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II **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: 2681–2693.

III **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: e0185482.

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My contribution to the publications is following:

Ref I – I participated in conducting the *in vivo* experiments.

Ref II – I participated in planning the experiments, construction of plasmids and strains, conduction of experiments and in the writing and editing of the manuscript.

Ref III – I participated in planning the experiments, construction of plasmids and strains, in determining the location of promoters, conducted all the *in vivo* experiments and wrote the manuscript.

ABBREVATIONS

(p)ppGpp	guanosine penta- or tetraphosphate
bp	base pair
BS media	barley seedling media in M9 buffer
Dps	DNA-binding protein from starved cells
eDNA	extracellular DNA
Fis	factor for inversion stimulation
H-NS	histone-like nucleoid structuring protein
HU	heat-unstable nucleoid protein
IHF	integration host factor
IPTG	isopropylthio-β-D-galactoside, inductor of *tac* promoter
LB	lysogeny broth
NAP	nucleoid associated proteins
RTX	repeats-in-toxins

INTRODUCTION

Biofilms are the prevailing lifestyle of bacteria in most natural environments. This is because living in a biofilm gives bacteria a number of advantages. When the environment is hazardous, residing in a biofilm protects them; and when conditions are favourable, biofilm is a way to settle down and not be carried away from the good life. Although biofilm is most certainly useful for bacteria, people seem to first associate it with economic loss, treatment-resistant diseases, clogged up pipes and dirty water. While biofilms are implied in all of those unwanted phenomena, many biofilm-forming bacteria are beneficial. Biofilms are employed in wastewater treatment to use up contaminants (Boltz *et al.*, 2017), in industrial water systems to inhibit corrosion (Zuo, 2007) and in agriculture as biocontrol agents and plant growth promoters (Emmert and Handelsman, 1999). In this thesis, I will focus on the biofilm formation of plant growth promoting bacterium *Pseudomonas putida*. More precisely, how its biofilm is regulated by the global regulator Fis.

P. putida is one of the known plant growth promoting bacteria (Espinosa-Urgel *et al.*, 2002). The cosmopolitan bacterium *P. putida* can be found in most soils and waters, but it prefers to live in the rhizosphere, where it forms biofilm on plant roots. *P. putida* can rapidly respond to the presence of root exudates and lysates in soils, colonize the root and establish a stable biofilm (Espinosa-Urgel *et al.*, 2000). This biofilm is an example of a potentially beneficial one for humans as it protects plants, including agriculturally important crops, against pathogens (Paulitz, 1991, Espinosa-Urgel *et al.*, 2002, Aksoy and Yilmaz, 2008, Gamalero *et al.*, 2010).

The matrix of *P. putida* biofilm is proteinaceous and known to contain two large extracellular adhesins LapA and LapF. LapA, the larger adhesin of the two, is a key factor for attachment and biofilm formation shown to be necessary in all tested conditions (Espinosa-Urgel *et al.*, 2000, Hinsa *et al.*, 2003, Yousef-Coronado *et al.*, 2008, López-Sánchez *et al.*, 2016). When the current study was started, *P. putida*'s biofilm regulation had not been very extensively studied. It was known that the removal of LapA from the cell surface is controlled by a posttranslational mechanism and that c-di-GMP regulates this process (Navarro *et al.*, 2011, Newell *et al.*, 2011). However, the precise location of *lapA*'s promoter(s) was not known until the current research. Later, the alarmone (p)ppGpp, as well as the transcriptional regulator FleQ and the two-component GacS/A system, were added to the list of factors that regulate *P. putida*'s biofilm (Martinez-Gil *et al.*, 2014, Jimenez-Fernandez *et al.*, 2016, Díaz-Salazar *et al.*, 2017).

We made the discovery that Fis upregulates *P. putida* biofilm by chance. While we were characterizing the Fis overexpression strain, which we use in our studies as *fis* is an essential gene in *P. putida*, we saw that excess of Fis decreases motility. This prompted the hypothesis, which we later on confirmed, that Fis may upregulate biofilm formation. Fis is a nucleoid-associated protein

and global transcriptional regulator known in *Escherichia coli* as an endorser of fast growth in nutrient abundancy (Ball *et al.*, 1992). Fis can regulate transcription either directly or indirectly. Direct regulation involves binding the promoter area of target genes: Fis recognizes specific binding sites and bends DNA while binding it (Pan *et al.*, 1996, Shao *et al.*, 2008) resulting in either up- or downregulation of the target genes.

The aim of this thesis is to elucidate how Fis regulates biofilm formation. As we saw that Fis upregulates *P. putida* biofilm by increasing the expression of LapA, we focused on determining the effect of Fis on the transcription of *lapA*.

REVIEW OF LITERATURE

1. Biofilm

Biofilms are matrix-enclosed microbial communities adhered to biological or non-biological surfaces. The majority of bacteria in most ecosystems, are in biofilms (Costerton *et al.*, 1978, McDougald *et al.*, 2012). Therefore, sessile lifestyle must be the natural phenotype for bacteria. To human societies, biofilms can be both beneficial and harmful. Beneficial biofilms are frequently used in wastewater treatment (Boltz *et al.*, 2017) for example in trickling filter systems (Von Sperling, 2007). They can also be employed to fight corrosion. Biofilms can stop corrosion in industrial water systems by using up corrosive oxygen, inhibiting the growth of corrosion-causing bacteria or generating protective layers on surfaces (Zuo, 2007). Biofilm-forming bacteria may act as biocontrol agents and promote plant growth (Emmert and Handelsman, 1999). Many possible methods of protection have been proposed: outcompeting pathogens for nutrients and niche (Lemanceau *et al.*, 1992, Yu and Lee, 2015), producing fungitoxic phenolics (Ongena *et al.*, 1999) and hydrogen cyanide (Flaishman *et al.*, 1996, Ramette *et al.*, 2003), inducing systemic resistance in the plant (Wei *et al.*, 1991, Matilla *et al.*, 2010) and injecting effector molecules via type IV secretion system (Bernal *et al.*, 2017).

On the other hand, biofilms cause problems in medicine, industrial production, and agriculture. Biofilm-forming bacteria are deemed to be 100 to 1,000 times more resistant to antibiotics and disinfecting agents than planktonic cells (Stewart and Costerton, 2001, Smith and Hunter, 2008). Therefore making biofilm-forming bacteria the main cause of chronic infections (Costerton *et al.*, 1999, Fux *et al.*, 2003) and contamination of medical devices and implants (Passerini *et al.*, 1992, Gristina *et al.*, 1994, Morris *et al.*, 1999). They cause billions of dollars of economic loss every year by clogging up and corroding industrial water systems (Little and Lee, 2014) and deteriorating the hygienic quality of drinking water (Wingender and Flemming, 2011). Similarly to clogging up water pipes, the plant pathogen *Xylella fastidiosa*'s biofilm blocks the transpiration stream flow in xylem and along with effector proteins causes Pierce's disease in grapevines (Chatterjee *et al.*, 2008).

1.1 Why do bacteria form biofilms?

There may be many reasons why bacteria have evolved to form biofilm and they may differ for species and environments. However, most of the reasons seem to fall into two broad categories: protecting against a hostile environment or staying in a favourable setting. Biofilm-forming bacteria are protected against many hazards: antibiotics and disinfecting agents (Mah and O'Toole, 2001, Stewart and Costerton, 2001), UV light (Espeland and Wetzel, 2001), unfavourable pH (Davey and O'toole, 2000, McNeill and Hamilton, 2003), de-

hydration and salinity (Le Magrex-Debar *et al.*, 2000, Chang *et al.*, 2007). This tolerance may not be caused solely by the benefits of being covered with biofilm matrix but rather by the different metabolic state of these bacteria (Spoering and Lewis, 2001). Also, it is difficult to pinpoint whether bacteria form biofilm as a response to stress signals or they are protected by the coincidence of being in a biofilm when the stress strikes. Although *E. coli* O517:H7 has been shown to form more biofilm in low nutrient media than in complex media (Dewanti and Wong, 1995) and *Agrobacterium tumefaciens* forms more in phosphate limitation than in phosphate abundance (Danhorn *et al.*, 2004), most bacteria seem to form biofilm in optimal nutrient concentrations (O'Toole *et al.*, 2000). For example, *E. coli* K-12 and *Vibrio cholera* do not form biofilm in minimal medium with no amino acid supplementation (Pratt and Kolter, 1998, Watnick *et al.*, 1999) and phosphate limitation negatively affects biofilm formation of *Pseudomonas aureofaciens* and *Pseudomonas fluorescens* (Monds *et al.*, 2001, Monds *et al.*, 2007). However, sometimes too many nutrients also seem to hinder biofilm formation. For example, *P. fluorescens* has an optimal glucose concentration which increases biofilm formation whereas both higher and lower concentrations have a deleterious effect on biofilm formation (Chen *et al.*, 2005). Similar results have been obtained in studies on *P. putida,* which also has an optimal concentration of glucose and phosphate that increases biofilm mass accumulation the most (Rochex and Lebeault, 2007). This suggests that while biofilm is a universal phenomenon, different bacteria have very specific reasons to utilize this mode of life to their advantage.

1.2 Biofilm development

Proposed biofilm formation steps vary between authors but generally contain (i) attachment, (ii) development and (iii) dispersal (O'Toole *et al.*, 2000, Sauer *et al.*, 2002, Stoodley *et al.*, 2002, Stanley and Lazazzera, 2004, Kirisits and Parsek, 2006, Simões *et al.*, 2010).

1.2.1 Attachment

The first step of biofilm formation is attachment. Bacteria must overcome repulsive electrostatic and hydrodynamic forces to approach the surface. This process is aided by flagellar motility and pili (O'toole and Kolter, 1998a, Klausen *et al.*, 2003b, Friedlander *et al.*, 2013). The attachment of free-floating bacteria to a surface can be either specific or nonspecific. Specific receptor-ligand binding has been described for bacteria attaching to biotic surfaces. *Staphylococcus epidermidis* adhesin SdrG binds blood plasma protein fibrinogen via a dock, lock and latch mechanism involving a dynamic conformational change of the adhesin (Ponnuraj *et al.*, 2003). This receptor-ligand binding is equivalent to the strength of a covalent bond, making it the strongest

of any adhesin investigated so far (Herman *et al.*, 2014). *E. coli* adhesin FimH, which is located on the tip of type I pili, binds the terminal mannoses on epithelial glycoproteins via a catch-bond mechanism (Pratt and Kolter, 1998, Proft and Baker, 2009, Sauer *et al.*, 2016). Catch bonds are receptor-ligand interactions that are enhanced by a mechanical force pulling bacteria away from the surface (Sokurenko *et al.*, 2008).

Nonspecific adhesins bind by hydrogen bonding, hydrophobic, Van der Waals, electrostatic or macromolecular forces (Busscher *et al.*, 2008). In both specific and nonspecific binding, the physicochemical interactions originate from the same fundamental forces, but the difference is whether a specific ligand is recognized. Nonspecific adhesins as the name suggests bind a wide array of both abiotic and biotic surfaces. Examples on nonspecific adhesins are *Staphylococcus aureus* Bap, *Enterococcus faecalis* Esp and *P. fluorescens* LapA, which are all huge multidomain proteins that contain a core of tandem repeats (Cucarella *et al.*, 2001, Toledo-Arana *et al.*, 2001, Hinsa *et al.*, 2003, El-Kirat-Chatel *et al.*, 2013). It has been proposed that the multiple domains of LapA enable it to bind to such a wide array of surfaces and different regions are used to bind different surfaces (El-Kirat-Chatel *et al.*, 2013, Boyd *et al.*, 2014).

1.2.2 Development

After the cells have attached, they form compact microcolonies. Biofilm formation model organism *Pseudomonas aeruginosa* has been shown to form microcolonies through clonal growth (Klausen *et al.*, 2003a, Klausen *et al.*, 2003b), but some authors have proposed that microcolonies also arise partially by bacterial aggregation to one another (Lappin-Scott and Bass, 2001, Pace *et al.*, 2005). Thereafter depending on the species and conditions microcolonies either merely grow bigger or go through a transition involving cells dissociating and moving between microcolonies before forming the mature biofilm structure. Structural rearrangements have been mostly studied in the genus *Pseudomonas* (Tolker-Nielsen *et al.*, 2000, Klausen *et al.*, 2003b). For example, *P. aeruginosa* mushroom-shaped biofilm arises from a non-motile subpopulation growing on certain foci and a motile subpopulation migrating from an initial monolayer to form mushroom caps (Klausen *et al.*, 2003a).

