INTRODUCTION

Biofilm formation is a complex bacterial process influenced by diverse environmental signals (Danhorn & Fuqua, 2007; Lugtenberg et al., 1999; O’Toole & Kolter, 1998; Rudrappa et al., 2007; Sauer & Camper, 2001). Switching from planktonic to sessile growth requires reorganization of bacterial physiology and thus the expression of a large number of genes is changed during the process of attachment to abiotic or biotic surfaces (Mathiesius, 2009; Matilla et al., 2007, 2011; Ramos-González et al., 2005). Indeed, we have shown that the global regulator Fis (factor for inversion stimulation) is involved in Pseudomonas putida PaW85 biofilm formation (Jakovleva et al., 2012). In addition, Fis reduces motility and recolonization of new areas on barley roots, showing that Fis is involved in the regulation of P. putida sessile and planktonic lifestyles (Jakovleva et al., 2012).

Fis is a small homodimeric nucleoid-associated protein that is quite well conserved in the families Enterobacteriaceae and Pseudomonadaceae (Azam & Ishihama, 1999; Beach & Osuna, 1998; Boswell et al., 2004). Although Fis is well-studied in enterobacteria, the role of this protein in pseudomonads has only been examined in a few published studies (Jakovleva et al., 2012; Kugelberg et al., 2005; Perron et al., 2005; Teras et al., 2009; Yeung et al., 2009; Yuste et al., 2006). Fis binds and bends DNA, and thereby it participates in several important processes for Escherichia coli, such as modulating DNA supercoiling (Schneider et al., 1999, 2001; Travers et al., 2001), direct regulation of transcription (Bradley et al., 2007; González-Gil et al., 1996; Xu & Johnson, 1995) and regulation of certain site-specific DNA recombination events (Dorgai et al., 1993; Finkel & Johnson, 1992; Johnson et al., 1986).

Both positive and negative effects of Fis on biofilm formation have been reported. However, the involvement of Fis in biofilm formation is documented quite briefly. For instance, Fis activates the expression of the aaf fimbrina genes, which are essential for biofilm formation in the enterocaggregative E. coli strain 042 (Chaudhuri et al., 2010; Czezulin et al., 1997; Sheikh et al., 2001). Contrarily, Fis represses the expression of the csg curli (a type of fimbria) gene in the enteropathogenic E. coli strain E2348/69 (Saldaña et al., 2009) and is involved in the repression of cellulose production in Dickeya dadantii (formerly Erwinia chrysanthemi) (Prigent-Combaret et al., 2012).

Contrary to enterobacteria, it is supposed that Fis is an essential protein in pseudomonads (Bradley et al., 2007;
Liberati et al., 2006; Osuna et al., 1995; Teras et al., 2009; Yeung et al., 2009), which makes it difficult to study Fis’s involvement in P. putida biofilm formation. Indeed, we can only examine Fis’s impact on P. putida biofilm using the fis-overexpression strain F15, since all our attempts to reduce the amount of Fis by fis gene deletion or conditional gene expression resulted in a lethal genotype or unexpected recombination of the native fis to somewhere in the P. putida genome (Jakovleva et al., 2012).

The matrix of bacterial biofilm can consist of several groups of macromolecules, such as nucleic acids, exopolysaccharides and proteins (Allesen-Holm et al., 2006; Chang et al., 2007; Martinez-Gil et al., 2010; Nilsson et al., 2011). The most important component for the biofilm matrix of P. putida, as well as Pseudomonas fluorescens, is proteinaceous. Both species require a similar central factor for biofilm formation – the large adhesion protein LapA (Duque et al., 2013; Hinsa & O’Toole, 2006; Hinsa et al., 2003). However, P. putida has an additional adhesin, LapF (Martinez-Gil et al., 2010), which is absent in P. fluorescens (Fuqua, 2010). LapA and LapF are the two largest proteins of P. putida, measuring 888.2 and 615.7 kDa, respectively (Winsor et al., 2011). These adhesins are cell-surface-localized secreted proteins that contain expansive repeat domains (Espinosa-Urgel et al., 2000; Fuqua, 2010; Hinsa et al., 2003). It has been demonstrated that LapA is required for cell-surface interactions and therefore is responsible for biofilm initiation, whereas LapF provides cell–cell interactions that lead to the formation of a mature biofilm (Fuqua, 2010; Martinez-Gil et al., 2010). Moreover, it has been shown that LapA is a key factor of adhesion to plant roots (Yousef-Coronado et al., 2008) or corn seeds for P. putida (Espinosa-Urgel et al., 2000).

We have shown that overexpression of Fis in P. putida causes enhanced biofilm formation in rich media like LB, King B and barley seedling medium (Jakovleva et al., 2012). In this work, we carried out transposon mutagenesis to find possible Fis target genes that could affect P. putida biofilm formation. The experiment mostly returned hits in the lap genes and therefore we assessed the influence of Fis on the quantity of adhesins LapA and LapF in P. putida cells. We found that Fis participates in biofilm formation through lapA and lapF regulation.

**METHODS**

**Bacterial strains, plasmids, oligonucleotides and media.** The bacterial strains and plasmids used in this study are described in Table 1, and oligonucleotides are described in Table S1 (available in the online Supplementary Material). Bacteria were grown in complete LB medium (Miller, 1992) and on the solid Fe-deficient King B medium (King et al., 1954). Solid LB medium contained 1.5% Difco agar and solid King B medium used for motility assessment contained 0.8% agar. Antibiotics were added at the following concentrations: 100 μg ampicillin ml⁻¹, 10 μg gentamicin ml⁻¹, 50 μg kanamycin ml⁻¹, 1.5 mg benzylpenicillin ml⁻¹, 200 μg streptomycin ml⁻¹. E. coli was incubated at 37 °C and P. putida at 30 °C. Bacteria were electro-transformed as described by Sharma & Schimke (1996).

