LapF and Its Regulation by Fis Affect the Cell Surface Hydrophobicity of *Pseudomonas putida*

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Abstract

The ability of bacteria to regulate cell surface hydrophobicity is important for the adaptation to different environmental conditions. The hydrophobicity of cell surface can be determined by several factors, including outer membrane and surface proteins. In this study, we report that an adhesin LapF influences cell surface hydrophobicity of *Pseudomonas putida*. Cells lacking LapF are less hydrophobic than wild-type cells in stationary growth phase. Moreover, the overexpression of the global regulator Fis decreases surface hydrophobicity by repressing the expression of lapF. Flow cytometry analysis revealed that bacteria producing LapF are more viable when confronted with methanol (a hydrophilic compound) but are more susceptible to 1-octanol (a hydrophobic compound). Thus, these results revealed that LapF is the hydrophobicity factor for the cell surface of *P. putida*.

Introduction

The ability to regulate cell surface hydrophobicity is important for bacterial adaptation to different environmental conditions [1,2]. Despite the fact that hydrophobicity of cell surface regulates physiology only passively, the influence to competitiveness of bacteria is remarkable. Cell hydrophobicity dictates the interaction to biotic and abiotic surfaces, the consumption of aromatic compounds, the protection against toxic compounds and even the effectiveness of bacterial motility in soil [3–7]. Additionally, the hydrophobic bacterial cell surface can provoke aggregation of cells in soil [8] promoting degradation of aromatic compounds such as phenol, pyridine and its derivatives via various metabolic pathways [9]. The hydrophobicity of surface may also have a protective role for bacteria. For example, an increase of cell surface hydrophobicity protects bacteria against several kinds of environmental stressors including osmotic stress, heat shock, and solvents [2,7,10].

The hydrophobicity of bacterial cell surface can be determined by several factors such as outer membrane proteins, lipopolysaccharides, S-layer proteins, lipoteichoic acids, and fimbrial adhesins [11–14]. For instance, enteric bacteria use fimbriae for adhesion to host cells via interaction of fimbrial lectin to carbohydrate of host cells [15]. These fimbriae contain a high
number of hydrophobic amino acids that probably help overcome the initial electrostatic repulsion barrier that exist between the host cell and the surface of bacteria [16,17].

*Pseudomonas putida* promotes plant growth by colonizing plant roots and protecting crop plants against pathogenic organisms [18]. Therefore, the adhesion and biofilm formation of *P. putida* has been investigated already for decades [19]. Colonization of plant roots starts with adhesion of bacteria to root surfaces. Once the initial weak interaction turns into irreversible attachment, bacteria start dividing and producing a biofilm matrix [18,20]. The biofilm matrix of *Pseudomonas putida* consists mainly of proteins [21,22]. Two large proteins LapA and LapF, which act as adhesins, were shown to be essential for *P. putida*’s biofilm formation [22–24]. It has been demonstrated that LapA is required for cell-surface interactions and is responsible for biofilm initiation [22,25], whereas LapF is participating in mature biofilm, providing cell-cell interactions [25,26]. In 2014, Boyd et al. showed that LapA can bind to hydrophilic as well as hydrophobic surfaces [27], but there has been no report so far linking LapF and hydrophobicity in *P. putida*.

The phenotypes needed for successful root colonization like motility, chemotaxis, endurance of oxidative stress, composition of outer membrane, quorum sensing and biofilm formation are complexly controlled by global regulators [18,28–30]. Therefore, it was no surprise that the global regulator Fis is involved in regulation of the migration, biofilm formation and competitiveness of *P. putida* on the barley roots [22,31,32]. Fis is a small DNA-binding homodimeric protein, which is participating in several important processes including regulation of transcription and recombination [33–36]. Fis is well-studied in enterobacteria where fis knockout mutants are viable; however, in pseudomonads it seems to be an essential protein, as fis deletion is lethal [33,37–39]. Thus, for studying the involvement of Fis in regulation processes, the options are limited to using fis overexpression.

We have previously shown that fis overexpression enhances *P. putida* biofilm formation most probably caused by an increase in the abundance of LapA of about 1.6 times compared to *P. putida* wild-type cells [22,31]. However, we have seen that fis overexpression represses the amount of LapF about 4 times. The Fis binding site Fis-F2 is mapped 150 bp upstream of the lapF gene coding sequence and the binding of Fis to this sequence represses the transcription of lapF [32]. Therefore, it was intriguing to study whether the two largest adhesins of *P. putida* LapA and LapF take part in regulation of cell surface hydrophobicity, as it was previously shown that cells growing in biofilm are usually more hydrophobic [7,40,41].

In this study, we measured the cell surface hydrophobicity, analysed as water contact angles (θw), of *P. putida* cells, when lacking the adhesins LapA and/or LapF. Whereas the absence of LapA had no effect, the lack of LapF significantly reduced the surface hydrophobicity in stationary-phase cells. In addition, the involvement of Fis in the regulation of *P. putida*’s hydrophobicity was shown, as Fis repressed the level of LapF in stationary phase and thereby decreased the hydrophobicity of *P. putida*. The effect of LapF on *P. putida*’s adaptive properties towards hydrophobic and hydrophilic compounds was studied by flow cytometry analysis.

**Materials and Methods**

**Bacterial strains, plasmids, oligonucleotides and media**

Bacterial strains and plasmids used in this study are described in Table 1 and oligonucleotides are described in Table 2. Bacteria were grown in complete LB medium. Antibiotics were added at the following concentrations: 10 μg gentamicin ml⁻¹, 200 μg streptomycin ml⁻¹, 50 μg kanamycin ml⁻¹, 1500 μg benzylpenicillin ml⁻¹. *E. coli* was incubated at 37°C and *P. putida* at 30°C. Bacteria were electrotransformed as described by Sharma & Schimke [42]. *E. coli* strain CC118 λpir [43] was used as a host strain for DNA cloning procedures and a donor strain in conjugation experiments.
Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype or description | Source/ reference |
|-------------------|-------------------------|-------------------|
| **E. coli**  |  |  |
| CC118 hpir  | Δ(ara-leu) araDΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE (Am) recA1 hpir phage lysogen | [43] |
| **P. putida**  |  |  |
| PSm            | PaW85, isogenic to KT2440; chromosomal mini-Tn7ΔSm1 (Sm\(^\text{\textsuperscript{r}}\)) | [31] |
| F15            | PaW85, isogenic to KT2440; chromosomal mini-Tn7ΔGm-term-lac\(^\text{\textsuperscript{r}}\)-P\(_{lac}\)-fis-T1T2 (Gm\(^\text{\textsuperscript{r}}\)) | [31] |
| PSmΔlapA       | PSm; ΔPP0168 (Sm\(^\text{\textsuperscript{r}}\)) | [22] |
| PSmΔlapF       | PSm; ΔPP0806 (Sm\(^\text{\textsuperscript{r}}\)) | [22] |
| PSmΔlapAΔlapF  | PSm; ΔPP0168 ΔPP0806 (Sm\(^\text{\textsuperscript{r}}\)) | [22] |
| F15ΔlapA       | F15; ΔPP0168 (Gm\(^\text{\textsuperscript{r}}\)) | [22] |
| F15ΔlapF       | F15; ΔPP0806 (Gm\(^\text{\textsuperscript{r}}\)) | [22] |
| F15ΔlapAΔlapF  | F15; ΔPP0168 ΔPP0806 (Gm\(^\text{\textsuperscript{r}}\)) | [22] |
| PSmKm          | PSm; Km gene is located 355 bp upstream of lapF coding sequence (Sm\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| F15Km          | F15; Km gene is located 355 bp upstream of lapF coding sequence (Gm\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| F15KmFm        | F15; Fis binding site Fis-F2 is mutated (Gm\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| PSmΔlapF3      | PSm; native DNA of 200 bp (including Fis-F2 binding site) upstream of lapF gene is replaced by 1.9 kb DNA of lac\(^\text{\textsuperscript{r}}\)-P\(_{lac}\) (Km\(^\text{\textsuperscript{r}}\), Sm\(^\text{\textsuperscript{r}}\)) | This study |
| F15ΔlapF3      | F15; native DNA of 200 bp (including Fis-F2 binding site) upstream of lapF gene is replaced by 1.9 kb DNA of lac\(^\text{\textsuperscript{r}}\)-P\(_{lac}\) (Km\(^\text{\textsuperscript{r}}\), Gm\(^\text{\textsuperscript{r}}\)) | This study |

