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Supervisor: Riho Teras, PhD, Docent, University of Tartu, Estonia

Opponent: Carla C. C. R. de Carvalho, PhD, Principal Investigator, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

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LIST OF ORIGINAL PUBLICATIONS

I. **Moor H, Teppo A, Lahesaare A, Kivisaar M, Teras R.** Fis over-expression enhances *Pseudomonas putida* biofilm formation by regulating the ratio of LapA and LapF. Microbiology. 2014 Dec;160(Pt 12): 2681–93.

II. **Lahesaare A, Moor H, Kivisaar M, Teras R.** *Pseudomonas putida* Fis binds to the *lapF* promoter in vitro and represses the expression of LapF. PLoS One. 2014 Dec 29;9(12).

III. **Lahesaare A, Ainelo H, Kivisaar M, Heipieper HJ, Teras R.** LapF and Its Regulation by Fis Affect the Cell Surface Hydrophobicity of *Pseudomonas putida*. PLoS One. 2016 Nov 3;11(11).

My contribution to the publications is following:
Ref I – Performed some of the experiments (flow cytometry analysis and purification of *P. putida* Fis) and contributed to the manuscript editing.

Ref II – Participated in construction of plasmids and strains, performing *in vitro* experiments and contributed to the writing and editing of the manuscript.

Ref III – Constructed plasmids and strains, performed most of the experiments and wrote the manuscript.

ABBREVIATIONS

Å	ångström (SI-unit equal to 10^{-10} m)
Bap	biofilm-associated protein
c-di-GMP	cyclic di-GMP
EPS	exopolysaccharides
Fis	<u>f</u>actor for <u>i</u>nversion <u>s</u>timulation
IPTG	isopropyl β-D-1-thiogalactopyranoside
LapA	large adhesion protein A
LapF	large adhesion protein F
LB	lysogeny broth
LPS	lipopolysaccharides
LTA	lipoteichoic acids
RNAP	RNA polymerase
WT	wild-type

INTRODUCTION

Bacteria have developed several mechanisms to cope with the changing conditions of the surrounding environment. The charge and other characteristics of cell surface can passively help to deal with unfavourable conditions or can be involved in the switch between two different life-forms: free-swimming aka planktonic lifestyle and surface-attached aka biofilm aka sessile lifestyle. The transition from planktonic to sessile happens when bacteria attach to the surface and/or each-other. In this process, the adhesion abilities to different surfaces play an important role in bacterial survival. In most cases, the adhesion properties of bacteria are defined by cell surface hydrophobicity. The hydrophobicity of cell surface may also play a protective role for bacteria. For example, the surface of *P. putida* becomes more hydrophobic in the presence of different kind of environmental stressors including toxic organic solvents (Heipieper *et al.* 2007). In this way the cells increase the aggregation with each-other, thus minimizing accessibility of their surface to toxic compounds. Cell surface hydrophobicity can be affected by several factors including lipopolysaccharides (LPS), lipoteichoic acids (LTA), S-layer proteins and adhesins. In gram-negative bacteria these components are mostly located on the outer membrane, which is the first barrier of bacteria to its surrounding environment. The components and structure of outer membrane can be reorganised in response to environmental factors. For enabling all these changes, bacteria must rearrange its physiology by altering a large number of genes. That kind of regulation is often executed by global transcription regulators. In this thesis, I will focus on the global transcription regulator Fis' (factor for inversion stimulation) role in affecting the transcription of biofilm-related gene *lapF* and influencing the hydrophobicity of soil bacterium *Pseudomonas putida*.

P. putida is a cosmopolitan bacterium able to colonize the rhizosphere of plant roots and dislodge the pathogenic microbes also inhabiting the same environment. The overexpression of *fis* is shown to increase *P. putida* biofilm formation and therefore it may be an important factor for plant root colonization (Jakovleva *et al.* 2012).

The initial aim of this study was to determine the factors through which Fis affects the biofilm of *P. putida*. After finding the regulative connection between Fis and the biofilm-related adhesins LapA and LapF, the aim of this thesis further focused on LapF – the second largest adhesin of *P. putida*. The role of LapF in *P. putida*'s biofilm formation has been briefly studied before. The involvement of LapF in cell-cell interaction in mature biofilm has been shown previously (Martinez-Gil *et al.* 2010), although, we did not see any effect of LapF in the Fis-induced biofilm of *P. putida*. As cells growing in a biofilm are shown to be more hydrophobic, we decided to investigate the possibility of LapF being involved in the cell surface hydrophobicity of *P. putida* and further study the role of Fis in the regulation of this process. Thus the aim of this study was driven by the initial finding of the connection between Fis and LapF, which

surprisingly did not involve biofilm formation by *P. putida*. Therefore the intriguing questions were raised: how Fis regulates *lapF*, could LapF be engaged in the surface hydrophobicity and if yes, then what would be the purpose of it regarding the survival and adaptation of soil bacterium *P. putida*?

In the first part of this thesis, I give an overview of the current knowledge about the bacterial response to environmental changes, cell surface hydrophobicity and its factors, biofilm formation, including the role of adhesins LapA and LapF in it and transcription regulator Fis. The experimental part of the thesis focuses on the characterization of the mechanisms involved in the regulation of hydrophobicity of *P. putida* by Fis via regulating the transcription of adhesion protein LapF. Additionally, I will propouse a model of biofilm formation, and hydrophobicity regulation systems in *P. putida* in light of my experimental results and previously published data.

REVIEW OF LITERATURE

1. Bacterial adhesion

A critical approach for bacteria to adapt to the surrounding environment is to switch between planktonic and sessile lifestyles. Planktonic cells move freely; however, appropriate conditions can trigger attachment to abiotic or biotic surfaces, and bacteria switch over to sessile growth, also called biofilm. The variety of surfaces and environments that bacteria are able to occupy are almost infinite. The planktonic bacteria can adhere to metal, plastic, soil particles, medical implant materials or eukaryotic tissues (Costerton *et al.* 1999). The first colonists usually form a weak reversible attachment, which can become stronger and more permanent, when cells start to anchor themselves more and more on the surface and to one another, using surface proteins to bind other cells in a process called cell adhesion (Garrett *et al.* 2008).

The physicochemical interactions between bacteria and surface involve attractive forces which usually can be either electrostatic or chemical forces such as van der Waals bonds, hydrogen bonds or hydrophobic interactions. More closely the interactions between cell and its substrate depend on the attractive and repulsive forces between the ion layer of bacteria and the charge of the surface. Therefore, the attachment of a cell with a negative charge is stronger to a positively charged surface and *vice versa* (Montville and Schaffner 2003). Several bacteria possess large surface proteins with a net negative charge contributing to adhesion to different substrates, thereby initiating biofilm formation (Soni *et al.* 2008).

1.1. Importance of bacterial cell surface hydrophobicity

Cell surface hydrophobicity has been shown to affect the interaction to abiotic surfaces for species like *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus* (Yousefi Rad *et al.* 1998). Despite the fact that hydrophobicity regulates bacterial physiology only passively, its influence on the competitiveness of bacteria is remarkable. For example, the *P. putida* toluene tolerant strain IH-2000, which surface has a lower hydrophobicity than the surface of toluene-sensitive mutant, exhibits decreased consumption of aromatic compounds *p*-xylene and cyclohexane (Kobayashi *et al.* 1999). Furthermore, some gram-positive bacteria, like *Bacillus licheniformis* S86 decrease the level of their hydrophobicity in the presence of toxic organic compounds like 3-methyl-1-butanol and therefore show low affinity against this compound (Torres *et al.* 2011). Bacterial surface hydrophobicity can influence even the effectiveness of bacterial motility in the soil. For example, compared to hydrophilic strain LAM1 the *Pseudomonas fluorescens* hydrophobic strain LAM2 shows a much higher adherence and migration abilities in different types of soils: clay loam, sandy loam and sandy soil (van Loosdrecht *et al.* 1987,

Singh *et al.* 2002). On the contrary, the hydrophobic bacterial cell surface has been shown to conduce to cell aggregation in a mixed population habitating soil (Liu *et al.* 2009). This can facilitate degradation of aromatic compounds such as phenol, pyridine or its derivatives via different metabolic pathways (Adav *et al.* 2008). In 1987 van Loosdrecht *et al* found that cell surface hydrophobicity is the main dominant characteristic of bacteria that assure adhesion (van Loosdrecht *et al.* 1987). Meaning that the hydrophobicity of the cell surface and the ability to regulate it is an essential factor for bacterial adaptation to different environmental conditions (Segura *et al.* 1999, Heipieper *et al.* 2007).

The hydrophobicity of cell surface may play a protective role for bacteria. For example, in response to different kind of environmental stressors including osmotic stress, heat shock, and solvents, the bacterial surface becomes more hydrophobic (Heipieper *et al.* 2007). In gram-negative bacteria the stress causing agents usually strike the outer membrane first. Therefore it is logical that surface changes are involved in stress response.

One of the mechanisms related to the changes in the surface of gram-negative bacteria is the formation of outer membrane vesicles (Baumgarten *et al.* 2012). *P. putida* strain DOT-T1E has been shown to release membrane vesicles within 10 minutes after encountering different stressors: toxic concentrations of long-chain alcohols, EDTA, NaCl and heat shock (Neumann *et al.* 2006, Heipieper *et al.* 2007, Baumgarten *et al.* 2012). The vesicles are often associated with the release of virulence factors of *P. aeruginosa*, which help the bacteria to survive in human lung epithelium (Kulp and Kuehn 2010). Another function of membrane vesicles occurs in the cell aggregation, where it helps bacteria to become more tolerant against antibiotics and biocides (Beveridge *et al.* 1997). The stress-induced release of membrane vesicles and increase of cell surface hydrophobicity consequently enhance the aggregation of bacteria (Neumann *et al.* 2006, Heipieper *et al.* 2007, Baumgarten *et al.* 2012). A potential mechanism or phenomenon for this is called depletion attraction, which means the aggregation of large particles (bacteria) increases the free movement of small particles (Dorken *et al.* 2012). The particles in a colloid cannot approach the surface of other particles no more than the distance of their own radius. Therefore every particle is surrounded by a so-called depletion zone and the free movement of particles reduces when the number of particles increases in the medium (Dorken *et al.* 2012). Thus, if two large particles get closer to each other, their depletion zones overlap, creating more space for smaller particles in a medium. In other words, the entropy of the smaller particles in a system increases and therefore the aggregation of hydrophobic bacteria is favored (Dorken *et al.* 2012).

To sum up, the hydrophobicity of cell surface plays an important role for microorganisms in adhesion and attachment to various surfaces, in biofilm formation as well as in tolerance against toxic organic compounds.