**DNA manipulations.** For the construction of deletion mutants, the DNA regions that flanked the deleted genes were cloned into the suicide vector pEMG using the protocol described by Martinez-Garcia & de Lorenzo (2011). The 501 bp long region located upstream of lapA (Up) was amplified by primers PP0168-1-fw and PP0168-1-rev (Table S1), and the 749 bp long region downstream of lapA (Down) was amplified by primers PP0168-2-fw and PP0168-2-rev. Upstream (Up) and downstream (Down) regions of 501 and 749 bp flanking the lapA gene were joined together by overlap extension-PCR (Horton et al., 1989). Thereafter, the 1.25 kb PCR fragment (Up + Down) was purified and cloned into pEMG using the EcoRI site, resulting in pEMG-LapA (Table 1).

To amplify the 495 bp fragment upstream of the lapBC operon (Up), oligonucleotides PP0167-1-rev and PP0167-1-fw were used. The oligonucleotides PP0167-2-rev and PP0167-2-fw were used for amplification of the 789 bp region downstream of the lapBC operon (Down). The 1284 bp joined fragment (Up + Down) was cloned into pEMG using the EcoRI and BamHI sites, resulting in pEMG-LapBC (Table 1).

For the amplification of the 695 bp long region upstream of the lapF gene (Up) the PP0806-1-rev and PP0806-1-fw oligonucleotides were used. The 729 bp long region downstream of the lapF gene (Down) was amplified using PP0806-2-rev and PP0806-2-fw oligonucleotides. The 1424 bp joined PCR fragment (Up + Down) was cloned into pEMG using EcoRI restriction endonuclease, resulting in pEMG-LapF (Table 1).

The pDOC-lapA-gfp vector (Table 1, Fig. 1) was employed for the generation of LapA-GFP fusion protein by inserting the gfp gene downstream of lapA in the P. putida chromosome. pDOC-lapA-gfp was constructed by using one-step ligation-independent cloning (SLIC) (Jeong et al., 2012) to insert three PCR fragments into pDOC-C (Lee et al., 2009) with EcoRI. The first PCR fragment was obtained by amplification of 389 bp from the 3’ end of the lapA gene without a stop codon using primers PP0168-10 and PP0168-2 (Table S1). The second PCR fragment was obtained by amplification of gfp and Km’ genes from pDOC-G (Lee et al., 2009) using oligonucleotides Emgfp-5 and Km6. The third PCR fragment was obtained by amplification of the 352 bp DNA sequence downstream from the lapA gene using primers PP0168-3 and PP0168-40. E. coli strain TG1 (Carter et al., 1985) was used for SLIC cloning.

All designed plasmids were sequenced in order to exclude PCR-generated errors in the cloned DNA fragments. The accuracy of recombination was controlled by sequencing the relevant chromosomal regions of P. putida.

**Construction of deletion mutants of P. putida and insertion of the lapA-gfp fusion cassette into the chromosome of P. putida**. Deletion mutants of P. putida strains PSm and F15 (Table 1) were constructed using the protocols of Martinez-Garcia & de Lorenzo (Martinez-Garcia & de Lorenzo, 2011). The ΔlapA mutants of PSm and F15 were used for the further construction of the double mutant ΔlapA ΔlapF.

To measure the cellular amount of LapA-GFP fusion protein, the corresponding lapA-gfp expression cassette was inserted into the chromosome of P. putida strains PSm ΔlapBC and F15 ΔlapBC. The plasmid pDOC-lapA-gfp, which is unable to replicate in Pseudomonas, was conjugatively transferred from E. coli TG1 into P. putida, using the helper plasmid pRK2013 (Figurski & Helinski, 1979), resulting in P. putida strains PSm ΔlapBC lapA-gfp and F15 ΔlapBC lapA-gfp, respectively (Table 1).

**Transposon mutagenesis and selection of F15 mutants with decreased biofilm formation.** For the identification of Fis-regulated genes that affect biofilm formation, the cells of P. putida
motility compared to F15 were selected. The presence of an intact
were grown for 48 h at 25°C. After growth, the DH5x strain was
IPTG. Four independent transposon mutagenesis experiments were
carried out, resulting in approximately 40,000 colonies. After colonies
were selected, they were sequenced to determine their genomic locations.