**Plasmids**

| Plasmid          | Description                                                                 | Source/ reference |
|------------------|------------------------------------------------------------------------------|-------------------|
| pSEVA-lacI tac-1 | RK2 expression vector containing in Ecl136II site a 1981-bp-long blunted BamHI fragment of P\(_{lac}\)-promoter and lac\(^\text{\textsuperscript{r}}\) repressor from pBRlactac (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pBlR lactac      | Expression vector containing P\(_{lac}\)-promoter and lac\(^\text{\textsuperscript{r}}\) repressor in pBR322 (Amp\(^\text{\textsuperscript{r}}\)) | [45] |
| pBluescript KS   | E. coli cloning vector (Amp\(^\text{\textsuperscript{r}}\))                       | Stratagene         |
| pBlc-Fp          | Cloning vector pBluescript KS containing in SmaI site a 438-bp-long region of lac\(^\text{\textsuperscript{r}}\) upstream and downstream DNA (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pBlc-Fm          | Cloning vector pBluescript KS containing in SmaI site a 438-bp-long region of lac\(^\text{\textsuperscript{r}}\) upstream and downstream DNA with mutated P-F2 binding site (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pBlc-Fy          | Cloning vector pBluescript KS containing in SmaI site a 506-bp-long region of lac\(^\text{\textsuperscript{r}}\) upstream DNA (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pBLKT-Fis-mut    | 177-bp-long promoter region of lapF gene with the mutated Fis-F2 site cloned into pBLKT-BamHI site (Km\(^\text{\textsuperscript{r}}\)) | [32] |
| pGP704-L         | Pir-dependent R6K replicon suicide vector (Amp\(^\text{\textsuperscript{r}}\)) | [46] |
| pGP-Fm           | Suicide vector pGP704-L containing in SacI and Sall sites a 438-bp-long region of lac\(^\text{\textsuperscript{r}}\) upstream and downstream DNA with mutated Fis-F2 binding site (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pGP-Fp           | Suicide vector pGP704-L containing in SacI and Sall sites a 438-bp-long region of lac\(^\text{\textsuperscript{r}}\) upstream and downstream DNA (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pGP-FmFy         | Suicide vector pGP704-L containing in Ecl136II and XbaI sites a 506-bp and 438-bp-long regions upstream and downstream of lac\(^\text{\textsuperscript{r}}\) gene (Amp\(^\text{\textsuperscript{r}}\)) | This study |
| pGP-FpFy-Km      | Suicide vector pGP704-L containing in Ecl136II and XbaI sites a 506-bp and 438-bp-long regions upstream and downstream of lac\(^\text{\textsuperscript{r}}\) gene, with Km resistance gene between them in Sacl site (Amp\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| pGP-FmFy-Km      | Suicide vector pGP704-L containing in Ecl136II and XbaI sites a 506-bp and 438-bp-long regions upstream and downstream of lac\(^\text{\textsuperscript{r}}\) gene, containing mutated Fis-F2 binding site and with Km resistance gene between them in Sacl site (Amp\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| pGP-FpFy-Km-lacI-tac-lapFSD | Suicide vector pGP704-L containing in Sacl and BglII sites a 506 bp and 527 bp long regions upstream and downstream of lac\(^\text{\textsuperscript{r}}\) gene, with Km resistance gene and lac promoter with lac\(^\text{\textsuperscript{r}}\) repressor gene. Shine-Dalgarno region replaced; Fis binding site Fis-F2 and the native promoter of lapF gene are deleted (Amp\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | This study |
| pUTmini-Tn5 Km2  | Suicide vector, source of Km resistance gene (Amp\(^\text{\textsuperscript{r}}\), Km\(^\text{\textsuperscript{r}}\)) | [47] |

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To examine the growth parameters, the strains of *P. putida* were grown overnight in LB medium. These cultures were used to inoculate fresh LB media so that the absorbance of the cultures at 580 nm was approximately 0.1. The bacteria were grown in 96-well microtiter plates (150 μl media per well) and \( A_{580} \) was measured at 7 minute intervals using a Sunrise-Basic Tecan microplate reader (Tecan Austria GmbH, Austria). Approximately 150 viable count data points were produced for each growth curve. Growth rate (\( \mu; h^{-1} \)) and the length of lag-phase of bacteria were estimated from growth curves by the Gompertz model [44]. The mean of the growth rate of different strains was calculated from 6 parallels.

### DNA manipulations and strain construction

The plasmids used for the construction of strains PSmKm, F15Km and F15KmFm were obtained by several sequential cloning steps. At first, the 506-bp-long DNA region of *P. putida* chromosome locating 695 bp to 189 bp upstream of the lapF start-codon was amplified by
using the primers PP0806-I-rev and lapF-Sacl. Thereafter the PCR product was cloned into pBluescript KS vector opened by SmaI restrictase resulting in pBlc-Fy (Table 1). Secondly, the 438-bp-long DNA region of \( P. \) putida chromosome at positions 198 bp upstream to 240 bp downstream of the \( lapF \) start-codon, which contained Fis-F2 binding site was cloned by the primers lapF-fw and lapF-RACE1. The PCR product was cloned into the pBluescript KS vector opened by SmaI restrictase resulting in pBlc-Fp (Table 1).