1.2. Bacterial surface hydrophobicity factors

Cell surface is the first barrier of bacteria. For example, the cell surface of all gram-negative bacteria consists of an outer membrane that can quickly react to environmental conditions and reorganise the structure of membrane components. Lipopolysaccharides (LPS), adhesins, lipoteichoic acids, S-layer proteins and other proteins are located on the outer membrane of cells and therefore are the first contact elements between bacteria and its surrounding environment. Surface hydrophobicity of cells is influenced by various factors. Their contribution and properties to hydrophobicity can also differ.

One of the main factors influencing bacterial surface hydrophobicity is lipopolysaccharides (LPS) on the outer membrane of gram-negative bacteria (Fig 1). LPS consist of hydrophobic fatty acid chain harbouring lipid A – a glucosamine disaccharide, which differs from a typical phospholipid by possessing six unsaturated fatty acid chains instead of two saturated or unsaturated chains. LPS also comprises of a heterogeneous core oligosaccharide linked to lipid A and the O-antigen, which is an immunogenic oligosaccharide consisting of 1-40 repeating subunits (Fig 1). These are the features that make the asymmetric outer membrane bilayer more hydrophobic compared to a typical phospholipid bilayer, by having strong lateral connections between LPS molecules and low fluidity (Neidhardt and Curtiss 1996). In *Legionella pneumophila*, the O-antigen of LPS forms a homopolymer lacking free hydroxyl-groups, making the surface more hydrophobic and therefore can assist the attachment of bacteria to target cells (Zahringer *et al.* 1995).
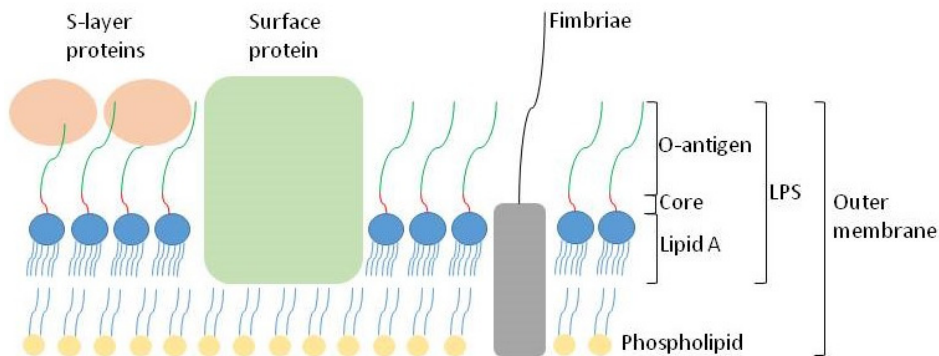
Figure 1. The localization of hydrophobicity factors (LPS, fimbriae, surface proteins and S-layer proteins) in the outer membrane of gram-negative bacteria.

In addition, lipid A also plays an important, yet indirect part in providing hydrophobic barrier to bacterial cells. For example, the repression of lipid A biosynthesis reduces the production of lipopolysaccharides, which therefore is shown to increase the permeability of hydrophobic antibiotics and can lead to

gram-negative bacteria becoming susceptible to them (Wyckoff *et al.* 1998). Consequently the role of LPS in cell surface hydrophobicity occurs mainly through changing the overall barrier of cell surface, which hinders the movement of different compounds between cell and its environment.

Similarly to LPS, S-layer proteins can also influence the hydrophobicity by contributing to the general surface hydrophobicity and protective barrier of the cells (Fig 1). Similarly to gram-negative bacteria most members of gram-positive *Bacillaceae* family are also covered with S-layer, which form a porous crystalline layer containing high amounts of hydrophobic amino acids (U. B. Sleytr *et al.* 1993). S-layers can function as protective coats or structural elements promoting adhesion and target surface recognition (Uwe B. Sleytr *et al.* 1994). Furthermore, the S-layer proteins have been reported to be involved in the surface hydrophobicity of *Lactobacillus acidophilus* ATCC 4356 (Smit *et al.* 2001) or *Bacillus cereus* (Kotiranta *et al.* 1998). Studies have shown that the changes in S-layer influence the surface hydrophobicity of *B. cereus*, which protect bacteria against the ingestion of polymorphonuclear leukocytes (Kotiranta *et al.* 1998).

Another factor demonstrated to be involved in general surface hydrophobicity and protection of bacterial cells is mycolic acids (MA). The cell envelope of gram-positive *Rhodococcus opacus* is described to possess long chain α-alkyl-β-hydroxy fatty acids, also known as mycolic acids (Asselineau and Lederer 1950). Mycolic acids, forming an outer lipid layer of the cell wall, are one of the main factors to provide tolerance for *R. opacus* and *Mycobacterium tuberculosis* against antibiotics and other stressful conditions (Alvarez *et al.* 2004, Barkan *et al.* 2009). Recently it was demonstrated that if grown in liquid medium and treated with toxic concentrations of NaCl or 4-chlorophenol the cells of *R. opacus* PWD4 respond by decreasing the average chain length of mycolic acids and the average amount of double-bonds in them. This was shown to be in correlation with higher saturation of membrane fatty acids and increased hydrophobicity of cell surface of *R. opacus* PWD4 (de Carvalho *et al.* 2016).

In addition to the factors influencing the general cell surface hydrophobicity, other factors, such as hydrophobic fimbrial adhesins can also affect the attathment of bacteria to surfaces (Fig 1) (Zahringer *et al.* 1995, Sidhu and Olsen 1997, Higashi *et al.* 1998). For instance, enteric bacteria use fimbriae for adhesion to host cells via interaction of fimbrial lectin to carbohydrate of host cells (Isberg and Barnes 2002). This interaction can be specific however adhesion can be reduced by electrostatic repulsion that exists between the host cell and the surface of bacteria. These fimbriae contain a high number of hydrophobic amino acids that probably help overcome the initial electrostatic repulsion barrier (Corpe 1980, Rosenberg and Kjelleberg 1986).

In common with fimbriae, several proteins have been shown to be connected with cell surface hydrophobicity which helps mediate the adhesion to surfaces. For example, a yeast protein, CSH1p was the first described hydrophobic protein on the surface of *Candida albicans*. The hydrophobicity of cells lacking *csh*

gene reduces 75% along with the adhesion to the extracellular matrix protein fibronectin (Singleton *et al.* 2005). However, it is suggested that CSH1p has a pleiotropic nature and its pathogenic contribution (e.g., adhesion to fibronectin) and lower cell surface hydrophobicity are independent of one-another (Singleton *et al.* 2005).

In *C. albicans* another hydrophobicity-related adhesin is Als3 – agglutinin-like sequence (ALS) family protein which contributes to yeast aggregation and adhesion to epithelium (Dranginis *et al.* 2007, Aoki *et al.* 2012). The enhanced exposure of Als3 on the surface of *C. albicans* provides hydrophobic hyphae and causes stronger adhesion to the hydrophobic substrate (Beaussart *et al.* 2012).

One more cell wall protein of yeast – Rbt1 is described to affect the adhesion of the cells via hydrophobicity. The N-terminal region of Rbt1 in *C. albicans* is demonstrated to be the key-factor for promoting the hydrophobicity of cell surface and therefore the adherence to polystyrene (Monniot *et al.* 2013). It is also demonstrated that in the central part of the protein, a domain consisting of 42 amino acids is responsible for the cell-cell interactions by promoting the aggregation of hyphea (Monniot *et al.* 2013). Furthermore, while Rbt1 protein shows similar anchorage in the cell wall of both cell types as yeast and as hyphae, then Rbt1 proteins are more exposed to the surface and unmasked in hyphae. This means that significant changes in the structure of cell walls, which could occur in the fimbriae layer, might allow to cover the surface proteins in yeast cells and expose in hyphae cells (Monniot *et al.* 2013). In 1997 Braun and Johnson characterized a gene, *TUP1*, which deletion caused *C. albicans* cells to grow in a filamentous state in the absence of any environmental inducing signal (Braun and Johnson 1997). A few years later it was demonstrated that *TUP1* represses the expression of hyphal-specific *rbt* gene family, including the cell-wall protein Rbt1 (Braun *et al.* 2000). Although these given examples of proteins involved in hydrophobicity and adhesion illustrate the mechanisms studied in yeast *C. albicans*, which cell wall structure is different from that of bacteria. Nevertheless, these examples enlighten the importance of hydrophobicity in microorganisms in general and explain the factors involved in this. However, in bacteria, there are also several proteins described to be involved in adhesion and hydrophobic interactions.

It was only recently demonstrated that *Helicobacter pylori* protein Hsp60 can function as a chaperone in acidic conditions, whereas the interactions between Hsp60 and other proteins undergoing acid-induced denaturation are occurring due to hydrophobic surfaces (Mendoza *et al.* 2017). A hydrophobic reporter probe 1,1`-bis(4-anilino) naphthalene-5,5`-disulfonic acid (bisANS) was used, which is non-fluorescent in an aqueous environment, but becomes fluorescent in contact with proteins having hydrophobic surfaces (Hawe *et al.* 2008). Measurements with bisANS showed high fluorescence in contact with Hsp60, demonstrating the hydrophobic surface of Hsp60. Furthermore, the fluorescence of bisANS was found to increase even more in moderately lower pH conditions (Mendoza *et al.* 2017). With this ability Hsp60, functioning as a

chaperone could protect other proteins from acidic environment, like in gastric epithelium of humans, which *H. pylori* colonizes.

The first large bacterial adhesion protein Bap (biofilm-associated protein) was described in *Staphylococcus aureus* (Cucarella *et al.* 2001). Disrupting the *S. aureus bap* gene decreases the surface hydrophobicity and subsequently reduces both, initial attachment to polystyrene and diminishes intercellular adhesion (Cucarella *et al.* 2001). *Pseudomonas fluorescens* and *Pseudomonas putida* have similar large adhesins: LapA in both species and LapF in *P. putida* (Hinsa *et al.* 2003). These large adhesins share common structural and characteristic features: high molecular weight, tandem repeats, cell-surface location and have relevant role in bacterial adhesion and biofilm formation (Lasa and Penades 2006).

P. putida utilizes these large extracellular adhesins, LapA and LapF, for attachment to abiotic and biotic surfaces (Yousef and Espinosa-Urgel 2007). LapF consists of 6310 amino acids and has only one large repeat domain, comprising of 64 repeating sequences, each of them with a length of 83-91 amino acids (Martinez-Gil *et al.* 2010). Whereas *P. putida* LapA consists of 8682 amino acids with two repeat domains, including 37 repeated hydrophobic sequences, each of comprising 100 amino acids. The accumulation of LapA protein on the cell-surface interface leads to the adhesion of bacteria to hydrophobic substrate (Espinosa-Urgel *et al.* 2000, Hinsa *et al.* 2003, Fuqua 2010, El-Kirat-Chatel *et al.* 2014). Therefore, it is suggested that LapA plays a crucial part in attachment and thus, is mostly responsible for biofilm initiation (Martinez-Gil *et al.* 2010). Whereas LapF is produced later, therefore, provides cell-cell interactions that lead to the formation of a mature biofilm (Martinez-Gil *et al.* 2010).