To localize mini-Tn5 insertions in the chromosome of P. putida,
arbitrary PCR of genomic DNA was performed. Arbitrary PCR consists of
two consecutive amplifications as described elsewhere (Martínez-García
et al., 2011; O’Toole & Kolter, 1998). A specific primer ME-I-intR and an arbitrary primer ARB6
were used (Martínez-García et al., 2011) for the first amplification. The
second amplification with primers ME-I-intR and ARB2 were used for
the second amplification (Martínez-García et al., 2011). The amplified fragments
were sequenced to determine their genomic locations.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli</td>
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<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (AcmT85 ind-B sam-7 nin-5 lacUV5-T7 gene 1)</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>CC118 Δpir</td>
<td>Δ (ara-levu araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 Δpir phage lysogen</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>DH5x</td>
<td>supE44 ΔlacU1-169(80 lacZAM15) recA1 endA1 hsdR-17 thi-1 gyrA-96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5x Δpir</td>
<td>Δpir lysogen of DH5x</td>
<td>Martínez-García &amp; de Lorenzo (2011)</td>
</tr>
<tr>
<td>TGI</td>
<td>supE hsdS5 thi Δ(lac-proAB) F′ (traD-36 proAB+ lacB lacZAM15)</td>
<td>Carter (1985)</td>
</tr>
<tr>
<td>P. putida</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaW85</td>
<td>Isogenic to KT2440</td>
<td>Jakley et al. (1977)</td>
</tr>
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<td>PSm</td>
<td>PaW85; chromosomal mini-Tn7-αSm1 (Sm′)</td>
<td>Jakovleva et al. (2012)</td>
</tr>
<tr>
<td>F15</td>
<td>PaW85; chromosomal mini-Tn7-αGm-term-lacP′-Plac-fis-TT2 (Gm′)</td>
<td>Jakovleva et al. (2012)</td>
</tr>
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<td>PSm ΔlapA</td>
<td>PSm; APP0168 (Sm′)</td>
<td>This study</td>
</tr>
<tr>
<td>PSm ΔlapBC</td>
<td>PSm; APP0166–0167 (Sm′)</td>
<td>This study</td>
</tr>
<tr>
<td>PSm ΔlapF</td>
<td>PSm; APP0806 (Sm′)</td>
<td>This study</td>
</tr>
<tr>
<td>PSm ΔlapA ΔlapF</td>
<td>PSM; APP0168 APP0806 (Sm′)</td>
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</tr>
<tr>
<td>F15 ΔlapA</td>
<td>F15; ΔPP0168 (Gm′)</td>
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<td>F15 ΔlapBC</td>
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<td>F15 ΔlapF</td>
<td>F15; ΔPP0806 (Gm′)</td>
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</tr>
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<td>F15; ΔPP0168 APP0806 (Gm′)</td>
<td>This study</td>
</tr>
<tr>
<td>PSm ΔlapBC ΔlapA-gfp</td>
<td>PSM; APP0166–0167 lapA-gfp (Sm′ Km′)</td>
<td>This study</td>
</tr>
<tr>
<td>F15 ΔlapBC ΔlapA-gfp</td>
<td>F15; ΔPP0166–0167 lapA-gfp (Gm′ Km′)</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pBAM1</td>
<td>Suicide vector carrying mini-Tn5 with kanamycin resistance gene (Km′ Amp′)</td>
<td>Martínez-García et al. (2011)</td>
</tr>
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<td>pDOC-C</td>
<td>Conjugative cloning vector (Amp′)</td>
<td>Lee et al. (2009)</td>
</tr>
<tr>
<td>pDOC-G</td>
<td>Conjugative cloning vector containing a gfp-Km′ cassette for fusion proteins (Amp′ Km′)</td>
<td>Lee et al. (2009)</td>
</tr>
<tr>
<td>pDOC-lapA-gfp</td>
<td>Conjugative pDOC-C with 389 bp of the end of the lapA gene without a stop codon, followed by 2045 bp of gfp and Km′ genes and 352 bp of the lapA downstream region, inserted in the EcoRI site (Amp′ Km′)</td>
<td>This study</td>
</tr>
<tr>
<td>pEMG</td>
<td>Suicide vector for knockout mutagenesis (Km′)</td>
<td>Martínez-García &amp; de Lorenzo (2011)</td>
</tr>
<tr>
<td>pEMG-AlapA</td>
<td>Suicide vector pEMG with 501 bp of upstream and 749 bp of downstream DNA of the lapA gene inserted at the EcoRI site (Km′)</td>
<td>This study</td>
</tr>
<tr>
<td>pEMG-AlapBC</td>
<td>Suicide vector pEMG with 495 bp of upstream and 789 bp of downstream DNA of the lapBC operon inserted between the EcoRI and BamHI sites (Km′)</td>
<td>This study</td>
</tr>
<tr>
<td>pEMG-AlapF</td>
<td>Suicide vector pEMG with 695 bp of upstream and 729 bp of downstream DNA of the lapF gene inserted at the EcoRI site (Km′)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW (Sce-I)</td>
<td>l-Sce-expressing plasmid (Amp′)</td>
<td>Wong &amp; Mekalanos (2000)</td>
</tr>
<tr>
<td>pUTmini-Tn5 Km2</td>
<td>Suicide vector, source of Km resistance gene (Amp′ Km′)</td>
<td>de Lorenzo et al. (1993)</td>
</tr>
</tbody>
</table>
Biofilm formation assay in multi-well plates. Fletcher’s method (Fletcher, 1977) with some modification (Jakovleva et al., 2012) was employed to measure the ability of P. putida to form biofilm. For proteinase K treatment, the 24 h old biofilm was treated with 0.8 U proteinase K for 2 h. No proteinase K treatment was performed for the controls.