For the construction of the strain F15KmFm two sequential PCRs were carried out to amplify the 438-bp-long DNA fragment containing mutated Fis-F2 Fis binding site. In the first PCR, the primers lapF-fw and lapF-down2 and the template plasmid pBLKT-Fis-mut \([32]\) carrying mutated Fis-F2 site were used for the DNA amplification. In the second PCR, lapF-RACE1 and the product of the first PCR were used as primers for the DNA amplification of the \( lapF \) promoter region from the \( P. \) putida PSm chromosome. The obtained DNA fragment was inserted into the pBluescript KS vector opened by SmaI restrictase resulting in pBlc-Fp (Table 1).

After that the plasmid DNA of pBlc-Fp and pBlcFm was cut with restrictases SacI and XhoI and 476-bp long DNA fragments, were cloned into pGP704-L opened by SacI and SalI restrictases, resulting in plasmids pGP-Fp and pGP-Fm, respectively (Table 1). Then the plasmid pBlc-Fy was cut with XbaI and EcoRV and the 552-bp-long DNA fragment was cloned into pGP-Fp and pGP-Fm opened with Ecl136II and XbaI restrictases, resulting in pGP-FpFy and pGP-FmFy, respectively (Table 1). Lastly, the Km-resistance gene from pUTmini-Tn5 Km2 was cloned into pGP-FpFy and pGP-FmFy into SacI site resulting in pGP-FpFy-Km with native Fis binding site and pGP-FmFy-Km with mutated Fis binding site, respectively (Table 1). The constructed plasmids were introduced by electroporation into \( E. coli \) strain CC118 \( \lambda \) pir. The obtained donor-strains were mated with the helper plasmid-carrying strain \( E. coli \) HB101 and the \( P. \) putida recipient strains PSm or F15. Triple mating resulted in the strains PSmKm, F15Km (Fig 1A), PSmKmFm and F15KmFm (Fig 1C).

The plasmids used for the construction of the strains PSmlapF3 and F15lapF3 were obtained by two sequential clonings. At first, the \( P. \) tac promoter with the lacI repressor gene was cloned from pSEVA-lacItac-1 into pGP-FpFy-Km opened by XbaI restrictase resulting in pGP-FpFy-Km-lacI-tac (Table 1). Thereafter, the 438-bp-long DNA region containing the Fis-F2 binding site was replaced by the 527-bp-long DNA region (locates at the positions 20 bp upstream to 507 bp downstream of the \( lapF \) start-codon) amplified from the \( P. \) putida chromosome by using the primers lapF-SD-SalI and lapF-rev4 and cloned into the plasmid pGP-FpFy-Km-lacI-tac opened with restrictases SalI and BglII, resulting in the construct pGP-FpFy-lacI-tac-lapFSD (Table 1). This plasmid carried downstream of the \( P. \) tac promoter a LacI-operator, a new artificial Shine-Dalgarno region and the first 240 nucleotides of \( lapF \) gene. The plasmid pGP-FpFy-Km-lacI-tac-lapFSD was introduced by electroporation into \( E. coli \) strain CC118\( \lambda \)pir and the triple mating was conducted as described previously. The obtained \( P. \) putida strains PSmlapF3 and F15lapF3 lacked the DNA region 200 bp upstream of the \( lapF \) start-codon including the \( lapF \) promoter elements and Fis-F2 binding site (Fig 1B). In these strains the \( lapF \) gene was transcribed under the control of IPTG-inducible \( P. \) tac promoter and the effects of Fis on the transcription of this gene were excluded.

All constructs were verified by the DNA sequencing in order to exclude PCR-generated errors in the cloned DNA-fragments. The accuracy of recombination in the desired \( P. \) putida strains was verified by the DNA sequencing of the relevant chromosomal regions of \( P. \) putida.

**Measurement of cell surface hydrophobicity**

Cell surface hydrophobicity was analysed by the measurements of the contact angle, \( \theta_w \), between water droplets and a filter (NC45, 0.45 \( \mu \)m pore size from Whatman, Maidstone, UK).
covered with bacterial cells and mounted on a glass slide [10]. DSA100 drop shape analyser system from Krüss GmbH (Hamburg, Germany) was used for measurements of contact angles.

Detection of the expression of *fis* and *lapF* in the constructed strains of *P. putida*

For the preparation of cell lysates, all studied *P. putida* strains were grown in 50 ml LB broth with or without 1 mM IPTG for 3 or 18 h. The cells were collected by centrifugation and lysed in RIPA buffer (50 mM Tris/HCl pH 7.4 buffer, 0.1% SDS, 1% NP-40, 1% Triton X-100, 0.5% DOC (sodium o xo cholate), 500 mM NaCl, 5 mM EDTA) and 100-fold diluted Halt protease and phosphatase single-use inhibitor cocktail (Thermo Scientific) as previously described [22]. The total amount of protein in the cleared supernatant was measured by the content of tryptophan and the proteins were separated in gradient (4–8%) polyacrylamide gel electrophoresis as previously described [22].

For the production of polyclonal mouse anti-Fis antibody, *P. putida* Fis(His) was overexpressed and purified with Ni-NTA agarose matrix (Qiagen) as previously described [37]. The polyclonal antibodies against Fis (PP4821) were produced and purified by LabAs. Western immunoblot analysis was carried out to detect the amount of Fis from the crude lysates of *P. putida*. Bacteria were grown to stationary-phase in LB medium in the presence or absence of 1 mM IPTG. The cells were collected by centrifugation and sonicated in Fis purification 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 [48]. Proteins were separated by Tricine-SDS-PAGE (10%) electrophoresis [49], transferred to a membrane and the membrane was probed with mouse anti-Fis as previously described [22].

Flow cytometry analysis

*P. putida* grown for 18 h in LB medium was treated with different concentration of methanol and 1-octanol for 30 minutes at 30°C. The bactericidal level of methanol and 1-octanol are
shown to be >50% and >0.05% respectively [50]. Therefore, methanol was added at final concentrations of 25 and 50% (v/v) and 1-octanol at 0.015 and 0.045% (v/v). Identical amounts of water were added to the control cells. Thereafter, the cells were stained using the LIVE/DEAD BacLight kit (Invitrogen). Staining of cells was performed as suggested by manufacturers and approximately 10,000 events from every sample were analysed with flow cytometer FACS Aria (BD Biosciences).

