \( \Delta \text{lapD} \) mutant is markedly different from that of the \( \text{lapA} \), \( \text{lapE} \), \( \text{lapB} \) and \( \text{lapC} \) mutants, which were unable to form a biofilm under any conditions tested, including when grown on either glucose + CAA or citrate medium (Hinsa et al., 2003). Therefore we propose that LapD is conditionally required for biofilm formation in the static biofilm system.

![Fig. 2. The \( \Delta \text{lapD} \) has a partial defect in reversible attachment.](http://mic.sgmjournals.org)

(a) Reversible and irreversible attachment. The percentage of cells reversibly attached (grey bars) and irreversibly attached (white bars) are shown for the wild-type (WT) and the \( \Delta \text{lapD} \) and \( \text{lapA} \) mutant strains. The bacteria were grown to mid-exponential phase on minimal medium with glucose + CAA then diluted 1:5 into the same medium and assayed for reversible and irreversible attachment (see Methods). (b, c) Kinetics of biofilm formation in glucose + CAA medium (b) and citrate medium (c). Biofilm formation was measured over a 24 h period in the 96-well plate assay. The attached bacteria were stained with crystal violet, the stain was solubilized in 30\% acetic acid and the absorbance measured at 550 nm. Wild-type, grey bars; \( \Delta \text{lapD} \) mutant, black bars.

Analysis of biofilm formation in a flow cell

To analyse the development of a mature biofilm, we grew the wild-type and the \( \Delta \text{lapD} \) mutant in a flow-cell system using minimal medium supplemented with 1 mM glucose. Flow cells provide a constant influx of fresh nutrients, thus sustaining the continued development of the biofilm over many days. Biofilms grown in the flow cell form the characteristic architecture composed of large macrocolonies surrounded by fluid-filled channels after 2 days incubation. The wild-type bacteria established a biofilm consisting of microcolonies and macrocolonies by day 2, while the \( \Delta \text{lapD} \) mutant bacteria were only able to attach individually or in small microcolonies to the surface (data not shown).

We also examined biofilm formation at 1 and 2 days in a flow-cell system using minimal medium supplemented with 1 mM sodium citrate. As shown in Fig. 3, the wild-type bacteria establish microcolonies at day 1 and then form microcolonies and macrocolonies by day 2. In contrast, for the \( \Delta \text{lapD} \) strain, only single cells were seen to attach and only a few small microcolonies formed at day 1. More microcolonies were apparent at day 2 but no larger macrocolonies were formed by the \( \Delta \text{lapD} \) strain. These results suggest that, while no defect was observed in the static system, under the more stringent flow conditions the \( \Delta \text{lapD} \) mutant is unable to form a mature biofilm in minimal citrate medium.

Localization of the LapD protein

To gain insight into the biochemical function of the LapD protein, we examined the subcellular localization of this protein. Fractionation of \( P. \text{fluorescens} \) inner and outer membranes, followed by Western blot analysis with an antibody produced to a LapD peptide, detected an \( \sim 70 \text{ kDa} \) band in the inner membrane of the wild-type strain, which matches the predicted \( \sim 71 \text{ kDa} \) size for LapD.
(Fig. 4a). This band was absent in both the inner- and outer-membrane fractions of the ΔlapD strain (Fig. 4a).

Using an antibody produced to the outer-membrane protein LapE, we were able to demonstrate that the inner-membrane fraction is not contaminated with outer-membrane components (Fig. 4a) (Hinsa et al., 2003). Furthermore, the LapE protein fractionates to the outer membrane in the ΔlapD mutant at wild-type levels, suggesting that the LapD protein is not required for the synthesis or the proper localization of the LapE protein.

As an additional control for the inner-membrane fractionation protocol, we generated a peptide antibody against SecY, a well-characterized inner-membrane protein (Akiyama & Ito, 1985). The ~48 kDa SecY protein fractionated almost exclusively to the inner-membrane fraction (Fig. 4b). Taken together, these data strongly support the conclusion that the LapD protein localizes to the inner membrane of P. fluorescens.

A mutation in lapD decreases extracellular levels of LapA

We previously reported that the LapA protein is localized to the supernatant and the cell-associated fraction of P. fluorescens. As described in Methods, the cell-associated fraction represents proteins weakly associated with the cell surface. We hypothesized that the LapD protein might have an impact on the ability of the LapA protein to localize to the cell-associated fraction. To test this hypothesis, we collected the cell-associated fraction and the cytoplasmic fractions from wild-type and ΔlapD mutant bacteria that had been grown overnight in minimal medium with citrate. There is very little LapA produced when bacteria are grown in glucose medium (data not shown); therefore, we used citrate-grown bacteria to quantify the extent of LapA secreted in the wild-type versus ΔlapD mutant.