Microcolonies and mature biofilm are associated with matrix production. The matrix appears to largely determine the structure of the mature biofilm (Flemming *et al.*, 2000). The resulting biofilm morphology can be smooth and flat, rough, fluffy or filamentous or have even more complex structures like mushroom-shape or fruiting bodies describe for *Myxococcus xanthus* (Flemming and Wingender, 2010). *P. aeruginosa* mature biofilm may range from a flat thin layer to a patchy pattern with interconnected microcolonies or even to mushroom-like formations depending on the growth conditions (Klausen *et al.*, 2003a, Barken *et al.*, 2008).

1.2.3 Dispersal

Staying in a biofilm also has its disadvantages. Whether environmental conditions become detrimental or high cell population locally uses up nutrients and produces toxic products, the end result is the same. Bacteria get trapped in the biofilm and lyse or are actively killed by other bacteria. Biofilm cell clusters of *P. aeruginosa*, *P. putida, Pseudoalteromonas tunicate* and *Actinobacillus actinomycetemcomitans* have been shown to become hollow, indicating the lysis of the "lower bunk cells" (Tolker-Nielsen *et al.*, 2000, Kaplan *et al.*, 2003a, Webb *et al.*, 2003, Mai-Prochnow *et al.*, 2004). Therefore, bacteria seem to have active mechanisms to escape biofilms. *Pseudomonas syringae*, *P. aeruginosa* and *A. actinomycetemcomitans* use polysaccharide lyases to dissolve the biofilm matrix (Boyd and Chakrabarty, 1994, Ott *et al.*, 2001, Kaplan *et al.*, 2003b). *S. aureus* uses a protease to degrade its biofilm (Boles and Horswill, 2008) and *P. fluorescens* seems to use both a polysaccharide lyase and a protease (Allison *et al.*, 1998, Newell *et al.*, 2011). Another explanation of biofilm cell clusters becoming hollow is seeding dispersal: hollow cavities become filled with non-aggregated planktonic cells, which are then released from the inside of the biofilm. This involves degradation of the matrix and probably some of the bacteria lysing (Webb *et al.*, 2003, Ma *et al.*, 2009). This has been described for the oral bacterium *A. actinomycetemcomitans* (Kaplan *et al.*, 2003a, Kaplan *et al.*, 2003b) and *P. aeruginosa* (Sauer *et al.*, 2002, Ma *et al.*, 2009). Whether seeding dispersal is the most important mechanism of partial dispersion is yet to be determined, but it is common for biofilms to regularly disperse some of its bacteria to colonize new surfaces. The released cells attach to new surfaces and form satellite colonies (Kaplan *et al.*, 2003a, Kirisits *et al.*, 2005) starting the cycle all over again.

1.2.4 *P. putida* biofilm formation

P. putida is a plant growth-promoting bacterium that often forms biofilm on plant roots. It attaches to both biotic and abiotic surfaces that also can be either hydrophobic or hydrophilic (Espinosa-Urgel *et al.*, 2000, El-Kirat-Chatel *et al.*, 2013). The surface attached *P. putida* starts to multiply forming compact microcolonies (Tolker-Nielsen *et al.*, 2000). As microcolonies have reached a certain size, structural rearrangements take place. Flow chamber grown *P. putida* irrigated with citrate minimal medium has been shown to dissociate from microcolonies and move inside and between the microcolonies via flagellum-driven motility (Tolker-Nielsen *et al.*, 2000). Similar structural rearrangements have also been described for *P. fluorescens* (Korber *et al.*, 1993, Korber *et al.*, 1994). Such movement is probably triggered by local carbon starvation (Gjermansen *et al.*, 2005) and transitions the microcolonies into mature biofilm (Tolker-Nielsen *et al.*, 2000). *P. putida* mature biofilm consists of loose irregularly shaped structures (Tolker-Nielsen *et al.*, 2000) and is known to dissolve rapidly in sud-

den carbon starvation (Gjermansen *et al.*, 2005). Protease LapG has been shown to be important for biofilm dispersal as it cuts the main adhesin LapA from cell surface allowing cells to detach (Gjermansen *et al.*, 2010).

1.3 Biofilm matrix

The biofilm is held together and protected by a matrix produced by sessile bacteria. Only up to 10% of the biofilm is thought to consist of bacteria, the rest is matrix (Flemming and Wingender, 2010). The biofilm matrix generally consists of water, exopolysaccharides, nucleic acids, proteins and lipids, although the exact compositions of biofilm matrixes differ significantly between microorganisms and growth conditions.

Polysaccharides are often considered to be the main structural components of the matrix and are present in most biofilms. Bacteria produce both homopolysaccharides and even more often heteropolysaccharides. Homopolysaccharides include glucans and fructans produced by the streptococci in oral biofilms and cellulose formed by *Gluconacetobacter xylinus*, *Salmonella enterica* serovar Typhimurium, *E. coli* and many others (Zogaj *et al.*, 2001). Heteropolysaccharides include xanthan formed by *Xanthomonas citri* subspecies *citi* (Guo *et al.*, 2010) and alginate produced by a wide variety of bacteria. However, more often than not the exact composition of exopolysaccharides remains unknown. For example, the biofilm model organism *P. aeruginosa* produces at least three different exopolysaccharides that contribute to biofilm formation: alginate, Psl and Pel (Jackson *et al.*, 2004, Matsukawa and Greenberg, 2004, Ryder *et al.*, 2007). While we know what alginate chemically looks like, Psl and Pel are recognized only by the operons encoding the proteins responsible for their synthesis (Ryder *et al.*, 2007).

In many bacteria, extracellular DNA (eDNA) plays an essential role in the establishment of biofilm structure (Whitchurch *et al.*, 2002). It has been shown to be structurally important in the biofilm of *P. aeruginosa* (Klausen *et al.*, 2003a, Webb *et al.*, 2003), *Streptococcus pneumoniae* (Moscoso *et al.*, 2006), *S. aureus* (Izano *et al.*, 2008), *Haemophilus influenza* (Jurcisek *et al.*, 2017) and many others. Extracellular DNA is generated through active (Heilmann *et al.*, 1997) or passive (Steinberger and Holden, 2005) cell lysis or specifically produced (Böckelmann *et al.*, 2006). Also, it is not randomly distributed in biofilm, but forms a filamentous scaffold (Böckelmann *et al.*, 2006) demonstrating that eDNA is an important biofilm component and not just a remnant of lysed cells.

Biofilm matrix also contains a considerable amount of proteins: secreted extracellular proteins, cell surface adhesins and subunits of flagella or pili. Out of those matrix proteins, adhesins affect biofilm formation the most. Proteinaceous adhesins are required for biofilm formation in many bacteria including *S. enterica* (Latasa *et al.*, 2005), *Enterococcus faecalis* (Toledo-Arana *et al.*, 2001), *P. aeruginosa* (Borlee *et al.*, 2010) and *P. putida* (Hinsa *et al.*, 2003).

Similarly to polysaccharides and eDNA, proteins contribute to biofilm structure and stability.

Lipids are also found in the biofilm matrix. *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* are known to form biofilms with a lipid-rich matrix (Ojha *et al.*, 2005, Ojha *et al.*, 2008) and *Serratia marcescens* produces extracellular lipids with surface-active properties (Matsuyama and Nakagawa, 1996).

1.3.1. *P. putida* biofilm matrix

P. putida biofilm matrix contains (in addition to water) all previously mentioned major components: protein, eDNA and polysaccharides (Jahn *et al.*, 1999). The prevailing component of *P. putida* biofilm is protein (Jahn *et al.*, 1999). That is probably because the two main adhesins of *P. putida* are proteins LapA and LapF (Fig 2). These cell surface localized proteins are the two biggest proteins of the bacterium (Martinez-Gil *et al.*, 2010, Ivanov *et al.*, 2012). LapA is the larger of the two and is considered to be the main factor for biofilm formation (Espinosa-Urgel *et al.*, 2000, Hinsa *et al.*, 2003). LapF has a role in mature biofilm formation and determines the surface hydrophobicity of the bacterium (Martinez-Gil *et al.*, 2010, Lahesaare *et al.*, 2016).

P. putida has been reported to also produce substantial amounts of eDNA in the sessile mode of growth (Steinberger & Holden, 2005). However, unlike for *P. aeruginosa*, extracellular DNA is not structurally important (Yousef-Coronado *et al.*, 2011). *P. putida* also produces four different polysaccharides: Pea, Peb, alginate and bacterial cellulose (Jackson *et al.*, 2004, Chang *et al.*, 2007, Nilsson *et al.*, 2011). Also unlike *P. aeruginosa*, none of the polysaccharides are absolutely necessary for biofilm formation but they play an important role in biofilm stability. Out of these four polysaccharides, Pea and Peb are more important for biofilm stability, while alginate and cellulose appear to be minor contributors (Nilsson *et al.*, 2011). Alginate seems to have a role in water-limiting conditions, where it maintains hydration (Chang *et al.*, 2007). Interestingly in the KT2440 strain (also used in this work), alginate is produced only in the rhizosphere and is undetectable in M9-citrate laboratory media (Ramos-González *et al.*, 2005). The importance of cellulose production is yet to be determined as some results indicate that it contributes to rhizosphere colonization (Nielsen *et al.*, 2011) and others show that it is not important (Martínez-Gil *et al.*, 2013). Still, it is probable that exopolysaccharides together with LapA function as a biofilm matrix and disrupting polysaccharide production is compensated by LapA to some extent (Gjermansen *et al.*, 2010).

2. Regulation of biofilm formation

Biofilm formation depends highly on environmental conditions. Osmolarity (O'toole and Kolter, 1998b, Jubelin *et al.*, 2005), carbon-source (Klausen *et al.*, 2003a, Barken *et al.*, 2008), availability of micronutrients such as calcium (Arrizubieta *et al.*, 2004, Boyd *et al.*, 2012), magnesium (Song and Leff, 2006, Mulcahy and Lewenza, 2011), phosphate (Monds *et al.*, 2007) and iron (Molina *et al.*, 2005), excess of copper (Baker *et al.*, 2010) and several other factors have been described to affect biofilm formation. This makes the regulation of this process very complex involving many global and specific regulators (Waite *et al.*, 2006, Fazli *et al.*, 2014).

2.1 Two-component systems

Bacteria rely on abundant two-component systems to process environmental or less often intracellular signals. Therefore, unsurprisingly more and more two-component systems are being linked with biofilm formation. Two-component systems in their simplest form consist of a sensor kinase and a response regulator. In response to the signal the sensor is activated and in turn activates the response regulator by phosphorylating it. Response regulators are often DNA binding proteins, which can participate in transcriptional control, but some response regulators bind RNA or proteins or even perform enzymatic activities (Stock *et al.*, 2000, Gao *et al.*, 2007).

Several known effectors of biofilm formation are sensed by two-component systems. For example, low phosphate levels are sensed by PhoB/PhoR (Makino *et al.*, 1986, Filloux *et al.*, 1988), low extracellular Mg^{2+} by PhoP/ PhoQ (McPhee *et al.*, 2006), excess of copper by CusS/CusR (Yamamoto and Ishihama, 2005) and oxygen levels by ArcB/ArcA two-component system (Spiro and Guest, 1991). The best described two-component system involved in biofilm formation is the GacS/GacA system (Fig 1).