Quantitative real-time PCR

RNA for the qRT-PCR reactions was extracted from \textit{P. putida} strains PSm and PSmΔlapF with NucleoSpin \textsuperscript{\textregistered} RNA II kit (Macherey-Nagel) according to the manufacturer’s protocol. The SYBR Green qPCR assay was performed on the Rotor-Gene Q system (QIAGEN) using SuperScript\textsuperscript{\textcopyright} III One-Step RT-PCR System with Platinum\textsuperscript{\textcopyright} Taq (Invitrogen). The reaction mixture, which was optimized as previously described [51], contained 5 μl of SYBR Green Reaction Mix, 4 pmol of each primer, 10 ng of RNA, 0.2 μl of SuperScript\textsuperscript{\textcopyright} III/ Platinum\textsuperscript{\textcopyright} Taq Mix and RNase-free water for a total volume of 10 μl. The amplification program included the reverse transcription step at 50°C for 3 min and the initial denaturation at 96°C for 5 min followed by 40 cycles of 15 s at 95°C, 30 s at 62°C and 20 s at 72°C. SYBR Green fluorescence was measured after each extension step and the specificity of amplification was evaluated by the melting curve analysis. The \textit{lapI} gene was amplified with the primers LapI-fw and LapI-rev and the reference gene \textit{rpoD} with the primers RpoDq-fw and RpoDq-rev. RT-qPCR data was analysed with Rotor-Gene Q software version 2.0.2 and LinRegPCR program version 11 [52]. The amount of \textit{lapI} mRNA in RT-qPCR was normalized against \textit{rpoD} mRNA. The mRNA of \textit{rpoD} was used as standard to reduce the random fluctuation of mRNA concentration. The fold difference between the \textit{lapI} mRNA level and the \textit{rpoD} mRNA level was estimated using the following formula:

\[
\left( \frac{N_{0,lapI}}{N_{0,rpoD}} \right) = \left( \frac{E_{Ct,rpoD}}{E_{Ct,lapI}} \right),
\]

where 

\( N_0 \) is the starting concentration of the amplicon, \( E \) the amplification efficiency and \( C_t \) the number of cycles needed to reach the threshold [52].

Statistical analysis

The ANOVA and \textit{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 13 software.

Results and Discussion

LapF increased the hydrophobicity of \textit{P. putida} cells

The hydrophobicity of cell surfaces of Gram-negative bacteria can be determined by several factors like outer membrane proteins, lipopolysaccharide composition and adhesins etc. [11–14]. It has been previously reported for \textit{P. putida} that LapA is the key factor for the early attachment of cells to abiotic and biotic surfaces, and LapF is important for cell-cell interaction in mature biofilms [25,26]. Although LapA is required for the adhesion of bacterial cells to both the hydrophobic and hydrophilic surfaces [27], participation of this adhesin in affecting the hydrophobicity of the cell surface of \textit{P. putida} has not been addressed prior to this work.

In order to investigate whether \textit{P. putida}’s two largest adhesins LapA and LapF could affect the hydrophobicity of cell surface, the cell surface hydrophobicity of PSmΔlapA, PSmΔlapF and the double-mutant PSmΔlapAΔlapF were compared with the wild-type strain PSm in both
exponential and stationary growth phase. \textit{P. putida} PSm wild-type cells harvested after 18 hours, in the stationary-phase, showed higher cell surface hydrophobicity ($\theta_w$ 76°, Fig 2A), which correlates with the previous findings that stressed bacteria have more hydrophobic cell surfaces [7]. Whereas stationary phase cells of the strain lacking LapA showed similar contact angles with the wild-type, the $\theta_w$ of the mutants lacking LapF (PSm$\Delta$lapF) and the double mutant of LapA and LapF (PSm$\Delta$lapA$\Delta$lapF) decreased to 47° and 44°, respectively (Fig 2A).

![Figure 2](https://example.com/f2.png)

**Fig 2.** The cell surface hydrophobicity of \textit{P. putida} PSm-originated strains. (A) Hydrophobicity of exponential phase (3h) and stationary-phase (18h) \textit{P. putida} wild-type strain (PSm), the \textit{lapA} knock-out mutant PSm$\Delta$lapA, the \textit{lapF} knock-out mutant PSm$\Delta$lapF and the double knock-out mutant PSm$\Delta$lapA$\Delta$lapF. (B) The growth curve of PSm, PSm$\Delta$lapA, PSm$\Delta$lapF and PSm$\Delta$lapA$\Delta$lapF grown in LB medium. The growth curves of the first 4.5 hours are shown. Three independent biological experiments were done with similar results. Data from six parallels from one experiment is shown. (C) Hydrophobicity of exponential phase (3h) and stationary-phase (18h) \textit{P. putida} strain expressing the \textit{lapF} gene at natural level (PSmKm) and the strain PSm$\Delta$lapF grown in the presence or absence of 1 mM IPTG in LB medium. The level of cell surface hydrophobicity is given as water contact angles, $\theta_w$ (in degrees) between water droplets and a filter covered with bacterial cells. Error bars denote 95% confidence intervals of the means. Letters a–d depict statistical homogeneity groups according to ANOVA post hoc Bonferroni test. Data from at least eight independent measurements is shown.

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Contrary to these observations, when cells were harvested in the exponential growth phase, all tested PSm knock-out mutants showed similar water contact angles varying between θ_w 64° and 66° (P = 1; Fig 2A). The cells of these mutants were approximately 1.2 times (P < 0.001) more hydrophobic than those of the wild-type cells (θ_w 56°, Fig 2A). As growth of bacteria can influence the result of cell surface hydrophobicity in exponential growth phase, we assessed the growth of P. putida PSm and the knock-out strains in LB medium (Fig 2B, Table 3). The strains grew similarly in stationary phase (data not shown), but differently in exponential growth phase. It is possible to distinguish two groups of strains by growth rate and lag-phase length: (i) PSm and PSmΔlapF, and (ii) PSmΔlapA and PSmΔlapAΔlapF (Fig 2B, Table 3). The growth parameters of PSm and PSmΔlapF were similar and differed from lapA null-mutant strains (Table 3). Whereas the lapF expression is strongly repressed in exponentially growing P. putida [22,53], the cell surface hydrophobicity of PSm and PSmΔlapF should be similar in exponential growth phase. Moreover, the absence of LapA in the surface of stationary phase cells did not affect the hydrophobicity of P. putida (Fig 2A). Thus, it seems that the cell surface hydrophobicity of knock-out mutants does not depend on either LapF nor LapA in exponential growth phase. However, the important factor for increasing P. putida cell surface hydrophobicity in the stationary phase is LapF and not LapA.

The protein hydrophobicity values in P. putida according to Kyte-Doolittle scale are -0.109 for LapA and 0.164 for LapF [54]. This scale is a measure for the hydrophobicity of amino acids [55], indicating that LapF is more hydrophobic than LapA. However, the total hydrophobicity of large proteins like LapA and LapF does not describe the surface properties of a protein. For example, LapA can bind to hydrophilic as well as hydrophobic surfaces [27]. According to the same database, two bacterial Ig-like domains are predicted in LapF locating in position 717 to 748 and 1158 to 1177 residues of amino acids [54]. Although, Ig-like domains are frequently found in the cell surface proteins of enterobacteria needed for adhesion, it is speculated that Ig-like domains are responsible for stabilization of protein rather than for adhesion [56]. For example, the Ig-like domains are found in the cytoplasmic enzymes SodC and LacZ that are not involved in adhesion [57]. Thus, the Ig-like domains in LapF probably stabilize the protein on the surface of P. putida.