To conclude, the factors influencing cell surface hydrophobicity can act via providing the general hydrophobic barrier to the cells mediated by LPS and S-layer proteins. Hydrophobicity factors can also act via adhesion and attachment of cells to the surface and each-other, in which adhesive fimbriae and different membrane proteins having hydrophobic characteristics are shown to be involved.

1.3. The regulation of cell surface hydrophobicity

Bacteria can quickly change cell surface properties by physiologically regulated mechanisms. However, the control of so-called hydrophobicity factors' expression is one potential regulation mechanism as well, although much slower compared to physiologically regulated mechanisms.

One of the physiological regulation methods for bacteria to change their surface hydrophobicity is to alter the molecular weight of the LPS layer. Baumgarten *et al.* in 2012 analysed the LPS composition of *P. putida* DOT-T1E in response to *n*-alkanols, which increase the cell surface hydrophobicity of DOT-T1E (Baumgarten *et al.* 2012). They used micellar electrokinetic chromato-

graphy (MEKC) to analyse the composition of LPS and found that decrease of LPS' molecular mass correlates to the increase of hydrophobicity and vesiculation. Similarly to *P. putida*, the loss of high-molecular-weight LPS has been linked to the formation of outer membrane vesicles in *P. aeruginosa* (Kadurugamuwa and Beveridge 1995, Makin and Beveridge 1996). The change of cell surface's hydrophobicity has been shown to be reversible. When *P. putida* DOT-T1E was treated with 1-octanol, the increase of cell surface hydrophobicity was quick. But the surface hydrophobicity started to gradually decrease when the cells were pelleted, washed and resuspended in fresh medium without 1-octanol (Baumgarten *et al.* 2012). It was proposed that the decrease in hydrophobicity is slow due to *de novo* synthesis of high molecular weight LPS.

In addition to physiological adjustment of LPS, the regulation can occur by alternative sigma factors controlling the expression of hydrophobicity-related genes. In 2014 Klein *et al* demonstrated that the lack of *yciM* (*lapB*) increases the envelope stress response of *E. coli*, which control the main steps of LPS biosynthesis and assembly (Klein *et al.* 2014). LapB is a lipopolysaccharide assembly protein B encoded by *lapB* gene, formerly known as *yciM* (Klein *et al.* 2014). The defects caused by lack of the LapB have been shown to be suppressed by alternative sigma factor RpoE and two-component system CpxRA, which contribute to envelope stress response (Klein *et al.* 2014). The heat-shock inducible alternative sigma factor σ^E (RpoE) and two-component signal transduction system CpxRA have both been demonstrated to be adjusted to extracytoplasmic stimuli (Mecsas *et al.* 1993, Snyder *et al.* 1995). For example, the level of proteins on the outer membrane influences the activity of RpoE (Mecsas *et al.* 1993). In *E. coli* the lack of *rpoE* can cause defects in outer membrane permeability, which has been linked to increased sensitivity to detergents and hydrophobic substances (Raina *et al.* 1995). One of the transcription targets for RpoE is *degP*, which product is a periplasmic protease specified for the degradation of various atypical extracytoplasmic polypeptides (Strauch and Beckwith 1988). The *degP* locus is also activated by the response regulator CpxR of the two-component system CpxRA (Raina *et al.* 1995). CpxR is activated by the inner-membrane sensor CpxA which in turn gets stimulated by the overproduction of NlpE – an outer-membrane located lipoprotein in *E. coli (Snyder et al. 1995)*. These examples suggest that both sigma-factor RpoE and two-component system CpxRA play a central role in envelope stress response, which could be the key factor in covering the defects of decreased hydrophobicity caused by the lack of LPS in the cell surface of bacteria.

Another example of hydrophobicity regulation involving alternative sigma factor is related to the expression control of large adhesion protein Bap, which is involved in adhesion, biofilm formation and virulence of *S. aureus* (Tormo *et al.* 2005, Tormo *et al.* 2007). Bap is shown to be positively regulated by SarA (Tormo *et al.* 2005, Tormo *et al.* 2007). SarA is a DNA-binding protein which generally activates the *agr* promoters, which produce Agr proteins. Agr in turn controls the expression of several virulence factors in response to increasing cell density (Novick 2003). Though, SarA is also able to activate some virulence

genes independently from *agr* (Novick 2003). For example, SarA binds the *bap* promoter independently from *agr*, therefore SarA is a positive regulator for Bap-mediated biofilm of *S. aureus* (Tormo *et al.* 2005, Tormo *et al.* 2007). One of the two alternative sigma factors described in *S. aureus* – σ^B affects the expression of multiple genes, including virulence genes (Horsburgh *et al.* 2002). It has been demonstrated that the lack of σ^B impairs the Bap-mediated biofilm (Marti *et al.* 2010). The extracellular proteases Aur and SspA, which are capable of degrading Bap, have been shown to be overexpressed in σ^B mutant, thus increasing the degradation of Bap protein and reducing protein-dependent biofilm formation of *S. aureus* (Marti *et al.* 2010). In the same work it was also demonstrated that in the σ^B gene and *agr* double-mutant, where the extracellular proteases are not being overexpressed, the biofilm formation is restored. This suggests that in *S. aureus* a protein-mediated biofilm could be controlled by a pathway, where global transcription regulators play a central role via regulating the expression of extracellular proteases (Marti *et al.* 2010).

Global transcription regulators have also been shown to be involved in the regulation of lipoteichoic acid (LTA)-related hydrophobicity in gram-positive bacteria. In addition, affecting hydrophobicity of *Streptococcus pyogenes,* LTA also helps to mediate the adhesion to the host cells (Hasty *et al.* 1992). The LTA of *S. pyogenes* forms complexes with surface proteins in a way where fatty acids of LTA can be exposed to the surface of bacteria (Ofek *et al.* 1983). The LTA complex with surface proteins is therefore responsible for mediating or inhibiting the adhesion of *S. pyogenes* to host cell receptors. A group of proteins of *S. pyogenes* called M-protein family has been demonstrated to be involved in anchoring LTA to the surface of the cells resulting in a more hydrophobic surface and enhanced biofilm formation (Courtney *et al.* 2009). M-proteins are in turn regulated by a global transcription regulator Mga (Caparon and Scott 1987). Specific binding of Mga to the promoter regions of genes encoding for M-proteins helps to strengthen the binding of RNA polymerase and therefore increases the transcription (McIver *et al.* 1995). The 45-bp Mga binding consensus (N_5 AGGTCAA(C)AAAGNT N_4 AA N_5 AAAAANCTGG(A)T(C)CTTTA) comprises of two highly conserved regions called C_L and C_R separated by an 11-bp sequence of more variable nucleotides with conserved adenine-pair in the middle (McIver *et al.* 1995). The C_R region of Mga binding site overlaps the -35 sequence of promoters, thus showing this region as a binding site for both Mga and RNA polymerase. Furthermore, *mga* promoter itself is positively auto-regulated by Mga (Okada *et al.* 1993). In a serotype M6 of Group A streptococcus strain JRS4 it is shown that the expression of *mga* and the genes under its control *emm* and *scpA* are active in logarithmic phase but are turned off upon entry into stationary growth phase (McIver and Scott 1997). One possible advantage for expressing these genes only in exponential phase is to save energy in starvation conditions, as proposed by the authors (McIver and Scott 1997). The products of *emm* and *scpA* genes could be very stable on the surface of Group A streptococcus, and the sufficient amount of these proteins may be

enough for the time when bacteria reach to stationary growth phase, and the further transcription may not be needed (McIver and Scott 1997).

Previous examples of the regulation of hydrophobicity factors included mostly the control on transcription level. However, the regulation of hydrophobicity factor's expression can also happen via modification of translational efficiency. For example, the translation of *slpA* mRNA encoding S-protein in *Lactobacillus acidophilus* ATCC 4356 depends on the secondary structure of 5`end of mRNA, which exposes the ribosome binding site (Boot *et al.* 1996). As shown by reporter gene analysis in *Lactobacillus casei*, the cutting of the leader sequence of mRNA leads to decrease in SlpA production (Boot *et al.* 1996). This leader sequence in 5' end of mRNA is untranslated; however, it is necessary to ensure the half-life of mRNA (Boot *et al.* 1996).

In general, the regulation of cell surface hydrophobicity could occur via quick physiological changes or through different gene expression pathways, involving alternative sigma factors, two-component signal transduction systems or global transcription regulators. All these complex regulation cascades depend on the environmental conditions and signals received by bacterial cells, which respond to it by activating or repressing the right genes at the right time.

2. Bacterial biofilm

Biofilm is a phenotype of bacterial population that has several functions. Bacteria can protect themselves from rough environment by forming biofilm or on the other hand the formation of biofilm could help them to settle down where conditions are better facilitating their existing as a community (Jefferson 2004). In biofilm bacteria can withstand hazardous abiotic factors like UV (Espeland and Wetzel 2001), high osmotic stress, dryness (Le Magrex-Debar *et al.* 2000), antibiotics and antimicrobial agents (Stewart and Costerton 2001) much better compared to planktonic cells. The matrix produced in biofilm acts as a barrier between bacteria and environment, which helps bacteria to tolerate stress better (Donlan and Costerton 2002).

On the other hand, biofilm formation does not occur only in response to stress conditions. An example would be bacterial biofilm on the roots of plants. In the rhizosphere, an ecological niche is formed, where nutrients are more freely available, and microorganisms have developed specific mechanisms to exploit this niche. Plant roots excrete a variety of components, such as amino acids, organic acids, simple sugars, carbohydrates and enzymes (Lynch and Whipps 1990). These exudates are released mainly through the tips of the roots, which are constantly growing. Therefore to take advantage of this nutritional paradise, in contrast to biofilm formation, microorganisms must be able to migrate along with the growth of roots. This means that for successful adaptation to different environmental conditions, bacteria have to be able to rapidly change their lifestyle and disperse from biofilm to recolonize new locations. For example, in *P. putida* it has been demonstrated that (among other factors)

nutrient deprivation, more specifically carbon starvation causes biofilm to disperse (Gjermansen *et al.* 2005).