2.1.1 GacS-GacA/RsmA pathway

The GacS/GacA two-component system regulates biofilm formation in many Gram-negative bacteria including *P. aeruginosa* (Fig 1), *E. coli* and *V. cholerae* (Parkins *et al.*, 2001, Suzuki *et al.*, 2002, Lenz *et al.*, 2005, Brencic *et al.*, 2009). GacS is a membrane-bound sensor histidine kinase and GacA a typical response regulator with a DNA binding domain (Lapouge *et al.*, 2008). GacA activates the transcription of small noncoding RNAs, which in *P. aeruginosa* are called RsmY and RsmZ (Kay *et al.*, 2006). These small RNAs bind and therefore inactivate the RNA binding posttranscriptional regulator RsmA (Liu *et al.*, 1997, Heurlier *et al.*, 2004). Free RsmA binds specific mRNAs containing the A(N)GGA sequence to repress biofilm formation and activate movement (Goodman *et al.*, 2004, Ventre *et al.*, 2006, Brencic *et al.*, 2009). Altogether the activation of GacS/A system leads to increased biofilm formation and repressed

motility. It also promotes the production of virulence factors (Reimmann *et al.*, 1997, Pessi *et al.*, 2001).

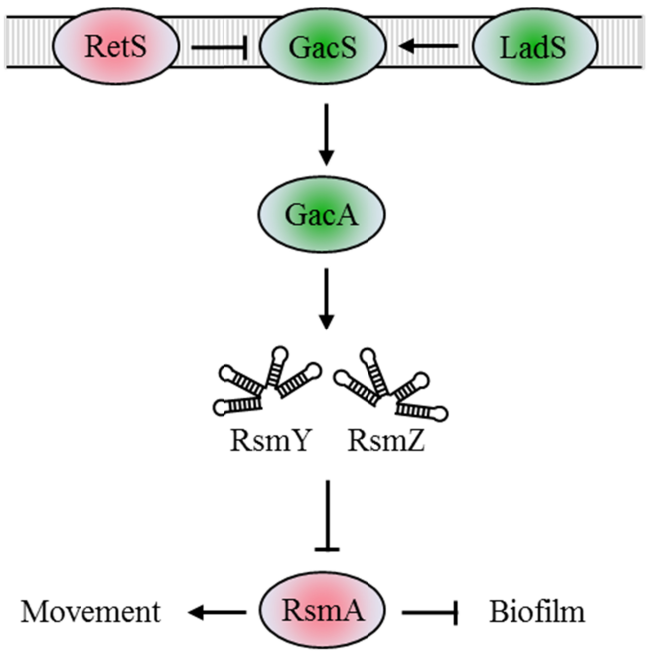

Figure 1. GacS-GacA/Rsm pathway in *P. aeruginosa*. Biofilm activators are depicted in green and repressors in red. GacS is a sensor kinase that recognizes an unknown signal and transmits it to GacA via phosphorelay. The signal transmission is activated by LadS and repressed by RetS. Phosphorylated GacA activates the transcription of small noncoding RNA-s RsmY and RsmZ, which bind and therefore inactivate posttranscriptional regulator RsmA. This stops RsmA from activating genes required for movement and facilitates the repression of biofilm genes.

GacS/A system in *P. aeruginosa* also interacts with RetS and LadS regulators. These are both membrane-bound hybrid sensors with inverse functions (Ventre *et al.*, 2006, Goodman *et al.*, 2009). RetS can form heterodimers with GacS, and block signal transduction to GacA (Goodman *et al.*, 2009) and LadS phosphorylates GacS to in turn promote GacA's phosphorylation (Chambonnier *et al.*, 2016). Both *ladS* and *gacA* deletion strains are impaired in biofilm formation while *retS* deletion results in increased biofilm mass (Parkins *et al.*, 2001, Ventre *et al.*, 2006, Goodman *et al.*, 2009).

2.1.2 The role of GacS-GacA/RsmA pathway in *P. putida* biofilm

P. putida GacS-GacA/RsmA pathway, although similar to the well-described *P. aeruginosa* system, is more elaborate as there are two additional RsmA homologues, RsmE and RsmI, and one additional small RNA, RsmZ. (Winsor *et al.*, 2015). RsmZ was first described in *P. fluorescens*, where it was also shown to be differently regulated from the redundantly acting RsmX and RsmY (Kay *et al.*, 2005).

The GacS-GacA/RsmA pathway seems to take part in *P. putida* biofilm formation. However, the connections are not quite clear yet. The disruption of the *gacS* gene does not affect adherence to corn seeds nor blue periwinkle (*Vinca major*) leaves but decreases biofilm formation (Duque *et al.*, 2013). This indicates that the pathway starting from GacS is not necessary for attachment but comes into play in later stages of biofilm development. The disruption of *gacS* also decreases the transcription of *lapA* and *lapF* (Martinez-Gil *et al.*, 2014) indicating that GacS can positively regulate biofilm formation by increasing the transcription of *lapA* and *lapF*. In this context it is controversial that the *gacS* mutant can adhere to seeds and leaves as well as the wild type, while LapA is crucial for adherence to the same seeds and leaves (Duque *et al.*, 2013). The effect on *lapF* transcription fits the model much better as LapF is needed in later biofilm development (Martinez-Gil *et al.*, 2010). Also, the GacS/A system positively regulates the alternative sigma factor RpoS (Whistler *et al.*, 1998, Martinez-Gil *et al.*, 2014), which is absolutely necessary for the transcription of *lapF* (Martinez-Gil *et al.*, 2010). Therefore, GacS/GacA most likely regulates the transcription of *lapF* indirectly via RpoS (Martinez-Gil *et al.*, 2014).

As the activation of the GacS/A two component system leads to sequestration of the Rsm proteins, it seems logical that while the disruption of *gacS* decreases biofilm formation, the simultaneous disruption of the *rsmA, I,* and *E* genes increases biofilm formation (Huertas-Rosales *et al.*, 2016). The system, however, is much more complicated as the deletion of one *rsm* gene at a time or the *rsmI* and *rsmE* together decreases biofilm formation on polystyrene (Huertas-Rosales *et al.*, 2016). Moreover, the deletion of *rsmAEI* does not affect the transcription of *lapA* (Huertas-Rosales *et al.*, 2016)

2.2 Alarmones

2.2.1 Cyclic-di-GMP

Cyclic-di-GMP (c-di-GMP) is a ubiquitous secondary messenger considered to be the main switch between motility and biofilm formation (Römling *et al.*, 2005, Hengge, 2009). C-di-GMP regulates cellular functions at multiple levels. C-di-GMP binding can allosterically regulate the activity or function of enzymes or regulate gene expression by either modulating transcription factors or by directly interacting with noncoding RNA molecules – riboswitches (Kulshi-

na *et al.*, 2009). C-di-GMP responding regulators include (in addition to riboswitches) proteins with c-di-GMP binding Plz domains (Amikam and Galperin, 2006, Hengge, 2009), catalytically inactive GGDEF/EAL domains (Newell *et al.*, 2011) and transcriptional regulators (Kalia *et al.*, 2013, Jenal *et al.*, 2017).

C-di-GMP is synthesized by diguanylate cyclases that contain GGDEF motifs and degraded by phosphodiesterases that contain EAL motifs (Simm *et al.*, 2004, Tischler and Camilli, 2004). Bacteria also have many catalytically inactive GGDEF and EAL domains which can act as c-di-GMP receptors (Newell *et al.*, 2011). C-di-GMP promotes biofilm formation in many Gram-negative bacteria: in *P. aeruginosa* and *V. cholera* c-di-GMP activates the transcription of exopolysaccharide synthesis genes (Beyhan *et al.*, 2006, Lee *et al.*, 2007) and in *Komagataeibacter xylinus* c-di-GMP regulates the activity of a cellulose synthase (Aloni *et al.*, 1982, Ross *et al.*, 1987). However, these are just a couple of examples as approximately 20 proteins containing GGDEF and/or EAL domain have been linked with biofilm formation in *P. aeruginosa* alone (Valentini and Filloux, 2016).

One of the global transcription regulators controlled by c-di-GMP is FleQ (Baraquet and Harwood, 2013). FleQ homologs are present in all *Pseudomonas* species and in many gamma-proteobacteria (Baraquet and Harwood, 2013). FleQ partakes in the switch between planktonic and biofilm lifestyle and its effects depend on the presence of c-di-GMP. When c-di-GMP levels are low, FleQ activates the transcription of flagellar genes σ^N-dependently and represses biofilm formation genes in *P. aeruginosa* (Dasgupta *et al.*, 2003, Hickman and Harwood, 2008). If c-di-GMP levels rise, it binds FleQ and changes its conformation. Now FleQ activates biofilm genes such as *pel*, *psl*, and *cdr* involved in exopolysaccharides production and no longer activates flagellar genes (Hickman and Harwood, 2008, Baraquet *et al.*, 2012).

2.2.2 (p)ppGpp

Stringent response alarmone (p)ppGpp is produced in response to a wide array of different nutritional limitations and stressors. It is necessary for bacterial cells to appropriately respond to stress (Potrykus and Cashel, 2008) and has been shown to affect biofilm formation in a number of bacteria. The deletion of (p)ppGpp synthases decreases biofilm formation in *Listeria monocytogenes* (Taylor *et al.*, 2002), *Streptococcus mutans* (Lemos *et al.*, 2004), *V. cholerae* (He *et al.*, 2012), *E. coli* (Åberg *et al.*, 2006) and reduces the capacity to sustain biofilm formation over an extended period of time in *Enterococcus faecalis* (de Paz *et al.*, 2012). Also, the overexpression the (p)ppGpp synthetase *relA* in *V. cholerae* increases biofilm formation. These results show that in most bacteria (p)ppGpp is a positive regulator of biofilm formation.

The mechanisms of the positive effects of (p)ppGpp on biofilm formation and stability have been described in *V. cholerae* (He *et al.*, 2012) and *E. coli* (Åberg *et al.*, 2006). In *V. cholerae* (p)ppGpp positively affects the trans-

criptional activators VspR and VspT, which activate the transcription of two *vsp* biofilm operons (He *et al.*, 2012). In *E. coli* (p)ppGpp activates one of the *fimB* promoters, from which FimB recombinase is produced (Åberg *et al.*, 2006). FimB recombinase mediates inversion of the *fim* promoter to the productive orientation, increasing the production of type 1 fimbria and thus biofilm formation (Gally *et al.*, 1996).

2.2.3 The role of c-di-GMP and (p)ppGpp in *P. putida* biofilm formation

Two alarmones with opposing effects have been shown to regulate biofilm formation in *P. putida*. C-di-GMP is a positive regulator and (p)ppGpp is unconventionally a negative regulator.

C-di-GMP increases biofilm formation and its decrease precedes biofilm dispersal in *P. putida* and its closely related species *P. fluorescens* (Gjermansen *et al.*, 2006, Monds *et al.*, 2007). In *P. putida*, nutrient starvation triggers c-di-GMP hydrolysis by the phosphodiesterase BifA (Jiménez-Fernández *et al.*, 2015). Studies performed in *P. fluorescens* show that the drop in c-di-GMP is sensed by the membrane-bound signal transduction protein LapD (Navarro *et al.*, 2011). LapD, when no longer in complex with c-di-GMP, releases the periplasmic protease LapG. LapG in turn cuts the adhesin LapA off the cell surface and releases cells from biofilm (Newell *et al.*, 2011). The drop in c-di-GMP does not only remove existing LapA but also decreases the transcription of *lapA* (Martinez-Gil *et al.*, 2014, Jimenez-Fernandez *et al.*, 2016). When there are plenty of nutrients again, the level of c-di-GMP rises. C-di-GMP inflicts conformational changes of LapD, which is then able to bind and inactivate LapG (Navarro *et al.*, 2011, Newell *et al.*, 2011). LapG is unable to cut LapA, which stays on the cell and enables biofilm formation. The transcription of *lapA* also increases, enhancing biofilm formation (Martinez-Gil *et al.*, 2014).

Many of c-di-GMP effects are executed by its responsive regulator FleQ, which is a master regulator of flagellar movement present in all *Pseudomonas* species. Disruption of *P. putida fleQ* diminishes flagellar motility and biofilm formation (Yousef-Coronado *et al.*, 2008, Jimenez-Fernandez *et al.*, 2016). Similarly to *P. aeruginosa,* in the presence of c-di-GMP *P. putida* FleQ inhibits flagellar genes and stimulates the transcription of biofilm genes (Jimenez-Fernandez *et al.*, 2016). *P. putida* FleQ increases the transcription of the adhesin *lapA* and exopolysaccharide production genes (Martinez-Gil *et al.*, 2014, Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016). Gel-shift analysis shows that FleQ binds the promoter regions of polysaccharide production genes *pea*, *peb* and *bsc* (Molina-Henares *et al.*, 2017) and adhesin *lapA* (Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016). C-di-GMP only affects *lapA* transcription through FleQ and their effect is synergistic (Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016). FleQ activates *lapA* transcription directly by DNA binding but its exact binding sites are yet to be determined. The activating effect of FleQ varies from

2 to 10 times between different authors and methods (Martinez-Gil *et al.*, 2014, Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016).