It has been previously shown, that in P. putida lapF is expressed only in stationary growth phase and no LapF is detectable in exponential phase cells [22,53]. In order to study the involvement of LapF in cell surface hydrophobicity, P. putida strain PSmlapF3 was constructed, in which lapF expression was under the control of IPTG-inducible P_tac promoter (Table 1, Fig 1B). In addition, P. putida strain PSmKm was constructed as a reference strain for PSmlapF3 by inserting a Km resistance gene 355 bp upstream of the lapF gene (Table 1). This strain had the native lapF promoter, although the chromosome of P. putida was interrupted by Km′-gene insertion similarly to PSmlapF3 (Fig 1A and 1B).

### Table 3. The growth parameters of P. putida strains grown in LB medium.

| Strain          | Growth rate, h^-1 | Lag-phase length, h |
|-----------------|-------------------|---------------------|
| PSm             | 0.513 (0.033)* a  | 1.416 (0.089) a     |
| PSmΔlapA        | 0.415 (0.013) b   | 0.502 (0.089) b     |
| PSmΔlapF        | 0.483 (0.022) a   | 1.261 (0.164) a     |
| PSmΔlapAΔlapF   | 0.398 (0.014) b   | 0.582 (0.180) b     |

* In the brackets are shown 95% confidence intervals of the means of 6 parallels. All R values were > 0.990. Letters a and b depict statistical homogeneity groups according to ANOVA post hoc Bonferroni test.

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The expression of lapF in these newly constructed strains was confirmed with silver-stained SDS-polyacrylamide gels, where proteins from cell lysates were separated by gel-electrophoresis (Fig 3A). LapF was present in 18 h stationary PSmKm both in the absence and presence of IPTG (Fig 3A) similarly to strain PSm [22]. Thus, the insertion of the Km-resistance gene in front of the lapF gene did not affect the expression of lapF. On the contrary, in stationary-phase cells of PSmlapF3, the lapF expression was under the control of IPTG as LapF was detected only in the presence of IPTG (Fig 3A). The expression of lapF in exponentially growing bacteria was not detectable due to technical reasons. The lysates of 3 h grown exponential cells decomposed in SDS-gels probably due to high level of protease activity in cell lysates despite of the addition of protease inhibitor cocktail during cell lysis preparation (data not shown).

The cell surface hydrophobicities of PSmKm and PSmlapF3 were measured in exponential and stationary cells both in the presence or absence of 1 mM IPTG (Fig 2C). The stationary-phase PSmlapF3 cells cultivated in the presence of 1 mM IPTG showed 1.3 times (P<0.001) higher contact angles (θw 56°) comparing to PSmlapF3 grown without IPTG (θw 43°, Fig 2C). In addition, the contact angles of PSmlapF3 with 1 mM IPTG (θw 59°) were comparable with that of PSmKm (P = 1). This proved that the transcription of the lapF gene from the P_tac promoter in the presence of IPTG can re-establish the hydrophobicity of P. putida cells to the level of the wild-type strain (Fig 2C). In exponential cells, when IPTG was added to the wild-type strain PSmKm, there was no significant change in the contact angles, as the θw value was approximately 56° for cells without IPTG as well (Fig 2C). However, the contact angle of P. putida cells increased 1.2 times (P<0.001) already in the exponential phase when the transcription of the lapF gene was induced in PSmlapF3 by IPTG (θw 65°) if compared to the contact angle of PSmlapF3 grown without IPTG (θw 54°) or wild-type PSmKm (θw 56°, Fig 2C). The increase in hydrophobicity of PSmlapF3 exponentially growing cells in the presence of IPTG implied that LapF is responsible for the increase in cell surface hydrophobicity of P. putida. These results further confirmed the hypothesis that the expression of LapF increases the level of hydrophobicity of P. putida cells.

Knocking-out the lapF gene did not influence the expression of the rest of genes in the lapFHIJ operon

To verify that the expression level of the other lapFHIJ operon genes has not changed by knocking out lapF gene, the lapl (PP_0804) mRNA level was measured with RT-qPCR (Fig 4). The mRNA level of lapl compared against the mRNA level of the reference gene rpoD was statistically insignificant (P = 0.3) between the wild-type and ΔlapF strain. Thus, the hydrophobicity of P. putida was most likely influenced by the lapF and not by the rest of the genes from the lapFHIJ operon.
Fis Regulates *P. putida* Hydrophobicity via LapF

Relative amount of *lapL* mRNA

|         | PSm       | PSmΔlapF  |
|---------|-----------|-----------|
| Value   | 0.9       | 1.5       |

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Fis overexpression reduced the hydrophobicity of stationary-phase cells by decreasing the level of LapF

The expression of lapF is controlled by two regulators. In stationary-phase cells, lapF expression is triggered by RpoS [26], the sigma factor required for starvation and general stress responses. The expression of lapF is additionally repressed by the global regulator Fis, which binds lapF promoter [32], resulting in the decline of the lapF expression [22].

To study the correlation between the cellular amount of Fis and hydrophobicity of *P. putida* cells, three strains originated from the fis overexpression strain F15 were constructed. First, the reference strain F15Km was constructed similarly to the PSmKm strain, which contained the native promoter in front of the lapF gene with the Fis binding site Fis-F2 (Table 1; Fig 1A). In addition, the artificial lapF expression strain F15lapF3 was constructed with the possibility to express the global regulator Fis and LapF by IPTG (Table 1; Fig 1B). Since the native promoter of the lapF gene was replaced by the lacI-P_tac cassette, we expected that the transcription of the lapF gene in this strain could be affected by the presence of IPTG but not by the level of fis overexpression. The third constructed fis overexpression strain F15KmFm carried a mutated Fis binding site Fis-F2 upstream of the lapF gene in *P. putida* chromosome (Table 1; Fig 1C).

In this strain, the substituted nucleotides in the Fis binding sequence Fis-F2 reduced the Fis binding to the lapF promoter in vitro as described previously [32] and therefore fis overexpression should regulate lapF transcription modestly or not at all.