In nature biofilm usually consist of bacteria from multiple species, which compete, cooperate and communicate with each other (Amann *et al.* 1995). For instance, the fermentative bacteria produce different acids and alcohols. These are in turn used as substrates by acetogenic bacteria, and after that methanogenic microorganisms can get energy by converting carbon dioxide, acetate, and hydrogen into methane (Davey and O'Toole G 2000). Another example of biofilm formation unrelated to stress has been demonstrated in *E. coli*, where bacteria can form biofilm in response to an increase in cell density through quorum sensing. A signal autoinducer 2 (AI-2) enhances the initial stages of biofilm formation in *E. coli* via stimulating the motility-related genes *motA* and *fliA* (Gonzalez Barrios *et al.* 2006). Therefore AI-2 increases the initial attachment of *E. coli* cells to the surface and thus improves the first steps needed for biofilm formation (L. A. Pratt and Kolter 1998).

In natural biofilms, microorganisms also compete with each-other for nutrients and try to inhibit the growth of other species in the community. For example, *Streptococcus oligofermentans* produces hydrogen peroxide (H_2O_2) from peptone by using L-amino acid oxidase to inhibit the growth of *Streptococcus mutans* in multi-species biofilms growing in the peptone-rich environment (Tong *et al.* 2008). Marine bacterium *Pseudoalteromonas tunicate* is shown to produce protein AlpP which has antibacterial properties and therefore living in the same environment can repress the growth of another marine bacterium such as *Cellulophaga fucicola* and *Alteromonas* sp (Rao *et al.* 2005).

The human society has learned to benefit from the bacterial biofilm. For example, microorganisms are used for bioremediation to remove contaminants like oil spills (Radwan *et al.* 2002), nitrogen compounds (Li *et al.* 2003) or industrial waste (Sekoulov and Brinke-Seiferth 1999). However, the formation of biofilms can also cause problems for humans. Hospital-related infections are one of the examples. Surgical instruments, like drips, scalpels, and catheters, are common sources of biofilm growth and subsequent infection. Biofilm forming methicillin-resistant *Staphylococcus aureus* (MRSA) is particularly important due to its resistance to multiple antibiotics, whereas frequent sources of MRSA are the patients themselves (Godwin *et al.* 2001, Salgado *et al.* 2003). Another critical area is the oil industry, where biofilm formation by sulphate-reducing bacteria (SRB) causes pipe and rig corrosion, blockage of filtration systems and oil spoilage (Santegoeds *et al.* 1998).

3. The regulation of biofilm-related adhesins LapA and LapF

Probably due to their different functions in biofilm, the synthesis of LapA and LapF are regulated differently (Fuqua 2010). The expression of *lapF* is controlled by stationary phase sigma RpoS in *P. putida* (Martinez-Gil *et al.*

2010), but the regulation of *lapA* expression seems to be more complicated. In *P. putida* the transcription of *lapA* is shown to be under control of 6 promoters, whereas only 3 of them display a moderate RpoS-dependence (Ainelo *et al.* 2017). Moreover, Fis activates the transcription of *lapA* by binding to 2 diffe-rent sites identified in the promoter region of *lapA* (Ainelo *et al.* 2017). In *P. putida* the two-component signal transduction system GacS/GacA seems to be involved in biofilm formation, influencing the expression of both – LapA and LapF (Fig 2) (Martinez-Gil *et al.* 2014). As shown in *P. aeruginosa*, when *gacS* is mutated, the detachment from biofilm increases (Petrova and Sauer 2009).

Initial attachment   Mature biofilm
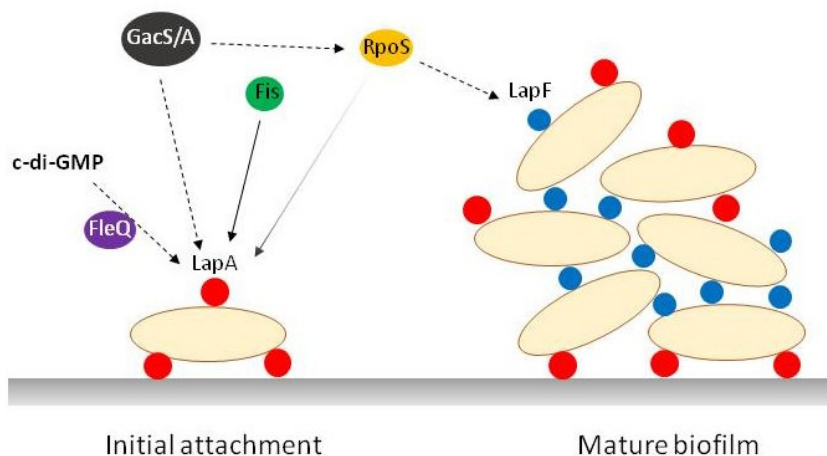
Figure 2. A potential model for a regulatory network of *lapA* and *lapF* transcription in the biofilm formation process of *P. putida*. Large ellipses demonstrate bacterial cells, red and blue circles on the surface of cells indicate LapA and LapF respectively. Direct positive effects are indicated with solid lines, dotted lines demonstrate activation of transcription without any indication of specific binding sites in front of genes. Gradient line represents partial regulation of *lapA* by RpoS, which means that only 3 out of 6 *lapA* promoters seem to be RpoS-dependent.

Part of the GacS/A signal transduction pathway is small RNA-binding trans-criptional regulators. In *P. putida* these small regulators, like RsmA and RsmE repress diguanylate cyclase *cfcR* translation through regulation of *rpoS* expres-sion (Huertas-Rosales *et al.* 2017). Furthermore, CfcR seems to be the key generator of the free pool of c-di-GMP in stationary phase *P. putida* and also a positive regulator for biofilm when Rsm proteins are absent (Huertas-Rosales *et al.* 2017). The secondary messenger c-di-GMP affects the expression of adhe-sine genes *lapA* and *lapF* in opposite ways. In response to artificially increased c-di-GMP, the transcription from *lapA* promoter increases (Martinez-Gil *et al.* 2014). On the other hand, the expression of *lapA* through c-di-GMP seems to require a flagellar regulator FleQ (Fig 2). In *P. aeruginosa* FleQ has been demonstrated to be involved in the expression of biofilm-related components,

like exopolysaccharides (Baraquet *et al.* 2012). C-di-GMP represses the ATPase activity of FleQ, therefore causing a down-regulation of flagellar gene expression. In contrast, the absence of c-di-GMP influences FleQ to activate Pel polysaccharide expression in *P. aeruginosa* (Hickman and Harwood 2008, Baraquet *et al.* 2012). In *P. fluorescens* F113, the swimming motility is shown to be regulated by GacS/GacA via controlling FleQ (Navazo *et al.* 2009). Furthermore, using β-galactosidase assay and RT-PCR Martinez-Gil *et al* in 2014 found that the GacS/A two-component system is modulating the expression of RpoS and is therefore indirectly involved in the regulation of *lapF* expression.

The expression of *lapF* is negatively regulated in early stages of biofilm of the *P. putida* strain, which overproduces c-di-GMP. This indicates that LapF is not needed in initial steps of biofilm formation (Fig 2). It is previously shown, that the lack of either adhesin LapA or LapF results in higher expression of exopolysaccharides, hinting that there might be some internal mechanism for balancing the biofilm structural components (Martinez-Gil *et al.* 2013). So far, the assessment of *lapF* transcription using β-galactosidase assay has demonstrated, that LapF is extensively expressed in the stationary phase but not in exponentially growing cells (Martinez-Gil *et al.* 2010, Martinez-Gil *et al.* 2014).

The expression of LapF and exposition to the surface of bacteria are suggested to be separate from LapA. LapF transportation to the cell surface is probably mediated by ABC-type transporter encoded by the other genes of the operon LapHIJ. However, there is no direct evidence that LapHIJ are involved in the transport of LapF (Fuqua 2010, Martinez-Gil *et al.* 2010).

LapF is important for *P. putida* to colonize plant roots. The *lapF*-mutants are unable to infect the roots properly: low infection ability appears both alone and in collaboration with the wild-type strain. Microscopy analysis shows that although *lapF* mutants are unable to form biofilm on plant roots alone, they are still viable in biofilm with the wild-type strain. This implies for the necessity of LapF in cell-cell interactions, as the LapF-nonproducing mutants can at least benefit from LapF expressed by wild-type strain (Martinez-Gil *et al.* 2010).


4. Global transcription regulator Fis

Fis (factor for inversion stimulation) is a small homodimeric protein found in both gammaproteobacteria families *Enterobacteriaceae* and *Pseudomonadaceae* (Beach and Osuna 1998, Boswell *et al.* 2004). Fis is thoroughly studied in *E. coli*, but there is a lot less information about Fis in genus *Pseudomonas.* Fis potein is conserved between Enterobacteria and Pseudomonases (Azam and Ishihama 1999). The protein identity is 64.8 %, and the similarity is 81.7 % between Fis in *E. coli* K12 and *P. putida* KT2440 (protein alignment tool http://cmr.jcvi.org). Despite the similarity of the proteins, the DNA-binding properties and therefore also the target genes can still differ in different orga-

nisms (Osuna *et al.* 1995, Bradley *et al.* 2007). For example, *Salmonella enterica* serovar Typhimurium LT2 and *E. coli* K12, which often share a similar environment and possess similar regulatory mechanisms, have different Fis regulons for their virulence genes (Wang *et al.* 2013). It has been demonstrated that *E. coli* Fis alone is responsible for the regulation of 76 genes directly and 15 genes in collaboration with other transcriptional factors (Martinez-Antonio and Collado-Vides 2003). According to microarray analysis performed by Bradley *et al*, *E. coli* Fis regulates indirectly the expression of 231 genes altogether. These genes can be divided into 15 different categories by their function, including motility, stress, biosynthesis of nucleotides and amino acids, energy metabolism, nutrient transportation etc (Bradley *et al.* 2007). Furthermore, Fis dictates the transcription of the genes encoding for four transcription factors and sigma-factors σ^{70}, σ^{38} and σ^{32} (Martinez-Antonio and Collado-Vides 2003).

In *E. coli* Fis regulates its transcription both directly and indirectly. Fis protein binds to the promoter of *fis* gene, blocking the binding of RNA polymerase and thereby the transcription of itself (Ninnemann *et al.* 1992). There are six different Fis binding sites in the promoter region of *fis* and two of them overlap the binding site for RNA polymerase (Ball *et al.* 1992). The indirect regulation of Fis takes place through the change of DNA topology, increasing the negative supercoiling (Schneider *et al.* 2000). Also, the expression of *fis* has been shown to be regulated by another global transcription factor – IHF. In *E. coli* IHF binds to the promoter of *fis* and increases the transcription from it up to 4 times (T. S. Pratt *et al.* 1997). Furthermore, cAMP-receptor protein (CRP) also directly regulates the expression of *fis.* In 2001 Nasser *et al* demonstrated the involvement of CRP in the formation of nucleoprotein complexes that are able to activate or repress the transcription of *fis* (Nasser *et al.* 2001).