Preparation of cell lysates for SDS-PAGE and protein quantification. For preparation of cell lysates, all studied P. putida strains were grown in 50 ml LB broth with or without 1 mM IPTG for 2.5 or 18 h. The cells were collected by centrifugation and suspended in RIPA buffer, containing 50 mM Tris/HCl pH 7.4 buffer, 0.1% SDS, 1% NP-40, 1% Triton X-100, 0.5% DOC (sodium deoxycholate), 500 mM NaCl (Mikelsaar et al., 2012b), 5 mM EDTA and 100-fold diluted Halt protease and phosphatase single-use inhibitor cocktail (Thermo Scientific). The cells were incubated for 4 h at 37°C. The cell lysates were centrifuged at 12,000 g for 15 min at 4°C. The total amount of protein in the cleared supernatant was measured spectrophotometrically by the content of tryptophan (http://www.biotek.com/resources/docs/Synergy_HT_Quantitation_of_Peptides_and_Amino_Acids.pdf) (Wisńiewski et al., 2009). Gel electrophoresis was carried out as described elsewhere (Mikelsaar et al., 2012a) with some modifications. A total of 40 mg total cell lysate was diluted 1:1 in 2× Laemmli sample buffer (Sambrook & Russell, 2001) and incubated for 30 min at 37°C. The proteins were separated in a gradient (4–8%) SDS-PAGE gel and the concentrating gel was omitted. Electrophoresis was carried out in a Hoefer electrophoresis system (Hoefer Scientific Instruments) at 30 V with the 10×12 cm glasses and the 0.75 mm spacers for 17 h at 4°C. The running buffer contained 37.5 mM Tris, 95 mM glycine and 0.05% SDS. The proteins in the SDS-polyacrylamide gels were silver stained (Chevallet et al., 2006), and the intensity of LapA and LapF bands was analysed by ImageQuant TL software. The following equation was used to calculate the amount of LapA molecules per one LapF molecule, where ’x’ is the intensity of a LapA band and ’y’ is the intensity of a LapF band on the silver-stained SDS-polyacrylamide gel, and ’m’ the molecular mass of a protein:

\[ N = \frac{X \times m_{\text{LapA}}}{y \times m_{\text{LapF}}} \]

The LapA/LapF ratio was separately calculated for each individual gel.

Overexpression and purification of P. putida Fis, production of mAbs against Fis, and Fis immunoblotting. For the production of monoclonal mouse anti-Fis antibody, P. putida Fis(His) was overexpressed and purified with a Ni-NTA agarose matrix (Qiagen) as described previously (Teras et al., 2009). The mAbs against Fis (PP4821) were produced and purified by LabAs. Western immunoblot analysis was carried out to detect the amount of Fis from crude lysates of P. putida. Bacteria were grown to stationary phase in LB medium with or without 1 mM IPTG. The cells were collected by centrifugation and sonicated in Fis buffer [100 mM Tris/HCl pH 7.5, 0.3 M NaCl, 5% (v/v) glycerol]. The cell lysates were centrifuged at 12,000 g for 30 min at 4°C. The total amount of protein in the cleared supernatant was measured spectrophotometrically by the
content of tryptophan (http://www.biotek.com/resources/docs/Synergy_HT_Quantitation_of_Peptides_and_Amino_Acids.pdf) (Wisniewski et al., 2009). Proteins were separated by Tricine-SDS-PAGE (10%) (Schägger, 2006) and transferred to a membrane (Hybond-ECL; GE Healthcare). For Western blotting, the membrane was probed with mouse anti-Fis purified mAbs at a final dilution of 1: 400, followed by alkaline phosphatase-linked goat anti-mouse IgG diluted 1:5000 (LabAbs). The blots were developed using 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium.

**Flow cytometry analysis.** The expression of the lapA-gfp translational fusion in *P. putida* was examined by the fluorescence of GFP. For cytoplasmic localization of the LapA-GFP fusion protein, GFP was fused to the C-terminus of LapA and the fluorescence was monitored in a *P. putida* ΔlapBC strain. *P. putida* ΔlapBC cells carrying the lapA-gfp fusion cassette in the chromosome were grown in LB medium with or without supplementation of 1 mM IPTG for 2.5 and 18 h. The cells were collected by centrifugation at 2000 g for 3 min and resuspended in M9 buffer (Adams, 1959). A total of 10 000 events from every sample were analysed by a FACSArray flow cytometer (BD Biosciences). GFP was excited at 488 nm and fluorescence was recorded at 530 nm. To assess the expression of *lapA-gfp* in *P. putida*, the basal emission of *P. putida* cells at 530 nm was subtracted from the fluorescence of PSm ΔlapBC and F15 ΔalapBC cells carrying the *lapA-gfp* cassette. This was required since *P. putida* belongs to the group of fluorescent pseudomonads that express a basal level of green fluorescence due to the production of pyoverdine (Meyer, 2000).

**Statistical analysis.** The ANOVA method and post hoc Bonferroni test at the significance level 0.05 were used to assess the variability of experimental data. The calculations were performed using Statistica 10 software.

**RESULTS**

**Overexpression of Fis reduces *P. putida* motility on the King B medium**

As a biofilm formation assay is unfeasible on a large scale, we used the opposite phenotype – bacterial motility – to search for Fis-regulated genes involved in *P. putida* biofilm formation. We assessed the motility of *P. putida* strains PSm (expresses Fis at the wild-type level) and F15 (carries an IPTG-inducible fis-overexpression cassette in the chromosome) on LB and King B media containing different concentrations of agar (data not shown). A drop of 2 μl of overnight bacterial culture was spotted on the LB and King B agar media, and cells were incubated for 48 h at 25 °C. Neither the PSm nor the F15 strain exhibited motility on LB medium containing 1.5% agar: only the inoculation spot, with a diameter of 8 to 9 mm, was visible (Fig. 2). At the same time, the movement of *P. putida* on the King B medium containing 0.8% agar was detectable as a lighter halo surrounding the dark inoculation spot (Fig. 2). However, the *lapA* deletion reduced the viscosity of bacteria, which resulted in the disappearance of the visible edges of inoculation drops (Fig. 2). The haloes of *P. putida* PSm cells were comparable on the King B medium and did not depend on the addition of IPTG. The diameters of the haloes were 12 mm (SD 0.1 mm) and 12.7 mm (SD 0.6 mm) in the absence and presence of 1 mM IPTG in the King B medium, respectively. Contrary to the PSm strain, the movement of *P. putida* F15 was reduced in the presence of IPTG in the King B medium. The diameters of the F15 haloes were 11 mm (± 1.8 mm) and 8 mm (± 0.6 mm) in the absence and presence of 1 mM IPTG (P<0.001), respectively. Although the motility of *P. putida* PaW85 derivatives PSm and F15 was poor on the King B medium, the motility retardation caused by Fis overexpression was visually detectable and the motility on the King B medium was usable for the screening of transposon mutants.