The alarmone **(p)ppGpp** is a negative regulator of *P. putida* biofilm and it functions by affecting the other alarmone c-di-GMP (Díaz-Salazar *et al.*, 2017, Liu *et al.*, 2017). During nutrient limitation, the level of (p)ppGpp increases and this induces biofilm dispersal (Díaz-Salazar *et al.*, 2017). It has been shown that (p)ppGpp decreases the transcription of *lapA* and its transport system genes and additionally induces LapA release from the cell surface (Díaz-Salazar *et al.*, 2017). Both of these effects can be the result of (p)ppGpp-induced drop in c-di-GMP levels (Díaz-Salazar *et al.*, 2017, Liu *et al.*, 2017). Moreover, (p)ppGpp stimulates the transcription of *bifA*, which' product BifA hydrolyses c-di-GMP (Díaz-Salazar *et al.*, 2017). Low c-di-GMP levels function in at least two pathways to decrease biofilm formation. Firstly, it releases the protease LapG, which cuts LapA away from the cell surface (Navarro *et al.*, 2011, Newell *et al.*, 2011). Secondly, low c-di-GMP levels disable FleQ from acting as a transcriptional activator for *lapA* thus inhibiting LapA production (Jimenez-Fernandez *et al.*, 2016). In addition, (p)ppGpp decreases the expression of the exopolysaccharide production genes *peb* and *bcs* (Liu *et al.*, 2017) that could also decrease biofilm formation. On the other hand, (p)ppGpp induces the expression of *pea* exopolysaccharide production gene and the *lapF* adhesin gene. LapF induction is caused by RpoS (Liu *et al.*, 2017).

2.3 Sigma factors

The σ factors are essential for RNA polymerase to recognize promoters and initiate transcription specifically at promoters. Therefore, σ factors determine which genes are transcribed. Bacteria typically have a housekeeping σ factor (*E. coli* as a model organism has σ^{70}) and several alternative σ factors. Housekeeping σ is always present in the cells and initiates transcription from the majority of genes in exponential growth phase (Murakami and Darst, 2003). Alternative sigma factors initiate the transcription of a specific set of genes, which may serve a common cause, e.g. entry into stationary phase (σ^{S}), heat shock response (σ^{H}), nitrogen assimilation and metabolism (σ^{N}), synthesis of flagella (FliA) or iron uptake (FecI). However, many of them regulate a broader set of genes than those that they are known for.

There are two divergent families of σ factors: the σ^{70} family and the σ^{54} family. Most bacteria only have one σ^{54} family member – the σ^{54} itself and all other sigma factors make up the σ^{70} family (Lonetto *et al.*, 1992, Gruber and Gross, 2003). σ^{70} family factors recognize two conserved elements named by their centred positions from transcription start site: -10 and -35 elements. In comparison, σ^{54} family factors recognize -12 and -24 elements (Morett and Buck, 1989). Inside the σ^{70} family, σ^{70} and stationary phase sigma σ^{S} are the most similar in sequence (Paget and Helmann, 2003). They recognize similar -10 boxes and can recognize the same promoters (Weber *et al.*, 2005).

However, σ^S has no conserved -35 sequence (Weber *et al.*, 2005, Typas and Hengge, 2006). Conserved promoter sequences recognized by *E. coli* sigma factors σ^{70}, σ^S and σ^N are shown in Table 1.

Extracytoplasmic function σ factors are a part of the σ^{70} family. They usually influence a smaller set of genes and have their own anti-sigma factors (Heimann, 2002). Anti-sigma factors bind their cognate sigma factors and inhibit their action.

Utilizing σ factors is another mechanism that enables bacteria to change their gene expression as a response to environmental conditions and therefore is linked to biofilm formation, although there are surprisingly few examples of described effects.

The sigma factor σ^N is involved in biofilm formation by activating polysaccharide production in *P. aeruginosa* (Goldberg and Dahnke, 1992), *Burkholderia cenocepacia* (Saldías *et al.*, 2008), *Vibrio fischeri* (Wolfe *et al.*, 2004, Yip *et al.*, 2005) and *Vibrio anguillarum* (Hao *et al.*, 2013). However, the particular pathways vary between these bacteria. In *P. aeruginosa*, σ^N regulates alginate expression (Goldberg and Dahnke, 1992), in *V. fischeri* it regulates the expression of *syp* polysaccharide genes involved in symbiosis (Yip *et al.*, 2005), in *V. anguillarum* it affects *wza* and *wbfD* involved in exopolysaccharide transport and biosynthesis and in *B. cenocepacia* it regulates the production of a biofilm-stabilizing exopolysaccharide via a c-di-GMP dependent mechanism (Fazli *et al.*, 2017).

Table 1 Conserved promoter sequences recognized by *E. coli* σ^{70}, σ^S and σ^N

σ^{70} family	**Factor**	**-35 element**	**-10 element***	**Bp between elements**	**Reference**
	σ^{70} (RpoD)	TTGACA	TATAAT	16–18	(Harley and Reynolds, 1987)
	σ^S (σ^{38}, RpoS)	Degenerate TTGACA	KCTATACTTAA	13–17	(Weber *et al.*, 2005, Typas and Hengge, 2006)
σ^{54} family	σ^N (σ^{54})	TGGCACG	TTGCW	4	(Morett and Buck, 1989, Barrios *et al.*, 1999)

*W is A or T and K is T or G

σ^S influences global gene expression and protein production in *E. coli* biofilms (Collet *et al.*, 2008, Ito *et al.*, 2009), however its effects are controversial. *E. coli rpoS* deletion mutant has been shown to have decreased and differently structured biofilm in minimal media (Adams and McLean, 1999, Collet *et al.*, 2008). On the contrary its transposon mutant has been shown to have increased

biofilm in rich media (Corona-Izquierdo and Membrillo-Hernández, 2002) and too much σ^S also seem to decrease biofilm formation in rich media (Ferrieres *et al.*, 2009). In *P. putida* σ^S has been shown to have a direct effect on a biofilm adhesin. The gene of the second biggest adhesin *lapF* has a strictly σ^S-dependent promoter (Martinez-Gil *et al.*, 2010).

P. aeruginosa extracytoplasmic sigma factor σ^E (AlgU) is involved in alginate production (Schurr *et al.*, 1996). AlgU controls the conversion of *P. aeruginosa* to the mucoid, alginate-overproducing phenotype associated with lethal infections in cystic fibrosis patients (Martin *et al.*, 1993). AlgU is also important for biofilm formation in non-mucoid *P. aeruginosa* where it is required for efficient attachment and formation of robust, shear-resistant biofilm (Bazire *et al.*, 2010). AlgU is proposed to increase biofilm formation by affecting Psl polysaccharide synthesis, production of LecA and LecB lectins and type IV pilus biogenesis (Bazire *et al.*, 2010).

2.4 Nucleoid associated proteins

Nucleoid associated proteins (NAPs) are small proteins that bind and bend DNA. They participate in several processes that all require changes in DNA topology. NAPs structure the bacterial nucleoid; partake in recombination, replication and transcription. Depending on the nature of the NAP, their interactions with DNA can be more or less sequence-specific (Table 2). For example Fis (factor for inversion stimulation) and IHF (integration host factor) recognize specific binding sites (Hales *et al.*, 1994, Shao *et al.*, 2008), HU (heat unstable nucleoid protein) binds DNA independently of the sequence (Bonnefoy and Rouviere-Yaniv, 1991) and H-NS (histone-like nucleoid structural protein) prefers an AT-rich sequence that usually has a curved structure (Zuber *et al.*, 1994). Most NAPs can be found on promoter areas/intergenic regions. Intergenic regions make up less than 10% of *E. coli* genomic DNA, but approximately 50% of IHF, H-NS and Fis, are bound with it (Grainger *et al.*, 2006).

Table 2. Conserved binding sequences of global regulators

Global regulator	Binding sequence*	Source
Fis	GNTYAWWWWWTRANC	(Finkel and Johnson, 1992, Shao *et al.*, 2008)
H-NS	curved DNA	(Zuber *et al.*, 1994)
IHF	WATCAANNNNTTR plus upstream A/T-rich elements	(Hales *et al.*, 1994)
HU	nonspecific	(Bonnefoy and Rouviere-Yaniv, 1991)

*W is A or T, R is A or G, and N is any nucleotide

Nucleoid associated proteins can regulate transcription directly by regulating the expression of target genes either through contacting RNA polymerase or by modulating the local conformation of DNA. Direct regulation always involves binding the promoter area of target genes. Nucleoid associated proteins can also regulate transcription indirectly via changing global superspiralisation or the physiological state of the cell. The most abundant nucleoid associated proteins in *E. coli* exponentially growing cells are Fis, HU, H-NS and IHF (Azam *et al.*, 1999). In stationary phase cells, Dps (DNA-binding protein from starved cells) becomes the most abundant nucleoid protein (Azam *et al.*, 1999).

NAPs as global regulators allow bacteria to adapt to ever-changing environmental conditions. Therefore, it is not surprising that they also play a role in biofilm regulation. More specifically, Fis, H-NS, IHF and HU have been shown to affect biofilm formation in bacteria.

2.4.1 Fis

Fis (factor for inversion stimulation) is a global transcription regulator and a nucleoid associated protein found in *Enterobacteriaceae* and *Pseudomonadaceae* (Beach and Osuna, 1998, Boswell *et al.*, 2004). It is a sequence-specific DNA binding protein (Table 2) that binds DNA in dimers and bends it between 50 to 90 degrees (Finkel and Johnson, 1992, Pan *et al.*, 1996, Shao *et al.*, 2008). In *E. coli* Fis is the most abundant in exponentially growing bacteria and its levels drop drastically in stationary phase (Ball *et al.*, 1992). It triggers the fast growth as a response to a sudden abundance of nutrients. Fis activates the transcription of genes involved in translation, nutrient transport, energy metabolism, flagellar biosynthesis and motility (Ball *et al.*, 1992, Bradley *et al.*, 2007). In *P. putida*, Fis mRNA levels are also highest in exponentially growing cells and drop approximately three times in stationary phase cells, but the levels never go as high up nor as down low as in *E. coli* (Yuste *et al.*, 2006).

Fis can repress or activate transcription. It can repress transcription by binding on the promoter and physically blocking RNA polymerase from binding or by trapping RNA polymerase and stopping it from forming an open complex (Schneider *et al.*, 1999, Grainger *et al.*, 2008). Fis activates transcription by binding near upstream of the promoter and interacting with RNA polymerase or from distance by changing the local topology. Fis can displace DNA twists to promote open complex formation (Opel *et al.*, 2004) or maintain local negative superspiralisation (Auner *et al.*, 2003). Additionally to described direct effects, Fis can, similarly to other NAPs, affect transcription indirectly by chancing global superspiralisation and the transcription of other regulator genes with global effects like *rpoS*, *gyrA*, *gyrB* and *topA* (Travers *et al.*, 2001, Hirsch and Elliott, 2005, Weinstein-Fischer and Altuvia, 2007).

Fis has been shown to have both negative and positive effects on biofilm formation in different bacteria. Fis decreases biofilm formation in *Dickeya dadantii* where cellulose is the main component of biofilm by repressing the

transcription of the cellulose operon (Prigent-Combaret *et al.*, 2012). In enteropathogenic *E. coli* E2348/69 Fis represses the expression of the main subunit of the *csg* curli therefore reducing biofilm formation (Saldana *et al.*, 2009). In contrary, Fis increases biofilm formation in *E. coli* 042, where it activates the expression of the *aaf* fimbria genes (Sheikh *et al.*, 2001). Additionally, it has been shown that Fis can activate biofilm formation indirectly by repressing signal transduction in *V. cholerae* quorum sensing regulatory pathway (Lenz and Bassler, 2007).