As seen in Fig. 5, there was less LapA protein in the cell-associated fraction of the ΔlapD strain compared to the wild-type strain. Using densitometry to quantify this difference revealed 7-3-fold less LapA in the cell-associated fraction of the ΔlapD mutant compared to the wild-type strain (P<0.009, n=8). As expected, the LapA-specific band was not detected in the lapA mutant. There was no observed difference in LapA level in the supernatant fractions between the wild-type and ΔlapD mutant (data not shown); however, LapA was difficult to detect by Western blot in the supernatant fraction.

![Fig. 3. Monitoring biofilm formation development in flow cells. Biofilm formation at day 1 and day 2 by wild-type (WT) and ΔlapD strain grown in M63 supplemented with 1 mM citrate is shown. All panels are top-down, phase-contrast images acquired at 945× magnification.](image)

![Fig. 4. Protein localization. Western blots developed with antibodies to LapD, LapE and SecY proteins were performed on the inner membrane (IM) and outer membrane (OM) cell fractions. (a) Western analysis using antibodies to LapD and LapE proteins against the subcellular fractions from the wild-type, the ΔlapD strain and the lapE mutant. A faint cross-reacting band not attributable to LapE is also present in all OM fractions. (b) The IM and OM fractions of the wild-type were used for Western analysis with the antibody to the SecY protein. In all experiments, 16 h cultures grown in minimal medium supplemented with citrate were used to prepare the fractions and proteins were resolved on gradient polyacrylamide gels (4–15 %).](image)

![Fig. 5. Localization of LapA. A Western blot developed with the antibody to LapA was performed on the cell-associated (CA) and cytoplasmic fractions. The Western analysis is shown for wild-type (WT), the ΔlapD strain and the lapA mutant. For this experiment, cells were grown for 16 h in minimal medium supplemented with citrate and the proteins resolved on a gradient polyacrylamide gel (4–15 %). The samples were normalized to optical density of the cultures.](image)
A lapD mutation does not detectably alter levels of cytoplasmic LapA or lapA transcription

The decreased level of LapA protein outside the cells in the ΔlapD strain could be attributable to a defect in the secretion of the LapA protein or in the production of the LapA protein. There was no detectable difference in the amount of LapA in the cytoplasmic fraction between the wild-type and the ΔlapD mutant (Fig. 5, right panel) as determined by densitometry (0-99-fold change, \( P > 0.49, n = 4 \)).

To determine whether the LapD protein has an impact on the transcription of the lapA gene, qRT-PCR analysis was performed. RNA was collected from late-exponential-phase wild-type and ΔlapD mutant cells grown in minimal medium plus citrate. Primers to the constitutively expressed rplU gene were used as a positive normalization control (Kuchma et al., 2005). An RNA-only control (lacking cDNA) was also used to ensure there was no DNA contamination in the RNA preparations – no PCR products were obtained from these control reactions (data not shown). Expression levels of lapA were quantified in picograms of input cDNA by using a standard curve method for absolute quantification as previously described by Kuchma et al. (2005).

There was no difference in the expression levels of the lapA transcript between the wild-type and ΔlapD mutant bacteria. The relative expression of lapA in the wild-type is 0.0173 + ±0.0019 versus 0.0216 + ±0.0049 in the ΔlapD mutant strain (\( P > 0.02, n = 12 \)).

A mutation in lapD does not affect several factors known to influence biofilm formation

Proteins containing a GGDEF domain have been shown to regulate cellulose production in bacteria including Gluconacetobacter, Salmonella and Pseudomonas (Garcia et al., 2004; Romling, 2005; Ross et al., 1991; Spiers et al., 2003; Tal et al., 1998). Furthermore, cellulose synthesis has been shown to be important for adhesion to surfaces and biofilm formation by a variety of micro-organisms (Gal et al., 2003; Garcia et al., 2004; Matthisse & McMahan, 1998; Spiers et al., 2003). To test for a defect in cellulose production, wild-type and ΔlapD strains were spotted on LB medium supplemented with 0.02% calcofluor and 5 μM FeCl₃ (Leigh et al., 1985). Iron was added to suppress production of fluorescent siderophores. There was no difference in calcofluor-dependent fluorescence between the wild-type and ΔlapD mutant strain.

We also investigated if there were changes in polysaccharide production between the wild-type and ΔlapD mutant. Polysaccharide production was monitored on LB medium and minimal medium plus citrate or glucose supplemented with Congo red and Coomassie blue using a range of agar concentrations (Friedman & Kolter, 2004). There was no difference in Congo red binding between the strains under the variety of conditions we tested. Finally, the ability of the ΔlapD mutant to produce EPSII was monitored by Sudan black staining (Liu et al., 1998). In Rhizobium meliloti, mutants unable to produce EPSII or succinoglycan are stained by Sudan black, whereas the wild-type remains unstained (Liu et al., 1998). P. fluorescens and P. putida have been shown to produce galactoglucans which have been implicated in biofilm formation (Read & Costerton, 1987). There was no difference in Sudan black staining between the wild-type and the ΔlapD mutant.