**Transposon mutants of the Fis-overexpressing *P. putida* strain F15 with reduced biofilm formation carry mini-Tn5 insertions in the *lap* genes**

In order to identify Fis-regulated genes that may affect biofilm formation, we performed transposon mutagenesis of the *P. putida* strain F15, which carries the Fis-overexpression cassette mini-Tn5-lacF-tac-flp-Δfis-ΔT1T2 in its chromosome. To find mutants with lowered biofilm formation, we searched for F15 transposon mutants exhibiting increased motility on the King B medium in the presence of IPTG.

We selected 155 colonies with improved motility and identified the insertion position of mini-Tn5 in the chromosome of 79 transconjugants. We excluded three colonies that had transposon insertions in the Fis-overexpression cassette. The insertions of mini-Tn5 in the remaining 76 colonies are represented in Table 2. Since 68 mutants had transposon insertions in the *lap* genes (Table 2), we focused our research on the *lap* genes and measured the biofilm formation ability of these mutants in comparison to the F15 and PSm strains. We found that the biofilm formation of the transposon mutants carrying mini-Tn5 insertions in the *lap* genes had decreased up to two times in comparison to the original Fis-overexpressing strain F15 (Table 2). These results indicated that Fis may regulate biofilm formation by affecting the expression of the *lap* genes.

**lapA and lapF deletions improve motility of *P. putida* cells with overexpressed Fis**

To further confirm the role of Fis in biofilm formation via regulating the expression of the *lap* genes, we deleted the *lapA* (PP0168) and the *lapF* genes (PP0806) from the chromosome of the PSm and F15 strains. Although no mini-Tn5 insertions to the *lapF* gene were selected, the ΔlapF mutant and ΔlapA ΔlapF double mutant were constructed to investigate the importance of both adhesins in Fis-overexpression conditions. Prior to the motility and biofilm experiments, the overexpression of the *fis* gene was confirmed by immunoblotting all the F15 lap knockout mutants. IPTG (1 mM) added to LB medium caused Fis overexpression in all our *lap* gene knockout mutants (Fig. 3).

The motility of the lap knockout mutants of *P. putida* was studied on the King B medium containing 0.8% agar...
**Fig. 2.** Movement of *P. putida* wild-type strain PSm and the Fis-overexpressing strain F15 and knockout mutants on King B medium containing 0.8 % agar and on LB medium containing 1.5 % agar. Bacteria were incubated for 48 h at 25 °C. The supplementation of 1 mM IPTG is shown by + at the top of the column. Arrows show the edge of the inoculation spot and the edge of the halo of *P. putida* movement on King B medium.

**Table 2.** *P. putida* genes for which disruption with mini-Tn5 increased motility and decreased biofilm formation of F15 cells in Fis-overexpression conditions

<table>
<thead>
<tr>
<th>Locus/strain</th>
<th>Gene symbol and name according to <em>Pseudomonas</em> database*</th>
<th>Localization according to <em>Pseudomonas</em> database*</th>
<th>No. of independent mutants</th>
<th>Biofilm†</th>
</tr>
</thead>
<tbody>
<tr>
<td>F15</td>
<td></td>
<td></td>
<td></td>
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<td>PSm</td>
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<td>PP0165</td>
<td>lapD orthologue; diguanylate cyclase</td>
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<td>Cytoplasmic membrane</td>
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<td>NM</td>
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<tr>
<td>PP4373</td>
<td>fleQ; Fis family transcriptional regulator</td>
<td>Cytoplasmic</td>
<td>1</td>
<td>NM</td>
</tr>
<tr>
<td>PP4519</td>
<td>tolC (lapE orthologue); TolC family type I secretion outer-membrane protein</td>
<td>Outer membrane</td>
<td>1</td>
<td>1.138</td>
</tr>
<tr>
<td>Intergenic region between PP5050 and PP5051</td>
<td>Intergenic region</td>
<td>–</td>
<td>1</td>
<td>NM</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>76</td>
</tr>
</tbody>
</table>

*Winsor et al. (2011).
†Relative biofilm formation with IPTG-induced Fis expression compared to no induction. Biofilm formation was measured as the absorbance in a microplate after 24 h incubation, as described in Methods. Data from at least 16 measurements are shown. In the case of transposon mutants, the biofilm of at least three independent mutants was measured if it was possible. NM, Not measured.
(Fig. 2). The movement of the knockout mutants constructed from PSm was similar to wild-type PSm without statistically significant differences (Fig. 2). The only exception was the motility of PSm ΔlapF in the presence of 1 mM IPTG. Namely, the halo diameter of PSm ΔlapF in the presence of IPTG was 14.8 mm (SD 0.5 mm) with a statistically significant difference from the halo diameter of wild-type PSm in the same conditions (P=0.011).