2.4.2 H-NS

H-NS (heat-stable nucleoid-structuring protein) is a global transcription repressor and a nucleoid structuring protein that is conserved among Gram-negative bacteria (Tendeng and Bertin, 2003). It is very abundant in the cells and appears to be maintained in a constant ratio to DNA (Free and Dorman, 1997, Doyle et al., 2007). H-NS does not have a conserved recognition sequence, but it recognizes the structure of DNA (Table 2). It binds to curved DNA, which is commonly associated with promoters and represses transcription (Yamada et al., 1990, Jáuregui et al., 2003). The described positive effects are probably indirect effects through other regulators (Dorman, 2004). To function as a transcriptional repressor, H-NS has to oligomerize (Rimsky, 2004). It has been shown to generate dimers, trimers and tetramers (Ceschini et al., 2000, Smyth et al., 2000).

H-NS decreases biofilm formation in *Actinobacillus pleuropneumoniae* (Dalai et al., 2009) and increase biofilm formation in *E. coli* K-12 (Belik and Tarasova, 2008). Only a single amino acid substitution is necessary in the N-terminal oligomerization domain of *E. coli* K-12 H-NS to strongly reduce biofilm formation (Hong et al., 2010). According to microarray analysis, H-NS regulates 19 genes related to biofilm formation (White-Ziegler and Davis, 2009). However, the effect is indirect: H-NS affects biofilm formation by regulating other nucleoid-associated proteins Cnu and StpA (Hong et al., 2010).

2.4.3 IHF and HU

IHF (integration host factor) and HU (heat unstable nucleoid protein) are abundant NAPs of the same protein family with many direct and even more indirect effects on transcription (Freundlich *et al.*, 1992, Arfin *et al.*, 2000). IHF and HU are expressed under various growth conditions and during different growth phases (Azam *et al.*, 1999). Both of them function as heterodimers consisting of two similar subunits, but the dimers of HU may also arrange into octamers (Guo and Adhya, 2007). The binding of either protein introduces bends into DNA: IHF introduces sharp bends of approximately 160° into DNA (Sugimura and Crothers, 2006), whereas HU bends DNA approximately 70° (Wojtuszewski

and Mukerji, 2003). IHF recognizes a specific consensus sequence (Table 2) with A/T-rich elements upstream of the core sequence (Hales *et al.*, 1994, Spurio *et al.*, 1997) while HU binds DNA independently of the sequence (Bonnefoy and Rouviere-Yaniv, 1991).

Firstly, IHF regulates biofilm formation indirectly by affecting the expression of other regulators. For example, IHF is known to upregulate the transcription of the global regulator Fis that is involved in biofilm regulation (Nasser *et al.*, 2002). Secondly, both IHF and HU have an unexpected effect on biofilm as structural proteins upholding the structure of extracellular DNA in the biofilm matrix (Goodman *et al.*, 2011). In *E. coli* U93 IHF and HU are specially released into the extracellular space in complex with double stranded DNA and help to form and maintain biofilm (Jurcisek *et al.*, 2017). Absence of these proteins makes extracellular DNA lose its structural stability and thereby disrupts the biofilm (Goodman *et al.*, 2011).

3. Extracellular adhesins LapA and LapF

P. putida has two known extracellular adhesins LapA and LapF, which affect biofilm (Fig 2). LapA is a key factor of *P. putida* and *P. fluorescens* biofilm formation (and absent from *P. aeruginosa*) involved in both initial attachment and mature biofilm formation (Gjermansen *et al.*, 2010). LapA is required for *P. fluorescens* to transition from reversible surface binding via its pole, to a more stable binding by its whole side (Hinsa *et al.*, 2003, Monds *et al.*, 2007). Mutants with insertion in *lapA* (mus-24) have severe seed adherence and biofilm formation defects while their chemotactic response is normal (Espinosa-Urgel et al., 2000). No conditions have been reported to efficiently rescue the *lapA* mutant's biofilm formation defect (Espinosa-Urgel *et al.*, 2000, Hinsa *et al.*, 2003, Yousef-Coronado *et al.*, 2008, López-Sánchez *et al.*, 2016).

LapA is conserved between *P. fluorescens* and *P. putida* strains, but the length of the protein varies due to flexible number of amino acid repeats (Fuqua, 2010). *P. putida* KT2440 LapA consists of 8682 amino acids and *P. fluorescens* Pf0-1 LapA of 5218 amino acids (Winsor *et al.*, 2015), making it the biggest protein in both species.

LapA consists of four domains: two different repeat domains, a short N-terminal domain and a C-terminal domain with 13 RTX (repeats-in-toxins) repeats (Satchell, 2011). LapA is a typical RTX family protein (Satchell, 2011). RTX family proteins are a heterogeneous group of proteins secreted by gram-negative bacteria. They contain glycine-rich nonapeptide repeats near the C-terminus and are transported by type I secretion system (Linhartová *et al.*, 2010). Similarly to other RTX proteins, the type I secretion system signal in LapA is also located in the C-terminus (Delepelaire, 2004).

LapA's role in both species is to provide cell-surface interactions enabling the cells to stick to a surface (Hinsa *et al.*, 2003, El-Kirat-Chatel *et al.*, 2013). LapA enables attachment to both hydrophilic and hydrophobic surfaces and

evidence suggests that it uses different mechanisms for the two. Firstly, the binding probability to both surfaces is not equal: LapA is more likely to bind to hydrophobic surfaces (El-Kirat-Chatel *et al.*, 2013). Secondly, only upon binding a hydrophilic surface, sequential unfolding of LapA, two repeats at the time, has been described upon mechanical stress (El-Kirat-Chatel *et al.*, 2013). Thirdly, LapA probably even utilizes different domains to attach to these substrates: C-terminus to attach to hydrophilic substrate and multi-repeat regions to bind to hydrophobic substrate (El-Kirat-Chatel *et al.*, 2013, Boyd *et al.*, 2014).

LapF with its 6310 amino acids is the second largest protein in *P. putida* (Hinsa *et al.*, 2003, Martinez-Gil *et al.*, 2010). It is not present in *P. fluorescens* (Fuqua, 2010). LapF is described as a necessary protein for cell-cell interactions contributing to mature biofilm, but irrelevant for attachment and early biofilm formation (Martinez-Gil *et al.*, 2010). *lapF* deficient mutants (mus-20) show a biofilm deficiency in glucose minimal medium, but no such effect can be observed in rich medium (Espinosa-Urgel *et al.*, 2000, Martinez-Gil *et al.*, 2010). Under flow conditions *lapF* mutation has a more dramatic effect as the mutants are unable to form microcolonies by themselves. However, they form a normal biofilm together with wild type cells, indicating its role in cell-cell attachment (Martinez-Gil *et al.*, 2010).

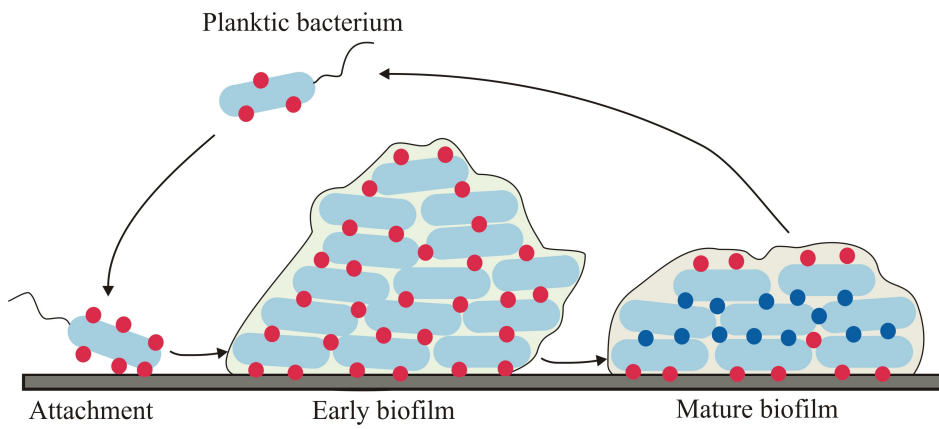

Figure 2. *P. putida* biofilm formation steps. LapA is depicted as red dots and LapF as blue dots.

3.1. Regulation of lapA and lapF expression

LapA is transcribed from the early lag phase to late stationary phase (Martinez-Gil *et al.*, 2014, Huertas-Rosales *et al.*, 2016). Curiously in the early logarithmic phase the transcriptional activity fluctuates about two-fold (Huertas-Rosales *et al.*, 2016). LapA is transported to the cell surface via the ABC

transporter LapEBC and the presence of LapA on the cell surface enables attachment and biofilm formation (Hinsa *et al.*, 2003). LapG is a posttranslational regulator of LapA that under conditions that do not favour biofilm formation (for example low P_i) cleaves LapA from the cell surface, thus removing the adhesin and preventing attachment (Newell *et al.*, 2011). The activity of the periplasmic cysteine protease LapG is regulated by the inner membrane c-di-GMP effector protein LapD. While LapD binds c-di-GMP, it undergoes a conformational change and is able to bind LapG, hindering its ability to cut LapA (Navarro *et al.*, 2011).

The transcription of *lapF* is low in the exponential phase and it increases greatly when bacteria enter the stationary phase (Martinez-Gil *et al.*, 2010). In accordance with that, the LapF protein is detectable only in stationary phase (Ref II). There are two reasons why *lapF* is expressed only in stationary phase: (i) its promoter is σ^S-dependent and (ii) in logarithmically growing cells Fis represses its expression (Martinez-Gil *et al.*, 2010, Lahesaare *et al.*, 2014). Fis binds the *lapF* promoter area overlapping the promoter and directly represses it transcription (Lahesaare *et al.*, 2014).

THE AIMS OF THE THESIS

The aim of this work was to elucidate the importance of the global regulator Fis in *P. putida* biofilm. As global regulators affect the transcription of many genes and biofilm formation in itself is a complex phenotype, the molecular link between Fis and biofilm cannot be easily deduced and needs thorough investigation.

After we saw that Fis is involved in *P. putida* biofilm formation through regulation the expression of *lapA*, our next aim was to ascertain the impact of Fis on the transcription of *lapA*. Additionally, as the location and number of *lapA* promoter(s) was unknown, it became a parallel goal to locate the promoter(s) of *lapA*.

RESULTS AND DISCUSSION

1. The *fis*-overexpression strain F15

In order to study the effects of Fis in *P. putida*, we first tried to delete the *fis* gene. Fis is known to be a non-essential protein in many bacteria, including *E. coli, V. cholera* and *S. enterica* serovar Typhimurium (Johnson *et al.*, 1988, Osuna *et al.*, 1995, Lenz and Bassler, 2007). However, we were unable to delete, disrupt or underexpress the *P. putida fis* gene (Ref I). Moreover, the closest anyone has come to obtaining a *fis* mutant in *Pseudomonas* species is a transposon insertion into the stop codon of the *P. aeruginosa fis* gene (Liberati *et al.*, 2006). Therefore, Fis is an essential protein in *P. putida* and probably also in other *Pseudomonas* species. As reducing the amount of Fis to study its effects was not an option, we increased its amount instead. To that end, we engineered *P. putida* PaW85 (isogenic to KT2440) to create an IPTG-inducible *fis*-overexpression strain F15, which has a *tac* promotor-controlled extra *fis* gene in its chromosome, delivered with the site-specific transposon mini-Tn*7* (Gm^R). Hence, adding IPTG to the growth medium will induce the over-expression of Fis in the F15 strain. As the wild-type control, we used the PSm strain throughout the experiments. PSm is also based on PaW85 but has only a mini-Tn*7* with Sm-resistance gene (Sm^R) inserted into its genome. Fis over-expression in F15 was confirmed with a Western blot analysis, which showed that Fis can be induced with IPTG in both stationary and logarithmic growth phase in LB and BS media (10% barley seedling extract in M9 buffer; Ref I, Fig 1). The growth rate measurements showed that *fis*-overexpression does reduce the growth rate of F15 approximately 1.6 times in the presence of 1 mM IPTG compared to wild type, but surprisingly only in LB media and not in BS media (Ref I, Table 2).