The expression of fis and lapF in these F15-originated strains was assessed. The overexpression of fis was confirmed by the immunoblotting of cell lysates prepared from newly constructed F15 strains. 1 mM IPTG added to the LB medium caused the fis overexpression in the all studied F15 originated strains (Fig 5). The lapF expression in the constructed strains was examined with silver-staining of SDS-polyacrylamide gels where proteins from cell lysates were separated by gel-electrophoresis (Fig 3B). LapF expression was downregulated in F15Km by the fis overexpression (Fig 3B) similarly to F15 [22]. Thus, the insertion of Km-resistance gene in front of the lapF gene did not affect the regulation of lapF expression by Fis. However, lapF was expressed in F15lapF3 and F15KmFm, in the strains missing correct Fis binding site Fis-F2, despite the fis overexpression (Fig 3B). These results confirmed that Fis repressed lapF expression via the Fis binding site Fis-F2. This is in good accordance with the previously
published data showing that Fis binds to the Fis-F2 site upstream of the lapF gene and represses the transcription of lapF [32]. The hydrophobicity of fis overexpression strain F15 was measured when cells were grown in LB medium for 3 h (exponential phase) and 18 h (stationary phase) in the presence or absence of 1 mM IPTG (Fig 6A). We observed that the fis overexpression in the presence of IPTG decreased hydrophobicity of cells in exponential growth phase 1.2 times ($P < 0.001$) from $\theta_w$ value of 56° to 48° and in stationary growth phase cells 1.6 times, from $\theta_w$ 74° to 46° ($P < 0.001$; Fig 6A). Similarly to the original strain F15, the contact angle of the strain F15Km ($\theta_w$ 71°) decreased 1.3 times in stationary phase by the addition of IPTG to the medium ($P < 0.001$) comparing to F15Km grown without IPTG ($\theta_w$ 53°; Fig 6B). At the same time, the contact angle of the strain F15KmFm ($\theta_w$ 75°) decreased minimally by the addition of IPTG to the LB medium ($P = 0.027$) comparing to F15KmFm grown without IPTG ($\theta_w$ 72°; Fig 6B). On the contrary, measurement of the hydrophobicity of the cells of the strain F15lapF3 carrying the lapF gene under the control of the IPTG-inducible P_{tac} promoter revealed the increased hydrophobicity in the presence of IPTG. The contact angles increased 1.4 times to $\theta_w$ 68° when IPTG was added to the medium compared to the F15lapF3 grown without IPTG ($\theta_w$ 49°, $P < 0.001$; Fig 6B). This data indicates that the hydrophobicity of P. putida depends on the presence of LapF on the cell surface. The expression of lapF is in turn repressed by Fis via binding to the Fis-F2 site at the lapF promoter region. Furthermore, this assures that fis overexpression can be directly related to only one certain phenotype—change of the hydrophobicity of P. putida by explicitly regulating the transcription of lapF gene. Contrary to the previously published studies about the relationships of P. putida biofilm and hydrophobicity, the findings of the present study suggest that biofilm, especially the Fis-enhanced, and the LapF-mediated hydrophobicity are not directly connected with each other in P. putida.
Flow cytometry analysis revealed protective role of LapF against methanol toxicity

Our results demonstrated that the expression of lapF increased the hydrophobicity of cell surface; therefore, we asked whether LapF could affect the viability of P. putida in the presence of hydrophilic and hydrophobic compounds like methanol and 1-octanol. Thus, the effects of methanol as an example for a hydrophilic solvent and 1-octanol as an example for a hydrophobic solvent to the membrane of P. putida wild-type strain PSm and the lapF knock-out strain PSmΔlapF were investigated. The strain PSm lapF3 grown with IPTG was used as a positive control to the wild-type strain PSm. Overnight grown cells treated with different concentrations of methanol and 1-octanol were stained using the LIVE/DEAD BacLight kit (Invitrogen) and flow cytometry analysis was used to determine the distribution of subpopulations.

When cells were treated with methanol at concentration of 25% and 50% (v/v) the survival of strains expressing LapF (PSm and PSm lapF3) was higher than the lapF knock-out mutant PSmΔlapF (Table 4, S1 Fig). Contrary to the results obtained with methanol, in cells treated with 1-octanol at concentrations of 0.015% and 0.045% (v/v), the viability of cells lacking LapF increased (Table 4, S1 Fig). As this method allowed us to observe cytoplasmic membrane integrity, we can see that LapF, increasing cell surface hydrophobicity, can be an important defensive factor for bacteria against methanol as an example of hydrophilic compounds.

Conclusions

This study demonstrates that LapF is the key factor of cell surface hydrophobicity of P. putida in the stationary phase, and that Fis regulates the hydrophobicity of cell surface in P. putida.

Table 4. Effect of LapF on cell membrane integrity in the presence of methanol and 1-octanol.

| Strain | IPTG | Methanol(%) | % of subpopulationsa | Strain | IPTG | 1-octanol(%) | % of subpopulationsa |
|--------|------|-------------|----------------------|--------|------|-------------|----------------------|
|        |      |             | Cells with intact membrane |        |      |             | Cells with intact membrane |
|        |      |             | Cells with permeable membrane |        |      |             | Cells with permeable membrane |
|        |      |             | Dead cells |        |      |             | Dead cells |
| PSm    | 0    | 0           | 93.6 (0.925) | Cells with intact membrane | 95.6 (2.199) | 1.22 (0.702) | 3.22 (1.504) |
|        | 0    | 25          | 20.9 (3.370) | Cells with permeable membrane | 60.9 (0.885) | 4.93 (0.155) | 34.2 (0.732) |
|        | 0    | 50          | 0.08 (0.062) | Dead cells | 66.6 (0.359) | 3.20 (0.091) | 30.2 (0.429) |
| PSmΔlapF | 0    | 0           | 97.2 (0.654) | Cells with intact membrane | 97.6 (10.57) | 0.47 (1.771) | 1.95 (10.39) |
|        | 0    | 25          | 6.71 (0.422) | Cells with permeable membrane | 86.7 (2.578) | 1.16 (0.322) | 12.1 (2.583) |
|        | 0    | 50          | 0.27 (0.417) | Dead cells | 81.5 (3.789) | 1.44 (0.323) | 17.1 (3.571) |
| PSm lapF3 | 1    | 0           | 97.2 (0.144) | Cells with intact membrane | 97.6 (10.57) | 0.47 (1.771) | 1.95 (10.39) |
|        | 1    | 25          | 11.4 (0.251) | Cells with permeable membrane | 76.4 (3.572) | 1.54 (0.401) | 22.1 (3.755) |
|        | 1    | 50          | 0.25 (0.280) | Dead cells | 70.4 (3.680) | 1.83 (0.360) | 27.8 (3.492) |

aIn the brackets are shown 95% confidence intervals of the means of at least three measurements.

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through the repression of lapF. We observed that the LapF-mediated hydrophobicity protects \textit{P. putida} cells against methanol as an example of hydrophilic solvents. On the other hand, bacteria expressing \textit{lapF} were more susceptible to 1-octanol as an example of a hydrophobic solvent.

**Supporting Information**

\textbf{S1 Fig. Visualization of subpopulations by flow cytometry analysis.} \textit{P. putida} strains PSm and PSm$\Delta lapF$ was grown for 18 hours in LB medium and thereafter treated with 25\% (v/v) methanol and 0.15\% (v/v) 1-octanol for 30 minutes. \textit{P. putida} strain PSm$lapF3$ was grown for 18 hours in LB medium amended with 1 mM IPTG and treated with chemicals similarly as other strains. Each dot represents an event, analysed by flow cytometer, that has been excited at 488 nm and respective fluorescence emission has been measured at 530 (30) and 616 (23) nm. Area of subpopulations of dead cells, cells with permeable membrane and intact membrane are shown.