The motility of all knockout deletion strains constructed from F15 was improved in Fis-overexpression conditions compared to the original F15 strain (Fig. 2). The halo diameter of F15 ΔlapA was 10.9 mm (SD 0.3 mm), F15 ΔlapF 10.0 mm (SD 0.8 mm) and F15 ΔlapA ΔlapF 11.4 mm (SD 0.9 mm) on King B medium supplemented with 1 mM IPTG. All these results revealed statistically significant differences from the motility of F15 (the corresponding P values were P<0.001, P=0.006 and P<0.001). In the absence of IPTG, the motility of F15 knockout variants was similar to that of F15. However, in the absence of IPTG the lapA deletion caused slightly increased motility compared to F15 (Fig. 2).

Fis-enhanced biofilm formation depends on functional LapA, but not LapF

We have previously shown that Fis overexpression enhances P. putida biofilm formation when bacteria are grown in microtitre plates for 24 h (Jakovleva et al., 2012). However, it is known that P. putida biofilm rapidly increases in quantity for about 6 to 10 h after attachment to the abiotic surface and thereafter starts to disintegrate (Gjermansen et al., 2010; Yousef-Coronado et al., 2008, 2011). We compared biofilm formation of the PSm and F15 strains at 4, 8 and 24 h after inoculation. Our data confirm that P. putida forms biofilm most efficiently at the beginning of cultivation. After 4 h of incubation, PSm biofilm was approximately 2.5 times higher than after 8 or 24 h (Fig. 4). Similarly to the wild-type, the F15 strain formed more biofilm at the beginning of cultivation. F15 cells grown for 4 h showed no IPTG effect; however, the positive effects of overexpression of Fis appeared starting from 8 h and were detectable at a higher level in the 24 h grown biofilm (Fig. 4).

To measure the effect of LapA and LapF on P. putida biofilm formation, PSm, F15 and their lap knockout derivatives were compared. In agreement with previous observations (Espinosa-Urgel et al., 2000; Gjermansen et al., 2010; Hinsa et al., 2003; Yousef-Coronado et al., 2008), the lapA mutant was deficient in biofilm formation (Fig. 4). For example, all lapA mutants derived from PSm and F15 formed 3.5 times less biofilm by the 4th hour compared to strains with a functional lapA gene (P<0.001; Fig. 4a). The reducing effect of the lapA deletion on the biofilm formation was retained in older biofilm as well, but was less obvious (Fig. 4b, c).

The deletion of the lapF gene did not influence the formation of biofilm in comparison to the wild-type cells (Fig. 4). This is in agreement with previous reports demonstrating the requirement of LapF in P. putida biofilm formation only in LB medium diluted 10 times and not in the regular LB medium (Fuqua, 2010; Martinez-Gil et al., 2010). Surprisingly, deletion of the lapF gene reduced biofilm of P. putida F15 cells (1.4 times, P<0.001) at the beginning of cultivation (cells cultivated for 4 h), but not in the mature biofilm at 8 and 24 h after inoculation. Moreover, the amount of biofilm increased similarly in both F15 and F15 ΔlapF when the expression of Fis was increased by the presence of IPTG, indicating that LapF is not needed for Fis-enhanced biofilm formation. Thus, our results confirm that LapA is more important for biofilm formation than LapF in a rich medium like LB, and LapA but not LapF is needed for Fis-enhanced biofilm formation.

The matrix of Fis-enhanced biofilm consists of proteinaceous component

In order to examine which matrix component is dominant in Fis-enhanced biofilm, we treated the 24 h old biofilm with either proteinase K, DNase I or cellulase. Neither DNase I nor cellulase degraded the mature biofilm (data not shown); only proteinase K was able to disrupt the 24 h old biofilm during 2 h incubation (Fig. 4c). The proteinase K treatment degraded Fis-enhanced biofilm, but also caused a statistically significant decrease in biofilm for all studied strains (all P values <0.001) except in ΔlapA ΔlapF...
strains. We observed a 1.4-fold decrease also for PSm ΔlapA ΔlapF and 1.2-fold decrease for F15 ΔlapA ΔlapF biofilm after proteinase K treatment, but the difference was not statistically significant (Fig. 4c). Thus, Fis-induced biofilm consists largely of proteinaceous component.

**Overexpression of Fis increases the amount of LapA and decreases the amount of LapF in P. putida cells**

Since Fis had a time-dependent effect on biofilm formation with biofilm being most strongly induced in a 24 h old culture and not at all in a 4 h culture, we were interested whether the amounts of surface adhesins vary correspondingly at the tested time points. Therefore, we measured the amount of LapA and LapF in P. putida cells grown in LB broth for 2.5 and 18 h by separating and quantifying proteins in silver-stained SDS-polyacrylamide gels (Fig. 5).

We selected the 2.5 h time point because cells grown for 4 h showed wildly fluctuating amounts of adhesins: LapF was sometimes detectable in the wild-type cells, but never in the Fis-overexpressing cells (data not shown). We suspect that the expression of the adhesins was being reorganized around the fourth growth hour. Furthermore, the growth times during the biofilm assay and the adhesion quantification experiment were not directly comparable due to different aeration in microplates and in shaken liquid medium. To compare the amounts of proteins in total cell lysates, we normalized the intensities of LapA and LapF bands against the corresponding bands of PSm grown without IPTG. In agreement with previously published β-galactosidase measurements (Martínez-Gil et al., 2014), LapF was undetectable in P. putida cell lysates prepared from bacteria grown for 2.5 h (Fig. 5a). At the same time, LapA was detectable, but overexpression of Fis in the fast-growing P. putida did not influence the amount of this...
protein (Fig. 5a, c). However, Fis overexpression increased the amount of LapA in the cell lysates approximately 1.6 times at the 18 h time point (Fig. 5b, c). Surprisingly, Fis overexpression decreased the amount of LapF approximately four times in the 18 h grown cells compared to the wild-type cells (Fig. 5b, d). Considering the molecular masses of LapA and LapF, 888.2 and 615.7 kDa, respectively (Winsor et al., 2011), Fis overexpression increased the number of LapA molecules per one molecule of LapF from 0.3 to 2.9 in the 18 h old cells.