We also tested swimming motility, which has been shown to be important for bacterial colonization of surfaces (de Weger et al., 1987; DeFlaun et al., 1990; O’Toole & Kolter, 1998b; Sauer & Camper, 2001). Swimming was monitored on LB medium solidified with 0.3% agar or M63 medium supplemented with citrate or glucose (plus 0.3% agar) – no differences in swimming motility were observed between wild-type and the ΔlapD mutant under any conditions tested.

DISCUSSION

We previously identified an ABC transporter and the large outer-membrane protein LapA that are required for the initial stages of biofilm formation, specifically the transition from reversible to irreversible attachment (Hinsa et al., 2003). In the studies presented here, we identified the LapD protein, which has a possible role in controlling LapA protein secretion and thus biofilm formation.

We determined that the sad-19 mutation, which confers a biofilm defect, mapped to an ORF that we have designated lapD. Using PCR and sequence analysis, we determined that lapD is located adjacent to lapA on the chromosome of P. fluorescens WCS365. The ΔlapA mutant is distinct from the other lap mutants because it can form a biofilm in minimal medium supplemented with citrate in the static 96-well assay at 8 h (O’Toole & Kolter, 1998b). The ΔlapD strain forms a less robust biofilm at 8 h in minimal medium supplemented with glucose + CAA compared to the wild-type strain; however, it is able to form a biofilm comparable to the wild-type by 24 h, suggesting that the ΔlapD strain is only delayed (and not completely blocked) for biofilm formation in glucose medium. Based on these data we propose that in the static 96-well biofilm assay the LapD protein is not absolutely required for biofilm formation. The ability of the ΔlapD strain to form a biofilm in the more stringent flow-cell system was tested and under these conditions it was only able to form microcolonies after 2 days incubation and could not progress to form a mature biofilm comparable to the wild-type strain. These results suggest that the ΔlapD mutation causes a conditional biofilm phenotype on citrate medium that is only apparent in the more rigorous flow-cell assay. Recent work by Gjermansen et al. (2005) also found that a mutation in the lapD homologue in P. putida (PP0165) is unable to form a biofilm comparable to the wild-type strain in flow-cell experiments. The differential requirement for LapD in glucose + CAA versus citrate may reflect the fact that more LapA is produced when bacteria are grown in citrate than when
grown on glucose + CAA (not shown). LapD may only be required in the static assay to facilitate the secretion of LapA when lower levels of LapA are produced.

We hypothesized that the LapD protein might affect the synthesis or function of the previously identified ABC transporter or the large adhesin LapA. The LapE protein is required in the static assay to facilitate the secretion of LapA when lower levels of LapA are produced. Further localization studies determined that less LapA protein is found in the cell-associated fraction in the ΔlapD strain compared to the wild-type. However, an equal amount of LapA protein is found in the cytoplasm of both strains (Fig. 5). One caveat of interpreting the data presented in Fig. 5, based on the smear below the LapA band in the Western blot, is that LapA in the cytoplasm appears to be proteolysed. Therefore, although our quantification of the ΔlapD strain in the Western blot, is that LapA in the cytoplasm appears to be proteolysed. Therefore, although our quantification of the Western blots indicates no change in LapA level in the cytoplasm, it is difficult to measure small changes in LapA cytoplasmic levels. The qRT-PCR analysis also showed similar lapA transcript levels for the wild-type and ΔlapD strain. Finally, we showed that there was no detectable effect of the ΔlapD mutation on several other biofilm-associated phenotypes, including motility and matrix production.

Based on our current data we propose that the LapD protein modulates the secretion of the LapA protein and thus biofilm formation. A low level of the LapA protein is able to reach the cell-associated fraction in the absence of the LapD protein and we predict that in the static biofilm assay system this is enough of the LapA adhesin for the bacteria to attach to the surface, but more LapA protein is required to initiate biofilm formation in the more stringent flow-cell system.

The precise mechanism by which the LapD protein effects secretion of the LapA protein is not known. Perhaps the LapD protein, which localizes to the inner membrane, can affect the function of the LapEBC ABC transporter and thus control secretion of the LapA protein. However, we did not see an accumulation of LapA in the cytoplasm as might be expected if a lapD mutant has decreased LapA secretion. However, as shown in Fig. 5, LapA in the cytoplasm seems to be susceptible to proteolysis as indicated by the smear below the main LapA band on the Western blot. This technical difficulty makes it difficult to accurately measure small changes in LapA levels in the cytoplasm. Thus it is possible that LapD could also affect production of LapA, or alternatively, the turnover of LapA in the cytoplasm. The focus of ongoing studies is to continue to improve our understanding of the effects of LapD on LapA localization, production and stability.

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