(TIF)

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**Author Contributions**

- **Conceptualization:** AL HJH RT.
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- **Formal analysis:** AL HJH RT.
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- **Validation:** AL HA HJH RT.
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- **Writing – original draft:** AL.
- **Writing – review & editing:** AL HA MK HJH RT.

**References**

1. Segura A, Duque E, Mosqueda G, Ramos JL, Junker F (1999) Multiple responses of gram-negative bacteria to organic solvents. Environ Microbiol 1: 191–198. PMID: 11207738
2. Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74: 961–973. doi: 10.1007/s00253-006-0833-4 PMID: 17262209

3. Goulter RM, Gentle IR, Dykes GA (2009) Issues in determining factors influencing bacterial attachment: a review using the attachment of *Escherichia coli* to abiotic surfaces as an example. Lett Appl Microbiol 49: 1–7. doi: 10.1111/j.1472-765X.2009.02591.x PMID: 19291206

4. Rodrigues DF, Elimelech M (2009) Role of type 1 fimbriae and mannose in the development of *Escherichia coli* K12 biofilm: from initial cell adhesion to biofilm formation. Biofouling 25: 401–411. doi: 10.1080/08927010902833443 PMID: 19306144

5. Torres S, Pandey A, Castro GR (2011) Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. Biotechnol Adv 29: 442–452. doi: 10.1016/j.biotechadv.2011.04.002 PMID: 21504787

6. Kobayashi H, Takami H, Hirayama H, Kobata K, Usami R, Horikoshi K (1999) Outer membrane changes in a toluene-sensitive mutant of toluene-tolerant *Pseudomonas putida* IH-2000. J Bacteriol 181: 4493–4498. PMID: 10419944

7. Baumgarten T, Sperling S, Seifert J, von Bergen M, Steiniger F, Wick LY, et al. (2012) Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. Appl Environ Microbiol 78: 6217–6224. doi: 10.1128/AEM.01525-12 PMID: 22752175

8. Liu XW, Sheng GP, Yu HQ (2009) Physicochemical characteristics of microbial granules. Biotechnol Adv 27: 1061–1070. doi: 10.1016/j.biotechadv.2009.05.020 PMID: 19464355

9. Adav SS, Lee DJ, Show KY, Tay JH (2008) Aerobic granular sludge: recent advances. Biotechnol Adv 26: 411–423. doi: 10.1016/j.biotechadv.2008.05.002 PMID: 18573633

10. Neumann G, Cornelissen S, van Breukelen F, Hunger S, Lippold H, Loffhagen N, et al. (2006) Energetics and surface properties of *Pseudomonas putida* DOT-T1E in a two-phase fermentation system with 1-decanol as second phase. Appl Environ Microbiol 72: 4232–4238. doi: 10.1128/AEM.02904-05 PMID: 16751536

11. Sidhu MS, Olsen I (1997) S-layers of *Bacillus* species. Microbiology 143 (Pt 4): 1039–1052.

12. Maroth S, von Aulock S, Hartung T (2005) Structure/function relationships of lipoteichoic acids. J Endotoxin Res 11: 348–356. doi: 10.1179/096805105X67328 PMID: 16303090

13. Zahringer U, Knirel YA, Lindner B, Helbig JH, Sonesson A, Marre R, et al. (1995) The lipopolysaccharide of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. Prog Clin Biol Res 392: 113–139. PMID: 8524918

14. Higashi JM, Wang IW, Shlaes DM, Anderson JM, Marchant RE (1998) Adhesion of *Staphylococcus epidermidis* and transposon mutant strains to hydrophobic polyethylene. J Biomed Mater Res 39: 341–350. PMID: 9468041

15. Isberg RR, Barnes P (2002) Dancing with the host: flow-dependent bacterial adhesion. Cell 110: 1–4. PMID: 12150990

16. Rosenberg M, Kjelleberg S (1986) Hydrophobic interactions: Role in bacterial adhesion. In: Marshall KC, editor. Advances in Microbial Ecology. New York: Plenum Press. pp. pp. 353–393.

17. Corpe WA (1980) Microbial surface components involved in the adsorption of microorganisms onto surfaces. In: Marshall GBaKC, editor. Adsorption of Microorganisms to Surfaces. New York: Wiley-Interscience. pp. pp. 105–144.

18. Lugtenberg BJ, Bloemberg GV (2004) Life in the rhizosphere. In: Ramos J, editor. *Pseudomonas*. New York: Kluwer Academic / Plenum Publishers. pp. pp. 403–430.

19. Haussler S, Parsek MR (2010) Biofilms 2009: new perspectives at the heart of surface-associated microbial communities. J Bacteriol 192: 2941–2949.

20. Lugtenberg BJ, Girard G (2014) Role of Phenazine-1-Carboxamide Produced by *Pseudomonas chlororaphis* PCL1391 in the Control of Tomato Fruit and Root Rot. In: Chincholkar SB, Thomashow L, editors. Microbial Phenazines Biosynthesis, Agriculture and Health: Springer-Verlag Berlin Heidelberg. pp. pp. 163–176.

21. Jahn A, Griebe T, Nielsen PH (1999) Composition of *Pseudomonas putida* biofilms: Accumulation of protein in the biofilm matrix. Biofouling 14: 49–57.

22. Moor H, Teppo A, Lahesaare A, Kivisaar M, Teras R (2014) Fis overexpression enhances *Pseudomonas putida* biofilm formation by regulating the ratio of LapA and LapF. Microbiology 160: 2681–2693. doi: 10.1099/mic.0.082503-0 PMID: 25253613

23. Gjermanssen M, Nilsson M, Yang L, Tolker-Nielsen T (2010) Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. Mol Microbiol 75: 815–826. doi: 10.1111/j.1365-2958.2009.06793.x PMID: 19602146
24. Hinsa SM, Espinosa-Urgel M, Ramos JL, O’Toole GA (2003) Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol 49: 905–918. PMID: 12890017

25. Fuqua C (2010) Passing the baton between laps: adhesion and cohesion in *Pseudomonas putida* biofilms. Mol Microbiol 77: 533–563. doi: 10.1111/j.1365-2958.2010.07250.x PMID: 20545855

26. Martinez-Gil M, Yousef-Coronado F, Espinosa-Urgel M (2010) LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture. Mol Microbiol 77: 549–561. doi: 10.1111/j.1365-2958.2010.07249.x PMID: 20545856

27. Boyd CD, Smith TJ, El-Kirat-Chatel S, Newell PD, Dufrene YF, O’Toole GA (2014) Structural features of the *Pseudomonas fluorescens* biofilm adhesin LapA required for LapA-dependent cleavage, biofilm formation, and cell surface localization. J Bacteriol 196: 2775–2788. doi: 10.1128/JB.01629-14 PMID: 24837281