Fis can be both, the activator or repressor of transcription, depending on the binding position on the promoter region of target genes. Prokaryotic transcription factors are generally classified into class I and class II activators (Fig 3) (Smolke 2009). Class I activator`s binding sites are usually located more than -61 bp from the transcription start site, and they secure the transcription activation by binding to the RNA polymerase (RNAP) α-subunit`s C-terminus (Fig 3) (Smolke 2009). Class II activators tend to bind the DNA near the -35 element of promoters and strengthen the RNAP binding via the N-terminus of RNAP α-subunit (Fig 3) (Smolke 2009). Class I and class II activators can also co-activate the same promoters. For example in *E. coli proP* P2 promoter is activated by CRP, acting as a class I activator by binding to the position at -121.5 bp from the transcription start site, while Fis binds to -41.5 position, acting as a class II activator (McLeod *et al.* 2002). Some transcription activators can also bind DNA far away from the transcription start site, thus activating the transcription by bending the DNA (Browning *et al.* 2004). Transcription repression usually occurs via binding between the -10 and -35 elements of promoter region and therefore blocking the binding of RNAP (Fig 3) (Smolke 2009).
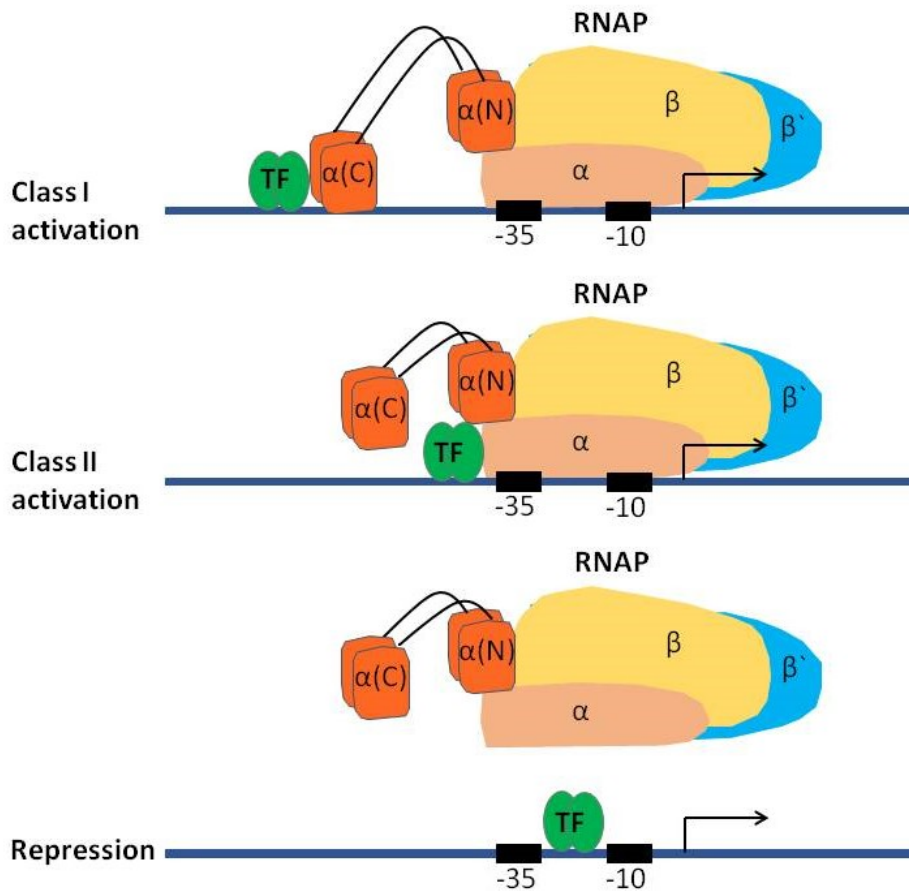
Figure 3. An illustration of class I and class II activation and repression mechanisms of transcription factors interacting with RNA polymerase (RNAP) inspired by Smolke *et al* 2009. Class I and II activation by TF occur via mediating RNAP to the right position and repression via TF happens by blocking the binding of RNAP. TF represents transcription factor, -10 and -35 mark the promoter elements, the arrow show the transcription start site and RNAP different subunits are α, β, β`, whereas α(C) and α(N) represent the C and N terminus of α-subunit.

Although Fis is important for bacteria in many processes, it does not seem to be essential for *Enterobacteriaceae* family members like *E. coli* or *S. enterica* serovar Typhimurium. Both bacteria have been shown to be viable and well growing after *fis* deletion, although the lag-phase of *fis* null-mutant may be prolonged (Johnson *et al.* 1988, Osuna *et al.* 1995, Bradley *et al.* 2007). Contrary to preceding members of *Enterobacteriaceae*, *fis* seems to be essential for the species from the genus *Pseudomonas*, as the cells cannot tolerate the lack of functional Fis protein (Liberati *et al.* 2006, Teras *et al.* 2009, Yeung *et al.* 2009). Liberati *et al* (2006) conducted an experiment, where they constructed a

non-redundant library of *P. aeruginosa* PA14 transposon mutants. This library consisted of nonessential PA14 genes with single transposon insertions chosen from an extensive library of transposon mutants. Insertions of 4468 genes were obtained, which make up 75 % of the genome of PA14. Only one mutant carried an insertion in the *fis* gene, and even this was located in a stop codon of the *fis* gene (Liberati *et al.* 2006). In 2009 Yeung *et al* used the very same transposon mutant in their experiments and demonstrated a significant reduction in growth rate in liquid swarming medium (Yeung *et al.* 2009). This suggests that *fis* is a potentially essential gene in *P. aeruginosa* PA14 (Liberati *et al.* 2006). Similarly, in our laboratory, the attempts for creating a *fis* knockout mutant in *P. putida* have not been successful (Teras *et al.* 2009).

The studies of expression regulation of *fis* in *E. coli* have shown, that the amount of Fis increases rapidly near to maximum of 50 000 molecules after inoculated with rich medium (Ball *et al.* 1992, Ali Azam *et al.* 1999). After growth in logarithmic phase for 90 minutes the amount of Fis starts to decrease till in stationary phase, there are less than 100 molecules of Fis in each cell (Ball *et al.* 1992, Ali Azam *et al.* 1999). Such rapid changes in protein level for bacteria could be important for growth phase-dependent regulation of different biological processes. The changes in the protein and mRNA level of *fis* appear similarly, but the changes in mRNA level start approximately 15 minutes earlier. Thus the most critical regulatory phase for *fis* expression in *E. coli* is transcription (Ninnemann *et al.* 1992).

4.1. DNA-binding properties of Fis

Fis is able to bind specific DNA by bending it up to 90° (Azam and Ishihama 1999). It has been showed that Fis participates in certain site-specific DNA recombination processes as well as suppressing DNA supercoiling in *E. coli*, which affects the under-winding of DNA strands and therefore indirectly the expression of genes. Fis can modulate the topology of DNA directly or indirectly via regulation of topoisomerase genes. For example, Fis binds the promoter of gyrase gene and represses the transcription from it (Travers *et al.* 2001).

In *E. coli* Fis, there are 4 tyrosine residues in positions 38, 51, 69 and 95, each of them is responsible for specific intra- and intermolecular interactions. Two of them – Tyr51 and Tyr95 are both participating in hydrogen bonding-salt bridge networking and therefore play a significant role in stabilization and flexibility of Fis protein (Boswell *et al.* 2004).

By the crystal structures of the Fis protein of *E. coli,* it is demonstrated, that Fis consists of 4 α-helixes and β-sheets, that form excrescent hairpin structures (Kostrewa *et al.* 1991, Hancock *et al.* 2013). The α-A helix in the N-terminus of the protein is essential for recombination and α-C, and α-D helixes in the C-terminus form a helix-turn-helix motif, needed for the binding with DNA (Yuan *et al.* 1991). Tyrosine residues responsible for recognizing specific DNA sequences are located in α-D helixes (Feldman-Cohen *et al.* 2006). In Fis-dimer

the distance between DNA-binding α-helixes is only 25 Å, which does not change in the binding process. Thus, Fis can only bind DNA by bending it (Kostrewa *et al.* 1991, Merickel *et al.* 2002).

The DNA-binding consensus of Fis in *E. coli* is GNTYAAAWTTTRANC, where Y = pyrimidine, R = purine, W = A or T and N = any nucleotide (Shao *et al.* 2008). The most critical nucleotides for high-affinity binding of Fis are G, A, T, and C in positions 1, 5, 11 and 15 respectively. Furthermore, the binding is shown to be stronger in the occurrence of A, and T nucleotide repeats in the middle of binding consensus and adjacent to it (Shao *et al.* 2008). In *E. coli* Fis, first of all, prefers to bind to DNA, which has very good similarity to consensus. When such sites are saturated with Fis, free Fis binds to less conserved DNA (Feldman-Cohen *et al.* 2006).

4.2. The role of Fis in regulating bacterial physiology

The whole process of adapting to different conditions in surrounding environment requires changes in bacterial physiology, which can be maintained by altering the expression of large number of genes (Ramos-Gonzalez *et al.* 2005, Matilla *et al.* 2007). Fis is a global regulator modulating the physiological state of bacteria by regulating the expression of multiple growth-related genes, chemotaxis, response to environmental changes, motility and biofilm (Ishihama 2009).

In *Enterobacteriaceae,* Fis is mainly known to be active in logarithmic phase. Therefore Fis has an opposite function to the stationary phase-specific sigma factor RpoS. In *E. coli* Fis controls the expression of rRNAs and tRNAs, which are needed in maximum quantities in fast-growing bacteria. On the other hand, the gene promoters needed for growth are inactivated in stationary phase, and RpoS upregulates the ones that are needed for more stressful conditions (Xu and Johnson 1995).

Fis can affect biofilm formation in both directions: activating or repressing it. In enteroaggregative *Escherichia coli* (EAEC) Fis has been shown to facilitate biofilm formation by regulating the biosynthesis of aggregative adherence fimbriae (AAF/II), which are important for EAEC to be able to form biofilm in the human intestinal mucosa. Fis regulates the transcription of 3 genes required for AAF/II fimbriae synthesis: *aggR* encoding for synthesis activator, *aafD* encoding for chaperon and *aafA* encoding for a subunit of fimbriae (Sheikh *et al.* 2001). The example of repression of biofilm by Fis is *Dickeya dadantii*. The main component of the biofilm matrix of *D. dadantii* is cellulose, produced by *bcs* operon, which transcription is repressed by Fis, therefore inhibiting the formation of biofilm (Prigent-Combaret *et al.* 2012). Fis also represses the synthesis of curli – adhesive fimbriae in enteropathogenic *E. coli* (EPEC) strain E2348/69. Fis inhibits the expression of the main subunit of curli and therefore reduces biofilm formation (Saldana *et al.* 2009). In *P. putida fis* overexpression has been shown to repress the motility of bacteria and enhance biofilm formation (Jakovleva *et al.* 2012, Ainelo *et al.* 2017).