2. Fis overexpression reduces motility and increases biofilm formation

Since Fis is known to enhance the flagellar motility of *E. coli* (Bradley *et al.*, 2007), we measured the motility of *P. putida fis*-overexpression strain F15 and wild type PSm in semisolid LB agar. Surprisingly, the effect was opposite to expectations: *fis*-overexpression decreased the swimming motility of *P. putida*. For example, 1 mM IPTG supplementation reduced the swimming ability of F15 2.8 times in LB and 2.5 times in BS (Ref I, Table 2). The reduction of the swimming motility was probably not caused by the slower growth rate in IPTG-induced F15, as the growth rate was reduced only in LB and the swimming ability was affected in both media. Also, as we observed that Fis-overexpressing cells retained their twitching motility (Ref I, data not shown), which is an energy-demanding process (Turner *et al.*, 1993), it indicates that an energy crisis does not cause the *fis*-overexpression phenotype.

In addition to reduced swimming motility, the Fis-overexpressing bacteria formed aggregates in semisolid LB agar that were visible in a light microscope (Ref I). The wild type strain did not produce visible aggregates. This prompted the idea that *fis*-overexpression may increase biofilm formation. Indeed, over-expressed *fis* increases the amount of 24-hour biofilm on polystyrene multi-well plates compared to F15 without IPTG supplementation in LB and BS media, up to 2.9 times and 1.9 times, respectively (Ref I, Fig 3). This indicates that Fis regulates *P. putida* biofilm formation, either directly or indirectly.

3. Biofilm timepoints

As both the expression of Fis and the amount of biofilm are growth phase dependent, we studied the effect of Fis on biofilm formation in different time points. Fis mRNA levels are highest in exponentially growing planktonic cells and drop approximately three times in stationary phase cells (Yuste *et al.*, 2006). The amount of *P. putida* biofilm, unlike many other bacteria, changes dynamically in time, reaching its peak somewhere between 4 and 10 hours of growth ("early biofilm") and decreasing more than twofold by approximately 24 hours ("mature biofilm") (Yousef-Coronado *et al.*, 2008, Gjermansen *et al.*, 2010, Yousef-Coronado *et al.*, 2011, Martinez-Gil *et al.*, 2014). We assessed the effect of *fis*-overexpression on 4-, 8- and 24-hours-old biofilm (Ref II, Fig 4). Our data confirmed that the amount of wild type *P. putida* biofilm reaches a peak early on and drops more that twofold by the 24th hour. The 4-hours-old biofilm was approximately 2.5 times higher than 8- or 24-hour biofilm (Ref II, Fig 4). *Fis*-overexpression has no additional effect on early biofilm: *fis*-over-expressing cells formed a similarly high amount of biofilm to wild type at 4 hours (Ref II, Fig 4A). The positive effect of Fis was visible on the 8-hours-old biofilm (Ref II, Fig 4B) and the strongest on the 24-hour biofilm (Ref II, Fig 4C). *Fis*-overexpression has no effect to 4-hour-old biofilm probably because Fis levels are naturally high in logarithmically growing bacteria and Fis binding sites are saturated.

4. Possible Fis target genes

Fis as a global regulator has a wide array of different possible pathways to affect biofilm. Firstly, it can change the expression of biofilm-related genes either directly or indirectly (by affecting other regulators). Fis itself can contact RNA polymerase or modulate local DNA conformation to regulate transcription (Schneider *et al.*, 1999, Opel *et al.*, 2004). Secondly, Fis can regulate trans-cription indirectly by changing global DNA superspiralisation or the physio-logical state of the cell.

We selected mini-Tn*5* transposon mutagenesis to find possible Fis target genes involved in biofilm formation as this method allowed us to assess

whether Fis regulates a specific set of genes or rather affects bacterial physiology. An even distribution of transposon insertions over the genome would indicate physiological effects and the opposite, many transposon mutants in certain genes, would indicate the regulation of a specific set of gene. As a biofilm formation assay would be unfeasible on a large scale, we opted for an experimental setup using the opposite phenotype – motility. We tested the motility of F15 mini-Tn*5* transposon (Km^R) mutants on King B medium with 0.8% agar in the presence of IPTG. In these conditions, the *fis*-overexpressing strain is unable to move (Ref II, Fig 2) and it is possible to visually detect transposon mutants with recovered motility. We analysed approximately 40 000 colonies and detected 155 with improved motility. We localized the mini-Tn*5* insertion site in 79 mutants and excluded three that had an insertion in the *fis*-overexpression cassette. Most transposon mutants with increased motility had an insertion into the *lap* genes (Ref II, Table 2). Out of the 76 transposon mutants with increased motility and functional *fis*-overexpression cassette, 68 had an insertion in the *lap* genes: 56 hits were detected in *lapA*, 6 in *lapD* and 5 in *lapB* and *lapC*, the LapA transport system genes. All the F15 *lap* mutants also exhibited reduced biofilm formation compared to F15 (Ref II, Table 2). These results prompted the hypothesis that (among other regulatory possibilities) Fis regulates biofilm formation through the *lap* genes.

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

To test whether Fis increases biofilm formation via *lapA* expression, we constructed a *lapA* deletion strain. Although our transposon assay identified no insertions in the *lapF* gene, we also included it in our experiments, as it is one of the two extracellular adhesins in *P. putida*. To test the effects of *lapF* we made *lapF* and *lapAlapF* strains from PSm and F15. Fis overexpression in all F15 *lap* mutants was confirmed with Western blot analysis (Ref II, Fig 3). We measured the biofilm formation of PSm and F15 *lapA* and *lapF* deletion strains in LB. Firstly, the disruption of *lapA* reduced wild type biofilm formation and the disruption of *lapF* had no effect (Ref II, Fig 4), which is in accordance with previously published results (Espinosa-Urgel *et al.*, 2000, Hinsa *et al.*, 2003, Yousef-Coronado *et al.*, 2008, Martinez-Gil *et al.*, 2010). The effect of *lapA* deletion was most obvious on 4-hour-biofilm but was retained in mature biofilm as well (Ref II, Fig 4AC). Our results confirm that in a rich medium, LapA is more important for biofilm formation than LapF. Secondly, *fis*-overexpression's positive effect on biofilm depended on functional *lapA* (Ref II, Fig 4BC). The deletion of *lapA* decreased the amount of Fis-induced 24-hour-biofilm to the level of F15 with no IPTG supplementation (Ref II, Fig 4C). The deletion of *lapF* from F15 did not decrease the Fis-enhanced biofilm (Ref II, Fig 4). This shows that Fis increases biofilm formation through *lapA* but not through *lapF*.

Since we observed that Fis-enhanced biofilm depends on the presence of LapA, an extracellular protein, we tested whether proteins are a major constituent of the Fis-induced biofilm. In order to do that, we treated the 24-hour-old biofilm with either proteinase K, DNase I or cellulase for 2 hours. DNase I and cellulase were unable to degrade the mature biofilm (Ref II, data not shown). Proteinase K did not only degrade the Fis-enhanced biofilm down to the level of F15 with no IPTG supplementation, but decreased the biofilm of all strains except for *lapAlapF* (Ref II, Fig 4C). This is in accordance with previous results demonstrating that *P. putida* biofilm is proteinaceous (Jahn *et al.*, 1999). Our results show that protein is also the major constituent in Fis-induced biofilm and in that sense, Fis-enhanced biofilm and wild type biofilm are similar.

Still, proteinase K did not degrade the strong Fis-enhanced biofilm of F15 and F15 *lapF* down to the same amount as it degraded the biofilm of PSm and PSm *lapF*. This might indicate that *fis*-overexpression promotes the production of a non-proteinaceous component in addition to proteinaceous component. It has even been described that the absence of LapA and/or LapF increases the amount of exopolysaccharides in the biofilm matrix (Martínez-Gil *et al.*, 2013). However, if *fis*-overexpression induced exopolysaccharide production, proteinase K should also be unable to degrade F15 *lapA* and F15 *lapAlapF* down to the level on PSm *lapA* and PSm *lapAlapF*, which is not the case. Therefore, *fis*-overexpression probably does not enhance biofilm by inducing the production of exopolysaccharides, but rather it is a technical issue of proteinase K being unable to degrade the strong Fis-enhanced biofilm at the same efficiency as PSm biofilm.

6. Fis overexpression increases the amount of LapA

To investigate whether *fis*-overexpression increases the amount of LapA, we measured the quantity of LapA and as a control also LapF in *P. putida*. For this, we ran crude cell lysates on SDS-PAGE and visualized the proteins by silver staining (Ref II, Fig 5AB). As LapA and LapF are the largest proteins in *P. putida*, they can be easily identified by comparing the topmost band patterns of the respective deletion strains. The intensities of LapA and LapF bands were normalized against corresponding bands of PSm grown without IPTG (Ref II, Fig 5CD). We first set out to measure the amount of LapA at 4 hours, the same timepoint we used for biofilm formation. However, we encountered strong sample-to-sample variation. This is in agreement with later transcriptional measurements (Huertas-Rosales *et al.*, 2016), which show that the logarithmic phase fluctuation of LapA amounts stems from highly variable transcription levels. Therefore, the fast growing cells were assayed at 2.5 hours, where the expression was more stable. In addition, we measured the amount of adhesins in stationary phase cells (18 hours).

We saw that LapA was present in both fast growing and stationary phase cells, while LapF only in stationary phase cells (Ref II, Fig 5A). Our protein

quantity results correlated with previously measured transcriptional activities showing that *lapA* is persistently transcribed and the transcription of *lapF* emerges in stationary phase (Martinez-Gil *et al.*, 2014).

Gel quantification revealed that *fis*-overexpression increased the amount of LapA 1.6 times in stationary phase cells (Ref II, Fig 5BC) but not in fast growing cells (Ref II, Fig 5AC). This correlates with *fis*-overexpression's effect on biofilm, which is also apparent in the stationary phase (Ref II, Fig 4C) but not in growing bacteria (Ref II, Fig 4A). Surprisingly, Fis overexpression also decreased the amount of LapF in stationary phase cells approximately 4 times (Ref II, Fig 5BD), indicating that Fis acts as a repressor for *lapF*.

The effect of *fis*-overexpression on the amount of LapA was verified by using FACS to monitor the fluorescence of LapA-GFP (green fluorescent protein) fusion protein. The FACS result supported the gel quantification results showing that the quantity of LapA depended positively on the amount of Fis (Ref II, Fig 6). Therefore, it seems probable that Fis is a positive regulator of LapA.

Altogether, the results confirm that Fis enhances biofilm formation through upregulating LapA. This is backed up by experiments showing that (i) Fis-induced biofilm is proteinaceous and depends on the functional *lapA* and that (ii) Fis increases the amount of LapA.

7. There are unusually many promoters in front of *lapA*

In order to ascertain whether Fis regulates the transcription of *lapA* directly or affects its expression indirectly, we first needed to map the positions of *lapA* promoters. Although the transcription of *lapA* had been studied, the amount and location of its promoters remained unknown. Therefore, previous works have used promoter areas that did not contain all the actual promoters. To find the promoters of *lapA*, we mapped the 5′ ends of its mRNA purified from exponential and stationary phase LB-grown *P. putida* by RACE. This gave us 8 possible transcription start sites altogether in exponential and stationary phase. The positions identified in the two growth phases were the same, except for 5′ ends corresponding to promoters P_{lapA5} and P_{lapA8}. We were unable to identify these in exponential phase samples, although this may have been due to technical reasons. Thereafter we predicted the -10 boxes of the eight putative promoters P_{lapA1} to P_{lapA8} (Ref III, Fig 1 and Fig 2) using the consensus sequence of *E. coli* σ^{70}-dependent promoters (Hawley and McClure, 1983).