28. Dekkers LC, van der Bij AJ, Mulders IH, Phoeilich CC, Wentwoord RA, Glандorf DC, et al. (1998) Role of the O-antigen of lipopolysaccharide, and possible roles of growth rate and of NADH:ubiquinone oxidoreductase (nuo) in competitive tomato root-tip colonization by *Pseudomonas fluorescens* WCS365. Mol Plant Microbe Interact 11: 763–771. doi: 10.1094/MPMI.1998.11.8.763 PMID: 9675892

29. Dulla GF, Krasileva KV, Lindow SE (2010) Interference of quorum sensing in *Pseudomonas syringae* by bacterial epiphytes that limit iron availability. Environ Microbiol 12: 1762–1774. doi: 10.1111/j.1462-2920.2010.02261.x PMID: 20553555

30. Mattilla MA, Espinosa-Urgel M, Rodriguez-Herva JJ, Ramos JL, Ramos-Gonzalez MI (2007) Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. Genome Biol 8: R179. doi: 10.1186/gb-2007-8-9-r179 PMID: 17784941

31. Jakovleva J, Teppo A, Saumaa S, Moor H, Kivisaar M, et al. (2012) Fis regulates the competitiveness of *Pseudomonas putida* on barley roots by inducing biofilm formation. Microbiology 158: 708–720. doi: 10.1099/mic.0.053355-0 PMID: 22222498

32. Lahesaare A, Moor H, Kivisaar M, Teras R (2014) *Pseudomonas putida* Fis binds to the lapF promoter in vitro and represses the expression of LapF. PLoS One 9: e115901. doi: 10.1371/journal.pone.0115901 PMID: 25545773

33. Bradley MD, Beach MB, de Koning AP, Pratt TS, Osuna R (2007) Effects of Fis on *Escherichia coli* gene expression during different growth stages. Microbiology 153: 2922–2940. doi: 10.1099/mic.0.2007/008565-0 PMID: 17768236

34. Browning DF, Grainger DC, Busby SJ (2010) Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. Curr Opin Microbiol 13: 773–780. doi: 10.1016/j.mib.2010.09.013 PMID: 20951079

35. Xu J, Johnson RC (1995) Identification of genes negatively regulated by Fis: Fis and RpoS modulate growth-phase-dependent gene expression in *Escherichia coli*. J Bacteriol 177: 938–947. PMID: 7860604

36. Finkel SE, Johnson RC (1992) The Fis protein: it’s not just for DNA inversion anymore. Mol Microbiol 6: 3257–3265. PMID: 1484481

37. Teras R, Jakovleva J, Kivisaar M (2009) Fis negatively affects binding of Tn4652 transposase by out-competing IHF from the left end of Tn4652. Microbiology 155: 1203–1214. doi: 10.1099/mic.0.022830-0 PMID: 19332822

38. Osuna R, Lienau D, Hughes KT, Johnson RC (1995) Sequence, regulation, and functions of fis in *Salmonella typhimurium*. J Bacteriol 177: 2021–2032. PMID: 7536730

39. Yeung AT, Torfs EC, Jamshidi F, Bains M, Wiegand I, Hancock RE, et al. (2009) Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. J Bacteriol 191: 5592–5602. doi: 10.1128/JB.00157-09 PMID: 19592586

40. Montag D, Frant M, Horn H, Liefeith K (2012) Dependence of the initial adhesion of biofilm forming *Pseudomonas putida* mt2 on physico-chemical material properties. Biofouling 28: 315–327. doi: 10.1080/08927014.2012.673219 PMID: 22452391

41. Ruhs PA, Bocker L, Inglis RF, Fischer P (2014) Studying bacterial hydrophobicity and biofilm formation at liquid-liquid interfaces through interfacial rheology and pendant drop tensiometry. Colloids Surf B Biointerfaces 117: 174–184. doi: 10.1016/j.colsurfb.2014.02.023 PMID: 24632390

42. Sharma RC, Schimke RT (1996) Preparation of electrocompetent *E. coli* using salt-free growth medium. Biotechniques 20: 42–44. PMID: 8770403

43. Herrero M, de Lorenzo V, Timmis KN (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol 172: 6557–6567. PMID: 2172216
44. Dalgaard P, Koutsoumanis K (2001) Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. J Microbiol Methods 43: 183–196. PMID: 11118653

45. Ojangu EL, Tover A, Teras R, Kivisaar M (2000) Effects of combination of different -10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in Pseudomonas putida. J Bacteriol 182: 6707–6713. PMID: 11073916

46. Pavel H, Forsman M, Shingler V (1994) An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of Pseudomonas sp. strain CF600 on para-substituted methylphenols. J Bacteriol 176: 7550–7557. PMID: 8002579

47. de Lorenzo V, Cases I, Herrero M, Timmis KN (1993) Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in Pseudomonas putida with lacZ-tet bicistronic reporters. J Bacteriol 175: 6902–6907. PMID: 8226632

48. Wisniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6: 359–362. doi: 10.1038/nmeth.1322 PMID: 19377485

49. Schagger H (2006) Tricine-SDS-PAGE. Nat Protoc 1: 16–22. doi: 10.1038/nprot.2006.4 PMID: 17406207

50. Ali Y, Dolan MJ, Fendler EJ, Larson EL (2001) Alcohols. In: Block SS, editor. Disinfection, Sterilization and Preservation. Fifth ed. Philadelphia: Lippincott Williams & Wilkins. pp. 229–254.

51. Nolvak H, Truu M, Truu J (2012) Evaluation of quantitative real-time PCR workflow modifications on 16S rRNA and tetA gene quantification in environmental samples. Sci Total Environ 426: 351–358. doi: 10.1016/j.scitotenv.2012.03.054 PMID: 22521102

52. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, et al. (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37: e45. doi: 10.1093/nar/gkp045 PMID: 19237396

53. Martinez-Gil M, Ramos-Gonzalez MI, Espinosa-Urgel M (2014) Roles of cyclic Di-GMP and the Gac system in transcriptional control of the genes coding for the Pseudomonas putida adhesins LapA and LapF. J Bacteriol 196: 1484–1495. doi: 10.1128/JB.01287-13 PMID: 24488315

54. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS (2016) Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res 44: D646–653. doi: 10.1093/nar/gkv1227 PMID: 26578582

55. Kyte J, Doolittle RF (1982) A simple method for displaying the hydrophobic character of a protein. J Mol Biol 157: 105–132. PMID: 7108955

56. Bodelon G, Palomino C, Fernandez LA (2013) Immunoglobulin domains in Escherichia coli and other enterobacteria: from pathogenesis to applications in antibody technologies. FEMS Microbiol Rev 37: 204–250. doi: 10.1111/j.1574-6976.2012.00347.x PMID: 22724448

57. Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, et al. (2003) Structure and biogenesis of the capsular F1 antigen from Yersinia pestis: preserved folding energy drives fiber formation. Cell 113: 587–596. PMID: 12787500