As Fis overexpression increases the quantity of LapA 1.6 times, which is a moderate effect compared to the 4-fold change for LapF, we controlled the Fis positive effect on LapA expression by another method, to be assured that the change of the LapA/LapF ratio depends on changes in the amounts of both proteins. Therefore, we assessed the impact of elevated Fis levels on the abundance of the LapA protein also by monitoring the fluorescence of a LapA-GFP fusion protein in 18 h grown cells. According to previous studies, the LapA C-terminus contains a signal sequence for a type I transport system and the transporter LapB is needed to transport LapA out of the cytoplasm (Boyd et al., 2014; Hinsa et al., 2003). To localize LapA-GFP in the cytoplasm, we deleted the 
\[ \text{lapB} \]
and 
\[ \text{lapC} \]
genes and fused the GFP protein to the LapA C-terminal end. Confirming the SDS-PAGE results, we found that the fluorescence of the F15 \( \Delta \text{lapBC} \) derivative carrying the lapA-gfp fusion positively depended on the amount of Fis (Fig. 6). Thus, overexpression of Fis in \( P. \ putida \) leads to an unbalanced ratio of adhesins in favour of LapA and the shift in the LapA/LapF ratio is caused by changes in the amounts of both proteins.

**DISCUSSION**

To elucidate the involvement of Fis in the regulation of biofilm formation, we carried out transposon mutagenesis of \( P. \ putida \) strain F15, which overexpresses Fis. Fis overexpression retards \( P. \ putida \) movement on King B medium containing 0.8 % agar (Fig. 2) and the derepression of motility by transposon mutagenesis was visually detectable. Thereafter, the ability of selected colonies to form biofilm was assessed and the insertions of mini-Tn5 were localized (Table 2). We searched for mutants with reduced biofilm formation and surprisingly we found that most of the studied insertions of mini-Tn5 were in the \( \text{lap} \) genes (Table 2). Since 74 % of the studied transposon mutants carried mini-Tn5 insertions in the \( \text{lapA} \) gene (Table 2), we focused our following studies on the adhesin genes \( \text{lapA} \) and \( \text{lapF} \). Although we did not select any colonies with a mini-Tn5 insertion in the \( \text{lapF} \) gene, we decided to elucidate the impact of Fis on LapF as well, since both adhesins are reported to be important for the development of \( P. \ putida \) biofilm.

**Fig. 5.** Quantification of the two largest proteins of \( P. \ putida \), LapA and LapF, in PSm and F15 cell lysates. (a, b) Bacteria were grown for 2.5 (a) and 18 h (b) in LB medium and total protein lysates were prepared. The cell lysates of \( P. \ putida \) PSm knockout deletion mutants used as controls were prepared from cells grown for 18 h. Supplementation with 1 mM IPTG is shown by + on top of the lane. (c) The quantity of LapA was normalized against wild-type cells (PSm) grown without IPTG supplementation. (d) The quantity of LapF was normalized against wild-type cells (PSm) grown without IPTG supplementation. It was not possible to quantify the amount of LapF in \( P. \ putida \) cells grown for 2.5 h as the band was not visible. Mean values from at least 12 measurements are shown with 95 % confidence intervals.
However, the screening of transposon mutants revealed that some other genes, for example PP2413, encoding a putative cytoplasmic GGDEF family protein (Winsor et al., 2011), may also participate in the regulation of biofilm formation. It has been reported that different c-di-GMP cyclases that contain the GGDEF domain regulate biofilm formation in P. fluorescens (Newell et al., 2011) and in P. putida (Martínez-Gil et al., 2014). Therefore, it is tempting to speculate that the product of PP2413 influences biofilm formation via synthesis of c-di-GMP, which regulates the presentation of LapA to the outer membrane of P. putida. It is known that LapD senses c-di-GMP in the cytoplasm and binds the periplasmic protease LapG, thereby inactivating proteolytic cleavage of LapA and inducing biofilm formation (Navarro et al., 2011; Newell et al., 2009). However, the impact of Fis on the development of biofilm is probably more complex. The role of PP2413, as well as other genes that were identified by transposon mutagenesis, in the Fis-mediated biofilm regulation will be examined in the future.

One of the most surprising results was the Fis-overexpressing lapA knockout strain’s recovered motility on the King B medium (Fig. 2). Additionally, the lapA deletion reduced the viscosity of bacteria, which is seen by the disappearance of the visible edges of the inoculation drops (Fig. 2). However, this phenotype could be explained by the adhesive nature of LapA. The overexpression of Fis results in an abundance of the major adhesin LapA on the surface of cells and this is probably the reason for the motility reduction on solid medium. The lapA deletion, in turn, probably reduces the stickiness of P. putida F15 cells and recovers the motility on solid medium. Similarly to the lapA deletion, the lapF deletion also caused recovered motility on solid medium, although we cannot explain why we did not detect any mini-Tn5 insertions in the lapF gene.