To confirm that the identified 5′ mRNA ends correspond to transcription start sites, we cloned successively longer *lapA* upstream fragments to a *lacZ* reporter system and measured the resulting β-galactosidase activities. The shortest fragment contained only the most proximal hypothetical *lapA* promoter and the successive extensions added one hypothetical promoter at a time (Ref III, Table 1). This experiment suggested that only P_{lapA3} and P_{lapA6} are *lapA*'s promoters. However, adding potential promoters to the construct did not always increase the activity of the promoter construct and adding one particular pro-

moter, $P_{lapA7,}$ even decreased the activity (Ref III, Table 1). Thus, we are probably adding regulator binding sites as well as promoters and additional regulators that bind longer fragments may mask the effect of weaker promoters in these constructs. Therefore, we decided to test the promoters individually by cloning all of them one by one in front of a *lacZ* reporter gene and measuring their β-galactosidase activity. All of the promoters except for P_{lapA1} induced β-galactosidase activity in PSm (Ref III, Table 1). To further investigate whether P_{lapA2} to P_{lapA8} are functional promoters, we mutated their potential -10 boxes. Disrupting the putative -10 boxes strongly decreased the activity of P_{lapA3} to P_{lapA8}, but did not affect the activity of P_{lapA2}, which we discarded as a non-functional σ^{70}-type promoter (Ref III, Table 1). Altogether, we identified six functional promoters for *lapA*, which were all negatively affected by the substitutions in potential -10 boxes. All of those promoters were active in both exponential and stationary phase cells (Ref III, Table 1). These experiments allowed us to determine the correct length of the *lapA* promoter area to be used in later transcription measurements.

Six promoters is an unusually high number, as most tested *E. coli* genes have probably one or two (Mendoza-Vargas *et al.*, 2009, Conway *et al.*, 2014). This many promoters indicates the complexity of *lapA* transcriptional regulation. The most proximal promoter of *lapA*, P_{lapA3}, seems to be the most important as it provides a high transcriptional activity in LB-grown exponential and stationary phase cells (Ref III, Table 1). Also, when we successively extended the upstream region of *lapA*, adding one hypothetical promoter at a time (Ref III, Table 1), P_{lapA3} was one of the two promoters that emerged as significant. The other one, P_{lapA6}, which' addition also increased the activity of the promoter area, surprisingly provided only a low transcriptional activity when measured individually. This means that by extending the promoter area to add P_{lapA6}, we may have also added a binding site of a positive regulator. P_{lapA6} was not the only promoter with low activity, P_{lapA7} also provided a low transcriptional activity. The contribution of these low-activity promoters to the expression of *lapA* might seem insignificant in LB media but it may be considerable in specific environmental conditions. For example, *P. putida* genes *algD*, *hsdM* and *gltX* display a very low basal level of transcription in M9-citrate medium and are strongly activated in the presence of root exudates (Ramos-González *et al.*, 2005). Therefore, it is possible that under different conditions, the transcription of *lapA* promoters changes.

8. Three *lapA* promoters are partially RpoS-dependent

We tested the RpoS-dependency of the *lapA* promoters by measuring the β-galactosidase activity of promoter constructs in stationary phase PSmΔ*rpoS* (Ref III, Table 1). Our results showed that the stationary phase sigma factor is involved in the regulation of three distal promoters: P_{lapA6}, P_{lapA7} and P_{lapA8} (Ref III, Table 1). As expected, rpoS, which' transcription and translation are down-

regulated in exponential phase (Kojic and Venturi, 2001, Yuste *et al.*, 2006, Jovcic *et al.*, 2008), had no effect to the transcription of P_{lapA6}, P_{lapA7} nor P_{lapA8} in logarithmically growing cells.

The effects of *rpoS* deletion were moderate, indicating partial σ^S-dependence of these promoters. As σ^S and the housekeeping sigma σ^{70} recognize a similar promoter consensus (Tanaka *et al.*, 1993, Gaal *et al.*, 2001), these promoters are probably controlled by both sigma factors.

9. Fis binds *lapA* promoter area in six specific positions *in vitro*

To elucidate how Fis increases the expression of LapA, we tested whether the *lapA* promoter area contains any Fis binding sites. *In silico* prediction revealed eight possible Fis binding sequences, Fis-A1 to Fis-A8 (Ref III, Table 2). Those sequences were subjected to DNase I footprint and gel-shift analysis (Ref III, Fig 4–7). Our results show that Fis binds six of these sites *in vitro* (Fig 3), with only Fis-A3 and Fis-A8 being false positive predictions. Mutating the Fis binding sites hindered Fis binding and enabled easier outcompetition by Fis-specific DNA (Ref III, Fig 4–7), further confirming Fis binding to these six specific sites in the *lapA* promoter area.

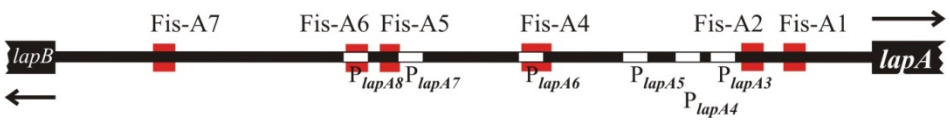

Figure 3. A schematic representation of *lapA* promoters and Fis binding sites. Promoters P_{lapA3} to P_{lapA8} are depicted as white boxes and Fis binding sites Fis-A1 to Fis-A7 (Fis-A3 is not depicted as no binding was detected) as red boxes. The back arrows show the beginnings of *lapA* and *lapB* genes.

10. The upregulation of *lapA* transcription depends on Fis binding sites Fis-A5 and Fis-A7

To ascertain whether Fis regulates the transcription of *lapA*, we measured the β-galactosidase activity of the 951 bp *lapA* promoter area containing all six promoters and six Fis binding sites, cloned into a low-copy-number promoter probe vector $p9TT_B$lacZ. We observed that *fis*-overexpression increases the transcription of *lapA* 1.4 times in stationary phase cells (Ref III, Fig 8A), revealing that elevated levels of Fis indeed activate the transcription of *lapA*.

To elucidate, which Fis binding sites are necessary for the transcriptional activation of *lapA* in the stationary phase, we mutated the Fis binding sites. Mutating Fis binding sites Fis-A5 and Fis-A7 abolished the positive effect of Fis on the transcription of *lapA* while mutating the rest of the binding sites did

not. This shows that Fis' positive effect on *lapA* transcription *in vivo* depends on the distal Fis binding sites, Fis-A5 and Fis-A7 (Ref III, Fig 8AFH). The rest of the Fis binding sites, Fis-A1, Fis-A2 and Fis-A6, have a redundant impact, if any, to *lapA* transcription. The importance of the Fis-A4 binding site in the regulation of *lapA* transcription stays unclear, as by mutating Fis-A4 site to the extent that Fis will no longer bind it, we also disrupted the overlapping P_{lapA6} promoter (Ref III, Fig 11AB). Therefore, we are unable to distinguish between the effects of disrupting the binding site and the promoter.

11. Fis-A7 binding upregulates its closest downstream promoter and Fis-A5 probably changes promoter area topology

As we saw that Fis-A5 and Fis-A7 binding sites were important for Fis-enhanced *lapA* transcription, we wanted to see whether Fis binding to these sites affects the transcription from their respective downstream promoters. Fis-A7 is located upstream of the P_{lapA8} promoter (Ref III, Fig 2). We saw that *fis*-overexpression increases the activity of P_{lapA8} 1.8 times compared to no IPTG supplementation and mutating the Fis-A7 binding site abolished the positive effect (Ref III, Fig 10). This indicates that Fis binds the Fis-A7 site and directly increases the transcription from P_{lapA8}.

Fis-A5 is located upstream of the P_{lapA7} promoter and overlaps it slightly (Ref III, Fig 2). *Fis*-overexpression seemed to repress the activity of the individual promoter P_{lapA7} (Ref III, Fig 11C), but mutations in Fis-A5 had no effect on the P_{lapA7} activity. Hence, Fis-A5 is not important for the transcriptional activation from P_{lapA7} (Ref III, Fig 11C), but it increases the transcription from the full-length *lapA* upstream region (Ref III, Fig 8F). Therefore, it probably takes part in creating a topology of the *lapA* upstream DNA that enables the transcriptional activation. Considering that P_{lapA7} is a weak promoter (Ref III, Table 1 and Fig 11C) and that the sum of effects is transcriptional activation, the main effect of Fis binding to Fis-A5 seems to be modifying *lapA* upstream DNA topology and not decreasing the transcription from P_{lapA7}.

Fis' positive effect on *lapA* transcription can be explained by two unexclusive mechanisms. Firstly, Fis directly increases the transcription from the most distal promoter P_{lapA8} by binding upstream of it on the Fis-A7 site (Ref III, Fig 10). Secondly, Fis can regulate *lapA* transcription by modifying the topology of the promoter area. One of the Fis binding sites important for Fis-enhanced transcription from *lapA*, Fis-A5 does not take part in the activation of its closest downstream promoter P_{lapA7} (Ref III, Fig 11C). Also, we cannot exclude the possibility that Fis-A1, Fis-A2, Fis-A4 and/or Fis-A6 binding sites could contribute to the *lapA* promoter area topology. Altogether, Fis has probably diverse effects: it directly activates the transcription from P_{lapA8} promoter and changes the DNA topology of the whole *lapA* promoter area.

12. The transcriptional regulation of *lapA*

In this work, we demonstrate that elevated levels of Fis upregulate the transcription of *lapA*. These results show that the positive effect of Fis directly depends on Fis binding to two sites: Fis-A5 and Fis-A7. The latter upregulates its nearest downstream promoter and Fis-A5 binding probably affects the topology of the promoter area (Fig 4). However, Fis is obviously not the only regulator affecting the transcription of *lapA*. The alarmones (p)ppGpp and c-di-GMP, the regulator FleQ and the two-component system GacS/A have all been shown to regulate the transcription of *lapA* (Martinez-Gil *et al.*, 2014, Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016, Díaz-Salazar *et al.*, 2017). The question remains, which of these other regulators directly affect *lapA* transcription and which function through other downstream effectors?

C-di-GMP is known to regulate the transcription of *lapA* through FleQ (Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016), but does FleQ affect the transcription of *lapA* directly? The experiments conducted in *E. coli* background with FleQ expressed from a plasmid showed no effect on the transcription of *lapA*, indicating either indirect regulation or the need for other *P. putida* specific factors (Jimenez-Fernandez *et al.*, 2016). On the other hand, FleQ has been shown to bind the promoter area of *lapA* by gel shift (Jimenez-Fernandez *et al.*, 2016, Xiao *et al.*, 2016). The exact positions of FleQ binding sites are yet to be determined, but FleQ has been predicted *in silico* to bind the *lapA* promoter area in three sites (Jimenez-Fernandez *et al.*, 2016). The two proximal sites overlap with Fis-A1 and Fis-A2. We saw that mutating Fis-A1 and Fis-A2 binding sites did not diminish the Fis effect, but decreased the overall transcriptional activity of the *lapA* promoter area (Ref III, Fig 8). By mutating Fis-A2 we also substituted three nucleotides in the predicted overlapping FleQ site and by mutating Fis-A1 we replaced 6 nucleotides adjacent to the predicted FleQ site. Therefore, it is probable that by mutating the Fis binding sites, the binding of FleQ was hindered. This indicates that the two proximal FleQ sites are roughly where predicted and that the effect of FleQ is probably direct. However, the direct binding of FleQ to these hypothetical sites still needs experimental verification.

The indirect effect of the alarmone (p)ppGpp is well described: (p)ppGpp increases the transcription of *lapA* by stimulating the transcription of the c-di-GMP hydrolase gene *bifA* (Díaz-Salazar *et al.*, 2017). The question is whether (p)ppGpp also affects *lapA* directly. (p)ppGpp is known to repress promoters with GC-rich discriminator sequences between the -10 box and transcription start site (Travers, 1980). Identifying the positions of *lapA* promoters made it is possible to look for discriminator sequences. However, only the most distal promoter, P_{lapA8} has a discriminator-like sequence. Therefore it is unlikely that (p)ppGpp directly represses the transcription of *lapA*, but to rule it out completely, it needs further experimental confirmation.

The effect of the GacS/A system on *lapA* transcription can be either direct or indirect, but as this two-component system is part of a larger pathway (Fig 1), indirect regulation seems more likely.