THE AIM OF THE THESIS

This work was initiated by the finding that *fis* overexpression enhances biofilm formation in *P. putida* (Jakovleva *et al.* 2012). The colonization experiments on barley roots demonstrated that *fis*-overexpressing *P. putida* is unable to move on the barley roots, instead the bacteria stay in the region where they were at the beginning of the colonization (Jakovleva *et al.* 2012). Motility being the opposite phenotype of biofilm formation raised a question, is Fis affecting biofilm indirectly or directly via regulating the transcription of biofilm-related genes. To answer the question we decided to study how Fis is taking part of regulation of genes involved in biofilm formation. We found that the gene expression of biofilm-related adhesins LapA and LapF are controlled by Fis. Regarding the circumstance that biofilm-forming cells have more hydrophobic surface, it was only logical to investigate the possibility that LapA and LapF are involved in influencing the cell surface hydrophobicity of *P. putida*. The aim of this work was focused more on studying the Fis regulation mechanism of adhesin-coding gene *lapF*, because of a controversial finding that the absence of *lapF* had no effect on Fis-induced biofilm formation of *P. putida*. Therefore we wanted to elucidate the potential purpose of LapF in the adaptation and survival processes of *P. putida*.

RESULTS AND DISCUSSION

1. The lack of LapF has no effect on *P. putida* biofilm grown in LB medium (Ref I)

To identify the biofilm-related genes that could be affected by Fis, mutagenesis with transposon mini-Tn*5* was conducted with *P. putida fis*-overexpression strain F15. This strain had an extra gene copy of *fis* under the control of *tac* promoter induced by IPTG (Jakovleva *et al.* 2012). Transconjugants with recovered motility were selected as the cells with opposite phenotype to biofilm formation. The improved motility was studied on the King B medium with IPTG in comparison with F15. The localization analysis of 76 colonies with mini-Tn*5* insertions in F15 strain revealed 68 independent hits in the *lap* genes (Ref I). Among them, 56 independent insertions were found in *lapA*, the gene of biggest adhesin in *P. putida*. Whereas no insertions were found in the gene of the second extracellular adhesin – *lapF*. In general, this test-system with mini-Tn5 could not ascertain the biofilm related genes which are repressed by *fis*-overexpression, as gene is already repressed and insertion did not activate genes.

Subsequently, we focused our research on *lap* genes and measured the biofilm formation of these transposon mutants compared with wild-type strain PSm and *fis*-overexpression strain F15. The biofilm formation of three independent *lapA*-transposon mutants in F15 exhibited approximately 2-times weaker biofilm formation compared with original F15 (Ref I, Table 2). To further confirm the *lap*-related biofilm regulation by Fis, we constructed *lapA* as well as *lapF*-deletion mutants of PSm and F15 strains (Ref I, Table 1). We decided to further investigate the biofilm involvement of both biggest adhesins in Fis-over-expression conditions, despite no findings of insertions in the *lapF* gene with transposon mutagenesis. When the deletion of *lapA* reduced biofilm formation in all constructed PSm and F15 mutants compared to wild-type strains, then the elimination of *lapF* had no effect on *P. putida* biofilm formation in LB-medium compared to wild-type PSm (Ref I, Fig 4). Also, the biofilm of the *lapF*-deletion mutant in F15 increased similarly to original F15, which further confirmed that the lack of LapF has no effect in Fis-induced biofilm formation of *P. putida* in LB medium. The results of wild-type strain are in good accordance with previously published data that LapF-deficiency has a negative impact on *P. putida* biofilm formation only in glucose minimal medium but not in complex medium LB (Martinez-Gil *et al.* 2010). Although, it is proposed that LapF is playing a role in cell-cell interactions in mature biofilm (Martinez-Gil *et al.* 2010).

2. Overexpression of *fis* represses the expression of *lapF* (Ref I)

Previous works have shown that *lapF* mutant was less capable of both individual and competitive plant root colonization, thus LapF could be important in the mature biofilm formation and can also contribute to colonization ability (Martinez-Gil *et al.* 2010). Therefore, the expression of *lapF* was assessed as a potential gene that can be involved in adhesion without Fis regulation.

At first, the amounts of adhesines LapA and LapF were quantified with the background of *fis* overexpression in different growth phases of *P. putida*. The cells were grown in LB medium for 2.5h and 18h, and the proteins from cell lysates were separated and quantified using silver stained SDS-polyacrylamide gels. In fast-growing cells, the amount of LapA was not affected and LapF was not detectable at all in wild-type nor *fis*-overexpression strains (Ref I, Fig 5). This corresponds to previously published data, where transcription activity measurements showed no expression of *lapF* in logarithmic growth phase *P. putida* cells (Martinez-Gil *et al.* 2014). In 18-hours-grown cells, the overexpression of *fis* increased LapA 1.6 times, but surprisingly the amount of LapF was decreased approximately 4 times by *fis* overexpression compared to the wild-type cells. This clearly suggests that Fis affects the expression of both adhesins LapA and LapF in *P. putida*. Also, it could indicate, that the mature biofilm of *P. putida* is mainly affected by LapA and the dispersion of wild-type biofilm, without *fis* overexpression, may be due to decreased levels of LapA. However, the results indicating the regulation of *lapF* expression by Fis centered the focus of this thesis to LapF and to the ascertainment of specific control of *lapF*`s transcription by Fis and LapF's role in *P. putida*.

3. Fis binds to the promoter of *lapF* (Ref II)

RpoS-dependency of *lapF*"s transcription was previously published by Martinez-Gil and coworkers (Martinez-Gil *et al.* 2010), although, without the exact position of *lapF*"s promoter. Thus, at first, the location of *lapF*"s promoter was needed to be identified. Therefore, the assumption was to find a recognisable -10 element of the RpoS-dependent promoter upstream of *lapF* gene. We used RACE method to map the *lapF* mRNA 5`ends and found 120 bp upstream of *lapF* starting codon one mRNA 5` end. Six nucleotides upstream of the founded mRNA 5` end we identified a putative -10 promoter sequence (Ref II, Fig 1).

For determination of potential Fis binding sequence(s), we used *in silico* prediction of Fis binding sites on upstream (-500 bp) and downstream (+100 bp) region of the *lapF* gene. Surprisingly no Fis-binding sites were found on an upstream region of *lapF*, but one binding site Fis-F1 was predicted approximately 65 bp downstream of *lapF* starting codon (Ref II, Fig 1). Verification of predicted Fis-binding site was conducted *in vitro* by DNaseI footprint

analysis, which did not confirm the binding of Fis to the predicted Fis-F1 sequence (data not shown). Instead, this assay revealed another Fis-binding site Fis-F2, upstream of the *lapF* gene (Ref II, Fig 1). Attractively the localization of Fis-F2 overlapped a putative -10 promoter sequence, which confirms the hypothesis of the binding of Fis to the promoter of *lapF* and by counteracting the binding of RNA polymerase. Therefore, Fis could directly repress the transcription of *lapF*. Fis binding to the Fis-F2 site was further confirmed by mutating the most critical nucleotides for Fis binding in Fis-F2 sequence. With these mutations, Fis was not able to bind to the Fis-F2-mut sequence *in vitro* (Ref II, Fig 1), which confirms the binding of Fis to the promoter region of *lapF*.

4. The overexpression of *fis* represses the transcription of *lapF* (Ref II)

To elucidate the direct effect of Fis on the transcription of the *lapF* gene, the promoter region of *lapF* was cloned into a promoter probe test system and the activity of *lapF* promoter was measured as β-galactosidase activity *in vivo*, where wild-type (PSm) and *fis*-overexpressing F15 strains were grown into stationary phase. The promoter region of *lapF* containing native Fis-binding site Fis-F2 or mutated version Fis-F2mut was cloned in front of reporter gene *lacZ*. F15 cells harbouring the native Fis-F2 binding site showed a drastic decrease in β-galactosidase activity when *fis* was overexpressed by the addition of 1 mM IPTG (Ref II, Fig 3). When Fis-binding site Fis-F2 was mutated, the over-expression of *fis* did not decrease the β-galactosidase activity anymore, compared with F15 without IPTG (Ref II, Fig 3), indicating the essential nature of this binding sequence for Fis-mediated repression. However, the mutations in Fis-F2 increased the LacZ activity in general. One possible explanation for this could be the introduced mutations in Fis-F2-mut, which diminished Fis binding but also changed the promoter sequence enough to result in higher transcription of *lacZ* gene and therefore β-galactosidase activity. Furthermore the transcription regulation of *lapF* could be carried out mutually by Fis and RpoS. As the trigger of exponential growth, Fis could be the repressor of *lapF* in fast-growing cells and RpoS could be the activator of *lapF* in stationary phase cells. Although this brings up a question of the necessity to repress the expression of *lapF* in bacterial logarithmic growth phase.

5. LapF provides the hydrophobicity to the surface of P. putida cells in stationary growth phase (Ref III)

From our studies, LapF has no *fis*-overexpression related effect on *P. putida* biofilm formation in LB medium, yet Fis still significantly reduces the amount of LapF. Moreover, the adhesin LapA seems to be the primary factor for *P. putida* biofilm formation. While LapA is shown to be important in the early attachment of cells to different types of surfaces, then LapF is described to be more significant in a mature biofilm for cell-cell connections (Fuqua 2010, Martinez-Gil *et al.* 2010). Also, *P. putida* cell surface growing in a biofilm is more hydrophobic (Baumgarten *et al.* 2012, Montag *et al.* 2012, Ruhs *et al.* 2014). Therefore it was intriguing to study the role of adhesins LapA and LapF in the regulation of *P. putida* cell surface hydrophobicity.

The hydrophobicity of the cells was measured as a contact angle between a water droplet and a filter paper covered with cell lawn. To elucidate the effect of adhesins on hydrophobicity, we measured the contact angles of deletion mutants of PSm Δ*lapA* and PSm Δ*lapF* strains grown for 3 h and 18 h in LB medium. As expected, the wild-type cells of stationary phase were more hydrophobic than cells in logarithmic phase (Ref III, Fig 2A), which correlates to the finding, that stressed cells exhibit more hydrophobic surface (Baumgarten *et al.* 2012). The most surprising result was the remarkable decrease in contact angles (from 76° to 47°) of the stationary phase cells lacking *lapF* comparing to wild-type strain PSm (Ref III, Fig 2A). At the same time, the lack of *lapA* did not show any difference in hydrophobicity compared to wild-type cells. Correspondingly, the role of LapA in attachment to surfaces has been shown to be independent of hydrophobicity, as it is capable of binding both hydrophilic and hydrophobic surfaces (Boyd *et al.* 2014).