The two largest P. putida proteins, LapA and LapF, play an important, if not the key role in P. putida biofilm development (Duque et al., 2013; Hinsa & O’Toole, 2006; Hinsa et al., 2003; Martínez-Gil et al., 2010). Our results are in accordance with previously published data: the lapA deletion reduced P. putida biofilm more than three times (Fig. 4a), but lapF deletion did not affect P. putida biofilm formation in the PSm strain, which expressed Fis at a natural level (Fig. 4). However, overexpression of Fis in P. putida (strain F15) enhanced biofilm at its 24 h incubated mature stage irrespective of the presence or absence of the functional lapF gene (Fig. 4b, c). Thus, although LapF has been described as the adhesin responsible for cell–cell attachment in mature biofilm, this protein seemed to be unnecessary to form Fis-induced biofilm in LB medium. However, we found that protease K is able to degrade all Fis-enhanced biofilms, showing that a proteinaceous matrix component is dominant in these biofilms (Fig. 4c). Still, protease K did not reduce Fis-enhanced biofilm of F15 and F15 ΔlapF down to the same amount as was detected in PSm and PSm ΔlapF. Therefore, it is possible that in addition to a proteinaceous component Fis also facilitates the production of some nonproteinaceous components like exopolysaccharides. It has been described that the absence of LapA and/or LapF increases the exopolysaccharide component in the matrix of P. putida biofilm (Martínez-Gil et al., 2013). However, Fis overexpression did not increase biofilm formation of the F15 ΔlapA strain, indicating that protease K degrades enhanced biofilms slower than the wild-type biofilm, since enhanced biofilm contains more matrix components to degrade.

To examine the quantity of adhesins in P. putida cells during growth, we quantified the amounts of LapA and LapF from total cell lysates. We found that the amounts of proteins in the wild-type cells correlate to recently published measurements of transcription activity (Martínez-Gil et al., 2014). LapA is already present in exponentially growing bacteria, whereas LapF is absent and accumulates in stationary phase cells (Fig. 5). However, the overexpression of Fis decreased the quantity of LapF in the stationary phase cells approximately 4 times and increased the amount of LapA 1.6 times compared to the wild-type cells. Thus, Fis overexpression results in an unbalanced ratio of the adhesins with LapA being in excess (Fig. 5). This indicates that LapA must have a central role in preserving P. putida mature biofilm. Thus, the overexpression of Fis either reverses biofilm development or prevents biofilm dispersion in late stages. In other words, the dispersion of P. putida wild-type (PSm) cells from mature biofilm may happen due to

![Fig. 6. Mean fluorescence of P. putida PSm ΔlapBC and F15 ΔlapBC strains with LapA-GFP fusion protein. The fusion protein was constructed by inserting the gfp gene downstream of lapA in the P. putida chromosome, resulting in a single ORF. The fluorescence of ΔlapBC knockout mutants of P. putida strains PSm and F15 was measured from cells grown for 18 h in LB medium. IPTG (1 mM) was used for overexpression of Fis in P. putida strain F15. The endogenous fluorescence of P. putida cells was subtracted from the fluorescence of cells carrying the lapA-gfp fusion cassette. Means of 12 independent measurements are shown with 95% confidence intervals. Asterisks represent a P value below 0.001.](image-url)
reduction of the relative amount of LapA on the surface of bacteria or due to the appearance of LapF on the surface of bacteria.

Our results indicate that Fis is involved in the regulation of lapA and lapF expression. Fis is actively expressed in the early stages of exponential growth of E. coli and thereafter the expression decreases approximately 100 times (Ali Azam et al., 1999). Fis expression does not change so drastically in P. putida. Compared to exponentially growing P. putida, the level of fis mRNA decreases approximately three times in stationary phase cells; however, it still depends on the growth phase (Yuste et al., 2006). In turn, the lapF gene is known to be under the positive regulation of RpoS, the sigma factor required for starvation and stress responses (Martinez-Gil et al., 2010). LapF is not expressed in fast-growing cells (Fig. 5) (Martinez-Gil et al., 2014). Therefore, Fis is an ideal candidate to be the transcriptional repressor of lapF in fast-growing bacteria when the amount of Fis in the P. putida cells is upregulated. In addition to this, we have found that Fis binds to the −10 hexamer of the lapF promoter and represses transcription from this promoter (data not shown). At the same time, Fis activates the expression of lapA and enhances biofilm formation at least partially through LapA.

In light of the foregoing information, it is likely that Fis mostly influences the early stages of P. putida biofilm development. Fis increases the quantity of LapA, thereby promoting the attachment to solid surfaces and initiation of biofilm. At the same time, Fis represses the expression of LapF.

In conclusion, in this study we have described the involvement of the global regulator Fis in biofilm formation. We found that Fis overexpression retards P. putida movement on King B medium and this movement is recoverable by mutations in the lapA gene. Higher amounts of Fis increase the quantity of LapA in P. putida, resulting in enhanced biofilm formation. At the same time, Fis strongly reduces the amount of LapF. Through these effects, Fis may prevent the dispersion of biofilm in LB-grown P. putida.

ACKNOWLEDGEMENTS

We thank Signe Saumaa and Andres Aineo for their comments on the manuscript, and Dmitrii Lubenets for his technical advice on the measurement of the fluorescence of bacteria. This work was supported by funding from the Estonian Ministry of Education and Research –Targeted Financing Project SF0180031s08 and Institutional Research Funding IUT20-19.

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Fis regulates biofilm through lap genes


Edited by: M. Whiteley