The finding of LapF`s participation in cell surface hydrophobicity of *P. putida* brought up a potential role of Fis in regulating hydrophobicity via LapF. For verifying that hypothesis, we constructed three types of strains carrying *lapF* promoter area modification in chromosome (Ref III, Fig 1). F15KmFm, where Fis binding site Fis-F2 upstream of the *lapF* gene was mutated, therefore decreasing the direct repression of *lapF* by Fis. PSmlapF3 and F15lapF3, where native promoter region of *lapF* was deleted from the chromosome and replaced by P$_{tac}$ promoter inducible with IPTG for *lapF* overexpression. And third, PSmKm and F15Km, as so-called wild-type control strains for the first two. The contact angle measurements of these strains confirmed that LapF is an important factor for hydrophobicity in stationary phase *P. putida* cells grown in LB-medium (Ref III, Fig 2 and 6). The wild-type originated strain PSmlapF3 is not able to express *lapF* without IPTG (Ref III, Fig 3) and is less hydrophobic than wild-type cells (Ref III, Fig 2). Though, the PSmlapF3 cell surface hydrophobicity is possible to induce by adding IPTG to medium. IPTG triggers *lapF* expression in PSmlapF3 cells, and the cells become more hydrophobic (Ref III, Fig 2C). Additionally, it is possible to induce *lapF* expression as well as the higher hydrophobicity of both logarithmic and stationary phase growing

PSmlapF via IPTG supplementation, when *lapF* is natively downregulated (Ref III, Fig 2).

In *fis* overexpression conditions as with F15-originated strains, it is clear that Fis regulates the hydrophobicity of *P. putida* via regulating the expression of *lapF*. This is in good agreement with our findings by Fis binding to the Fis-F2 site on the promoter of *lapF* and therefore suppressing the transcription from *lapF* gene (Ref II), whereas mutating the Fis-F2 abolishes Fis' repression. This is further confirmed by the contact angle measurement result of F15KmFm in stationary phase, where mutations in Fis-F2 binding site diminishes Fis binding, thus providing *P. putida* cells LapF-mediated hydrophobicity (Ref III, Fig 6). In other words, mutating the Fis-F2 binding site changes the phenotype of the cells, which confirms the involvement of Fis in direct regulation of hydrophobicity of *P. putida* via LapF.

In *S. aureus* a biofilm-associated protein Bap, which carries similar structural features with LapF, has also been shown to be involved in the regulation of hydrophobicity. *S. aureus* cells with a mutation in *bap* gene show decreased surface hydrophobicity compared to wild-type cells and therefore reduced the initial attachment to polystyrene as well as intercellular adhesion (Cucarella *et al.* 2001). In *S. aureus* Bap is involved in several stages of biofilm, starting from initial attachment, whereas LapF has been shown to be important only in mature biofilm (Cucarella *et al.* 2001, Martinez-Gil *et al.* 2010). Moreover, in *S. aureus,* the surface adhesion proteins are usually expressed in stationary and early exponential phase, while in *P. putida* the LapF is only expressed in stationary phase cells. This suggests that the two characteristically similar adhesins in different species have potentially different functions.

5.1. LapF-provided hydrophobicity as a potential defensive factor for *P. putida* (Ref III)

It is known that LapF is not essential for *fis*-induced biofilm in *P. putida* and the main adhesin for biofilm is LapA, which can bind to both hydrophobic and hydrophilic surfaces (Boyd *et al.* 2014, Moor *et al.* 2014). Cells growing in biofilm tend to be more hydrophobic, although the lack of *lapF* in *P. putida* makes cell surface more hydrophilic than wild-type cells, these hydrophilic cells can still form biofilm (Ref II, Ref III). What could be the reason of hydrophobic surface for *P. putida* and is LapF needed for biofilm formation or for something else? To answer these questions, we decided to test the viability of *P. putida* cells with and without *lapF* against hydrophilic and hydrophobic compounds. Methanol as a representative of hydrophilic and 1-octanol as a representative of hydrophobic chemicals was used in different concentrations to assess the effect on *P. putida* wild-type cells and cells lacking *lapF*. Stationary phase LB-grown bacteria, where LapF provides a hydrophobic surface, have more cells with intact membrane compared to cells lacking LapF, when treated with hydrophilic methanol (Ref III, Table 4). Similarly, when lacking *lapF,* there are more cells

with intact membrane when treated with hydrophobic 1-octanol, suggesting that LapF might play a role in defence mechanisms of *P. putida* against hydrophilic compounds. Furthermore, this means that Fis could have an important part in defensive regulation also, as the main repressor of *lapF* in *P. putida*.

P. putida is a rhizospheric bacterium (Lugtenberg and Bloemberg 2004) and therefore has to deal with reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) produced by plant roots (Apel and Hirt 2004, Matilla *et al.* 2007). Plants secrete ROS through their roots into rhizosphere for protection against pathogenic microorganisms. Plant growth promoting bacteria like *P. putida* also have to cope with this. Previously described results with methanol as an example of hydrophilic toxic compound, showed that *P. putida* cells overexpressing *lapF* had more hydrophobic surface and therefore were protected against methanol treatment compared with cells lacking *lapF* (Ref III). Thus these results suggest the possibility that LapF may protect the cell against other hydrophilic toxic compounds like H_2O_2. It has been previously described that the ability of LapF-deficient *P. putida* strain to colonize plant roots has dropped compared to the wild-type strain (Martinez-Gil et al. 2010). Therefore, LapF may also protect the rhizospheric *P. putida* against ROS and facilitate colonization, although, the most essential defensive factors against ROS are oxidoreductases that reduce ROS directly, for example, catalases (Kat), catalase-peroxidases (CP), superoxide dismutases (SOD) (Passardi *et al.* 2007). This possibility enlightens the role of adhesine LapF, not only being involved in mature biofilm formation (Martinez-Gil *et al.* 2010) but also playing a part in situations, where bacteria need to protect themselves against hydrophilic compounds or improve the ability to absorb nutrients from the environment for a better adaptation in changing conditions.


6. Model for the potential regulatory mechanism of LapF and Fis in *P. putida*

Here I propose a potential scheme for Fis-mediated regulation of LapF in *P. putida* cell aggregation and hydrophobicity regulation (Fig 4). Fis is a negative regulator of LapF, which expression is thereby repressed in the exponential growth phase. When cells enter the stationary phase, different stressors including nutrient deficiency start to set in. Stationary phase specific sigma factor RpoS activates and induces the expression of *lapF* as soon as Fis levels start to decrease and RpoS level increases. LapF provides *P. putida* a more hydrophobic surface that can passively protect cells against toxic hydrophilic compounds. When cells are confronted with toxic substances, the hydrophobicity of the cell surface increases and bacteria start to aggregate (Baumgarten *et al.* 2012). In this way, they could reduce the surface area of the cells exposed to the toxic compounds.

The ability of better defense against different kinds of chemicals and compounds found in the rhizosphere helps bacteria to compete with other micro-

organisms, especially plant pathogenic bacteria. In that way, bacteria acquire a better recolonization ability, which could improve the survival of bacteria on plant roots.
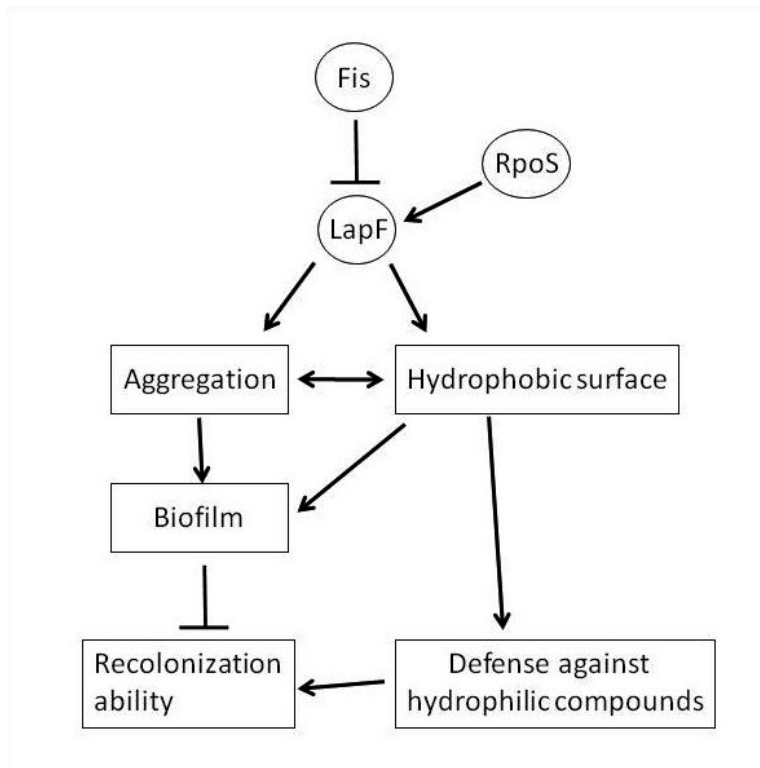
Figure 4. Proposed scheme for Fis-dependent regulation of *P. putida* biofilm formation and hydrophobicity via regulating the expression of the adhesine gene *lapF*. Solid lines with arrows indicate positive regulation and solid lines with blunt ends mark repression.

CONCLUSIONS

Pseudomonas putida is a soil bacterium mainly residing in the rhizosphere of plants. That kind of habitation is strongly dependent on the roots exudates, which include both nutrients and toxic compounds for bacteria. In order to survive and outcompete other microorganisms in the rhizosphere *P. putida* must be able to quickly adapt to the changes in its surrounding environment. For example staying put by forming biofilm or moving freely to recolonize new parts of plant roots for better conditions. Often, such extensive changes involve global transcription regulators, which are able to control the expression of several genes from different categories. Global regulator Fis is previously shown to enhance the biofilm formation of *P. putida* and reduce its recolonization ability on barley roots.

The first contact with environment happens through the surface of bacteria, which has developed the ability to change its composition and properties for better adaptation. One of the mechanisms for this is to alter the hydrophobicity of the cell surface. It can be done via different factors, like lipopolysaccharides, lipoteichoic acids, fimbriae, S-layer proteins or several surface proteins. Prior this work, a surface protein LapF, had only been described as a biofilm-related protein, having a role in cell-cell connections in the mature biofilm. Also, there was no detailed information published about the transcription regulation of *lapF* in *P. putida*. The aim of this research was to study and enlighten the role of LapF in *P. putida,* and it`s transcriptional regulation by global regulator Fis and the potential involvement of Fis and LapF in altering the hydrophobicity of *P. putida*`s surface. The main findings of this work can be summarized as follows:

- The absence of LapF does not affect the ability to form biofilm of *P. putida* in LB medium. This finding is in good accordance with previous work showing the LapF is needed for mature biofilm only in minimal medium and not in LB medium. However, it may suggest that LapF might have additional properties for *P. putida* besides biofilm formation.
- Fis regulates the expression of *lapF*. This thesis is the first to present that Fis binds to the promoter sequence of *lapF* by covering the -10 element of *lapF* promoter and therefore represses the transcription. In *P. putida lapF* is shown to be activated in stationary phase by sigma factor RpoS and no expression of *lapF* is detected in the logarithmic phase. Therefore it seems that Fis and RpoS might act counteractively in regulating the expression of *lapF* – repression by Fis in logarithmic phase and activation by RpoS in stationary phase.
- The stationary phase cells of *P. putida* expressing *lapF* are more hydrophobic than cells without LapF.
- LapF might have a potential role as a protective factor for *P. putida*. More hydrophobic bacteria can be more aggregative and therefore might help the cells reduce the surface exposed to the environment. This can be a passive protection mechanism against toxic organic compounds. The results of

measuring the ability of *P. putida* to tolerate different toxic compounds, like hydrophobic 1-octanol and hydrophilic methanol or hydrogen peroxide in the presence or absence of LapF, might suggest an additional role of LapF in *P. putida*.

SUMMARY IN ESTONIAN

Globaalse regulaatorvalgu Fis-i roll *lapF* geeni ekspressiooni reguleerimisel ja rakupinna hüdrofoobsuse mõjutamisel mullabakteris *Pseudomonas putida*

Bakteritel on pikaaegse evolutsiooni käigus välja kujunenud omadused ja mehhanismid selleks, et olla kohanemisvõimelised ja jääda ellu mitmesugustes keskkonnatingimustes. Vastavalt vajadusele muudavad bakterid oma eluvormi, ujudes vabalt keskkonnas ringi või olles paiksed ja moodustades koos teiste bakteritega biofilmi. Üleminek liikuvalt eluviisilt paiksele algab tavaliselt bakterite kinnitumisega elusale või elutule pinnale.

Üks peamisi bakteriraku pinna omadusi, mis aitab kinnitumisele kaasa, on hüdrofoobsus. Hüdrofoobsema pinnaga rakud agregeeruvad omavahel paremini, tänu millele nad vähendavad eksponeeritavat rakupinda kahjulikele ainetele. Näiteks mullabakter *Pseudomonas putida* muudab oma rakupinda hüdrofoobsemaks kokkupuutel erinevate toksiliste ainetega. Bakterite pinna hüdrofoobsust võivad mõjutada mitmesugused rakumembraaniga seotud faktorid, sh lipopolüsahhariidid, S-kihi valgud ja ka adhesiinvalgud. Nende faktorite olemasolu või puudumine rakupinnal aitab bakteril vastavalt vajadusele suurendada või vähendada oma pinna hüdrofoobsust. See omakorda soodustab keskkonnaga kohanemist ning vajadusel kaitseb rakku toksiliste ainete eest. Suuremahuliste füsioloogiliste muutuste läbiviimiseks on bakteril vaja aktiveerida või maha suruda palju geene, mida viivad läbi globaalsed regulaatorid. Käesolevas töös kirjeldatakse *P. putida* pinna hüdrofoobsufaktorit LapF-i ning selle geeni ekspressiooni regulatsiooni Fis-ga.

P. putida on kosmopoliitne bakter, mis tihti koloniseerib taimejuuri ja soodustab taimede kasvu. Üldiselt mullabakterid eelistavad elada risosfääris, sest taimed eritavad juurte kaudu bakterite jaoks kergesti kasutatavaid süsinikallikaid. Samas, taimed võivad juurte kaudu ümbritsevasse mulda eritada ka toksilisi ühendeid. Seega bakterid peavad risosfääris kohanema nii kasvu soodustavate kui ka pärssivate tingimustega. Näiteks, *P. putida* puhul oleme varem näidanud, et üleekspresseeritud *fis*-i korral jäävad bakterid paikseks, moodustades rohkem biofilmi, ja nende liikumine on pärsitud. Liikumine on omakorda oluline taimejuurte uute osade koloniseerimiseks. Seega, Fis mõjutab oluliselt *P. putida* konkurentsi- ja kohanemisvõimet. Uurides Fis-st sõltuvaid geene, mis osalevad biofilmi reguleerimisel, leidsime, et Fis represseerib *lapF*-i transkriptsiooni, samas oli teada, et LapF on oluline taime juure koloniseerimiseks. Võttes arvesse, et biofilmi moodustavad bakterid võivad olla hüdrofoobsemad, huvitas meid just LapF-i potentsiaalne roll *P. putida* pinna hüdrofoobsuse mõjutamisel. Enne käesoleva töö ilmumist ei ole varem avaldatud seoseid LapF-i ja *P. putida* pinna hüdrofoobsuse vahel ega ole ka teada detailsemat informatsiooni *lapF* geeni ekspressiooni regulatsiooni kohta. Saadud tulemused võib kokku võtta järgnevalt:

- LapF-i puudumine ei mõjuta *P. putida* biofilmi moodustamisvõimet toit-ainerikkas LB-söötmes. Saadud tulemus langeb hästi kokku varem avalda-tuga, kus on näidatud, et LapF on oluline ainult minimalssöötmes kasvanud rakkude biofilmi moodustumiseks ning seda biofilmi hilistes etappides.

- Globaalne regulaatorvalk Fis seondub *lapF* geeni promootorile, takistades sellega RNA polümeraasi seondumist ja *lapF*-i transkriptsiooni. Kuna Fis-i hulk on kõrge bakteripopulatsiooni eksponentsiaalses kasvufaasis, siis Fis käitub antagonistlikult statsionaarse sigmafaktori RpoS-ga, mis aktiveerib *lapF*-i transkriptsiooni.

- *P. putida*, mis toodab LapF-i, on statsionaarses kasvufaasis hüdrofoobsem võrreldes rakkudega, kus puudub LapF. LapF-st tulenev hüdrofoobsem rakupind võib omada potentsiaalset kaitsvat rolli *P. putida*'le. LapF-ga *P. putida*'l on soodustatud rakkude agregeerumine, tänu millele vähendatakse toksilistele ainetele kättesaadavat rakupinda. Kirjeldatud kaudne kaitse-mehhanism võib olla üks seni avastamata LapF-i rolle mullabakteris *P. putida*.

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PUBLICATIONS

CURRICULUM VITAE

Name: Andrio Lahesaare
Date of Birth: February 18, 1986
Citizenship: Estonian
Contact: Veerenni 53a, Tallinn, Estonia
                andrio.lahesaare@gmail.com
                +372 53982871

Education:
Since 2011   University of Tartu, PhD student (molecular and cell biology);
2009–2011   University of Tartu, MSc (gene technology);
2005–2009   University of Tartu, BSc (gene technology);
1993–2005   Pärnu Koidula Gymnasium

Language skills:
Estonian, English; Russian in intermediate level

Research interests:
Global transcription regulator Fis and its role in regulating the adaptation mechanisms of soil bacterium *Pseudomonas putida*.

List of publications:
1. Moor H, Teppo A, Lahesaare A, Kivisaar M, Teras R. Fis overexpression enhances *Pseudomonas putida* biofilm formation by regulating the ratio of LapA and LapF. Microbiology. 2014 Dec. 160(Pt 12): 2681–93.
2. Lahesaare A, Moor H, Kivisaar M, Teras R. *Pseudomonas putida* Fis binds to the *lapF* promoter *in vitro* and represses the expression of LapF. PloS ONE. 2014 Dec. 9(12):e115901.
3. Lahesaare A, Ainelo H, Teppo A, Kivisaar M, Heipieper HJ, Teras R. LapF and its regulation by Fis affect the cell surface hydrophobicity of *Pseudomonas putida*. PloS ONE. 2016 Nov. 11(11):e0166078.
4. Ainelo H, Lahesaare A, Teppo A, Kivisaar M, Teras R. The promoter region of *lapA* and its transcriptional regulation by Fis in *Pseudomonas putida*. PloS ONE. 2017 Sep. 12(9):e0185482.

Scholarships and research grants:
1. FEMS Research Grant, 2014 and 2015
2. Kristjan Jaak Scholarship, 2015
3. Olev and Talvi Maimets scholarship, 2016

Other organisational and professional activities:
1. Supervisor of Practical Course in Genetics, 2012–2014
2. Member of Estonian Society for Microbiologists, since 2013
3. Participated in several international conferences

ELULOOKIRJELDUS

Nimi: Andrio Lahesaare
Sünniaeg: 18. veebruar 1986
Kodakondsus: Eesti
Kontaktandmed: Veerenni 53a, Tallinn, Eesti
andrio.lahesaare@gmail.com
+372 53982871

Hariduskäik:
Alates 2011   Tartu Ülikool, doktorantuur (molekulaar- ja rakubioloogia);
2009–2011   Tartu Ülikool, MSc (geenitehnoloogia);
2005–2009   Tartu Ülikool, BSc (geenitehnoloogia);
1993–2005   Pärnu Koidula Gümnaasium

Keeleoskus:
Eesti ja inglise keel; vene keel kesktasemel

Teadustegevus:
Globaalse regulaatorvalgu Fis roll keskkonnatingimustega kohanemiseks vajalike mehhanismide reguleerimisel mullabakteris *Pseudomonas putida*.

Publikatsioonide loetelu:
1. Moor H, Teppo A, Lahesaare A, Kivisaar M, Teras R. Fis overexpression enhances *Pseudomonas putida* biofilm formation by regulating the ratio of LapA and LapF. Microbiology. 2014 Dec. 160(Pt 12): 2681–93.
2. Lahesaare A, Moor H, Kivisaar M, Teras R. *Pseudomonas putida* Fis binds to the *lapF* promoter *in vitro* and represses the expression of LapF. PloS ONE. 2014 Dec. 9(12):e115901.
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Saadud uurimistoetused ja stipendiumid:
1. FEMS`i noore teadlase stipendium, 2014 ja 2015
2. Kristjan Jaagu välissõidu stipendium, 2015
3. Olev ja Talvi Maimetsa stipendium, 2016

Muu teaduslik organisatsiooniline ja erialane tegevus:
1. Geneetika praktikumi juhendamine, 2012–2014
2. Eesti Mikrobioloogide ühenduse (EMÜ) liige, alates 2013
3. Osalemine mitmetel rahvusvahelistel konverentsidel

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