The regulators described so far (Fig 4) are probably not a complete list. When we extended the promoter area of *lapA*, we saw that adding the promoter P_{lapA7} decreased the transcriptional activity of the *lapA* upstream fragment (Ref III, Table 1). Therefore, we likely added the binding site of an undescribed direct repressor as part of that sequence. Also, Fis as a global regulator could affect the transcription of *lapA* through downstream effectors as well. We suspected that Fis may regulate the transcription of *fleQ*, but as we were unable to show *in vitro* Fis binding to the *fleQ* promoter region (data not shown), we can conclude that Fis is not a direct regulator of *fleQ*.

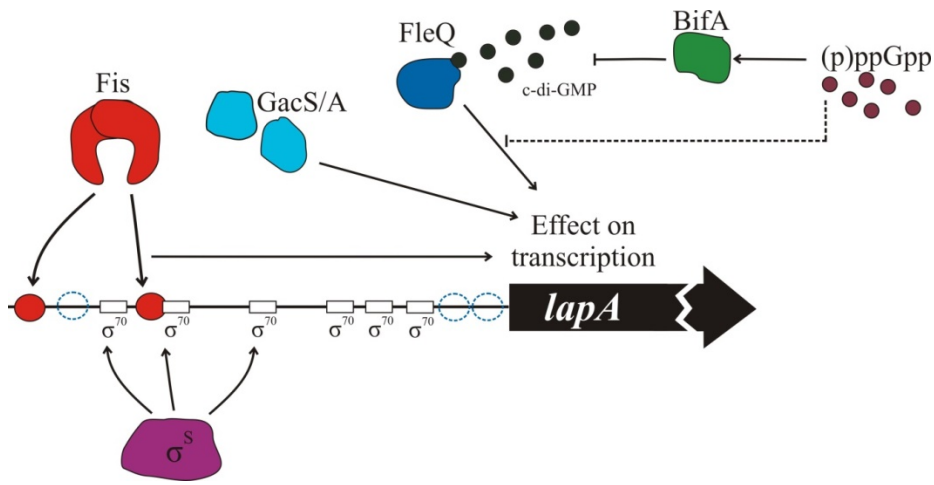

Figure 4. The transcriptional regulators of *lapA*. Promoters are depicted as white boxes, *lapA* gene as a black arrow, Fis binding sites as red circles and *in silico* predicted FleQ binding sites as dotted circles. Lines ending with arrows denote positive effects and lines ending with T-shapes denote negative effects. Dotted lines indicate possible effects.

13. *P. putida* biofilm as a well-being phenotype

The interesting thing about *Pseudomonas* species' biofilm is that it is quite clearly a response to well-being. *P. putida* forms the strongest biofilm in rich media and when the nutrients start to run out, the strong biofilm decays (Fig 2). We have seen that *P. putida* forms very strong 4-hour-old biofilm and that it decays about 3-fold in the next 4 hours (Ref II, Fig 4). Similar trends have been previously described by several authors (Yousef-Coronado *et al.*, 2008, Gjermansen *et al.*, 2010, Yousef-Coronado *et al.*, 2011). The idea that *P. putida*

biofilm is a well-being phenotype is backed up by recently published data demonstrating that the stress alarmone (p)ppGpp promotes biofilm dispersal (Díaz-Salazar *et al.*, 2017). (p)ppGpp regulates biofilm in many bacteria such as *L. monocytogenes* (Taylor *et al.*, 2002), *S. mutans* (Lemos *et al.*, 2004), *V. cholerae* (He *et al.*, 2012) and *E. coli* (Åberg *et al.*, 2006), but in these cases it instead leads to biofilm formation. This shows that in most bacteria, biofilm formation is more closely linked to the stress response, but *Pseudomonas* species stand out with their opposite biofilm regulation.

Our finding that Fis increases *P. putida* biofilm formation contributes to the theory that biofilm is a well-being phenotype. Fis is naturally produced in response to fast growth in nutrient-rich conditions (Yuste *et al.*, 2006), which coincides with strongest biofilm formation. By overproducing Fis in stationary phase, we have managed to artificially induce the signal for good conditions and trick the cells into producing high amounts of biofilm. *Fis*-overexpression increases biofilm formation by upregulating the transcription of *lapA* (Ref III), which in turn increases the amount of the LapA protein (Ref II). Therefore, Fis retains the early strong biofilm through to the stationary phase.

In fast growing bacteria, LapF is not produced (Ref II, Fig 5a). The transcription of *lapF* is repressed because of two reasons: its promoter is σ^S-dependent and Fis represses its expression (Martinez-Gil *et al.*, 2010, Lahesaare *et al.*, 2014). Our group's work has shown that Fis directly represses the transcription from the only *lapF* promoter (Lahesaare *et al.*, 2014). Therefore, LapF is not part of the well-being biofilm. LapA is not necessary for early biofilm nor is it important for biofilm formation in rich media (Espinosa-Urgel *et al.*, 2000, Martinez-Gil *et al.*, 2010). However, it has been shown to be useful for biofilm formation in minimal media (Martinez-Gil *et al.*, 2010). This raises the question, does *P. putida* also have a non-well-being biofilm, where it utilizes LapF? While the very strong biofilm appears as a response to abundant nutrients, a reasonable amount of biofilm is still present in LB media after 24 hours (Ref II, Fig 4). Also, *P. putida* forms some biofilm in almost any growth medium provided that a carbon source is present. Certain stressors can (to some extent) even increase the amount of mature biofilm (Baumgarten *et al.*, 2012). This indicates that *P. putida* also has a non-well-being biofilm. Whether LapF takes part in it, is still debatable, however, there are several links between poor conditions and LapF. In minimal media that have less abundant nutrients, LapF is necessary for biofilm formation (Martinez-Gil *et al.*, 2010). Also, the stringent response alarmone (p)ppGpp increases the amount of LapF, which further links this adhesion with stressful conditions (Liu *et al.*, 2017).

To conclude, *P. putida* seems to need biofilm formation in both favourable conditions and during stress. The early strong biofilm contains a lot of Fis-induced LapA and is a response to nutrient abundancy. The mature biofilm contains more LapF than LapA and is probably a response to oncoming stress. In rich media, LapF is never absolutely necessary, but in stress conditions it probably proves useful for bacteria.

CONCLUSIONS

It is no surprise that the most capable modulators of bacterial life, the global transcriptional regulators, are linked to one of the major choices that faces a growing bacterial culture: whether to swim around or stay put and form a biofilm. One global regulator that has been previously linked with biofilm is Fis. Its effect on biofilm in different bacteria is either negative or positive and the mechanisms seem diverse. We study the effects of Fis on *P. putida* biofilm and have seen Fis overexpression's positive effect on mature biofilm. *Fis*-overexpression has no additional effect on the strong early biofilm probably because Fis levels are naturally high in logarithmically growing bacteria and Fis binding sites are saturated. In the course of this work, we discovered that Fis' effect on biofilm depends on the extracellular adhesin LapA. We present evidence that Fis regulates biofilm formation via controlling the expression of *lapA*:

- Fis-induced biofilm is proteinaceous and depends on the functional *lapA*.
- Fis overexpression activates the transcription of *lapA* and increases the amount of LapA protein in stationary phase cells.
- Fis binds *lapA* promoter area in six specific positions *in vitro*.
- Two out of the six Fis binding sites, Fis-A5 and Fis-A7, are responsible for the positive effect of Fis on the transcription of *lapA*. Fis binding to Fis-A7 upregulates its closest downstream promoter and Fis binding to Fis-A5 probably changes promoter area topology.

As part of this work, we described the promoter area arrangement of *P. putida lapA*. Although the transcription dynamics over growth phases had been studied before, the molecular regulation of transcription along with the exact number and location of the promoters was unknown. Acquired knowledge on *lapA* promoters can be concluded as:

- The transcription of *lapA* in LB-grown bacteria is initiated from six promoters.
- The most proximal promoter seems to provide the strongest transcriptional activity.
- Three distal promoters are partially σ^S-dependent.

The amounts of biofilm and Fis are in good correlation, with both being most prominently found during exponential growth and the levels dropping upon entry into stationary phase. When we overproduce the fast-growth-associated Fis in the stationary phase, the cells are tricked to interpret it as a sign for good growth conditions and produce large amounts of biofilm. This ties in with the fact that extra Fis cannot induce a higher than normal level of biofilm in logarithmically grown cells. However, when the native amount of Fis decreases, artificial Fis overexpression can retain the biofilm at its peak level even when the nutrients start to deplete and growth slows down. These results summarized above lend support to the hypothesis that *P. putida* biofilm is a well-being phenotype. However, lesser amounts of *P. putida* biofilm remain while nutrients

are depleting and certain stressors can even increase the amount of mature biofilm to some extent. Therefore, it can be concluded that *P. putida* needs biofilm in good times and in bad, in sickness and in health.

SUMMARY IN ESTONIAN

Fis suurendab *Pseudomonas putida* biofilmi hulka, tõstes *lapA* ekspressiooni

Bakterid elavad looduses valdavalt biofilmis, pinnale kinnituvate bakterite kogumis. Biofilmis elamisel on bakterite jaoks mitmeid eeliseid. Biofilmis elavad bakterid on kahjulike keskkonnamõjude eest paremini kaitstud ja heades tingimustes aitab pinnale kinnitumine nendesse tingimusesse jäädagi. Biofilm on bakterite jaoks selgelt kasulik, kuid inimestele seostub esmalt suurte kahjudega tööstuslikus tootmises, ummistunud torude, saastunud joogivee ja resistentsete haigustega. Kuigi biofilmid on osalised kõigis eelmainitud hädades, siis paljud biofilmi moodustavad bakterid on ka kasulikud. Oma doktoritöös uurin ühe sellise kasuliku bakteri, *Pseudomonas putida*, biofilmi moodustumist. *P. putida* on kosmopoliitne bakter, mida leidub laialdaselt nii mullas kui vees, aga mis eelistab koloniseerida risosfääri ja juurtel biofilmi moodustada. *P. putida* biofilm kaitseb taimi, ka põllumajanduslikult olulisi kultuure, patogeenide eest ja soodustab nende kasvu (Espinosa-Urgel jt, 2000).

P. putida biofilmi maatriks koosneb suurel määral valkudest, sealhulgas suurtest pinnavalkudest LapA-st ja LapF-ist. Neist kahest suurem, LapA, on väga oluline nii pinnale kinnitumiseks kui biofilmi moodustamiseks (Espinosa-Urgel jt, 2000; Hinsa jt, 2003; López-Sánchez jt, 2016; Yousef-Coronado jt, 2008). Käesoleva uurimistöö alguses ei olnud *P. putida* biofilmi veel erilise põhjalikkusega uuritud, kuid oli teada, et LapA hulka raku pinnal saab bakter vähendada selle pinnalt lahti lõikamisega ning seda protsessi reguleerib alarmoon c-di-GMP (Navarro jt, 2011; Newell jt, 2011). Praeguseks on kirjeldatud, et ka teine alarmoon (p)ppGpp, transkriptsiooni regulaator FleQ ja GacS/A kahekomponentne süsteem mõjutavad *P. putida* biofilmi moodustumist (Martinez-Gil et al., 2014, Díaz-Salazar et al., 2017).

Meie avastasime juhuslikult, et globaalne regulaator Fis mõjutab *P. putida* biofilmi moodustamist. Kuna Fis on globaalne transkriptsiooni regulaator, siis võib see transkriptsiooni mõjutada nii otse kui kaudselt. Fis mõjutab geeni otseselt, kui seondub selle promootoralale ja kas aktiveerib või represseerib geeni transkriptsiooni. Fis-i üleekspressiooni tüve (mida kasutame, kuna *fis* on *P. putida*'s hädavajalik geen) kirjeldades avastasime, et suurenenud Fis-i hulk vähendab bakterite liikumist. Kuna biofilmi moodustumine on liikumise vastandfenotüüp, tekkis hüpotees, et Fis soodustab biofilmi moodustumist. Fis-i üleekspressioon soodustabki küpse biofilmi moodustumist, aga ei mõjutanud juba niigi väga tugeva noore biofilmi teket.

Käesoleva doktoritöö eesmärgiks oli selgitada, kuidas Fis mõjutab biofilmi moodustumist. Nähes, et Fis-i üleekspressioon suurendab LapA hulka rakus, uurisime süvitsi Fis-i mõju *lapA* transkriptsioonile. Meie järgnevalt kokku võetud tulemused näitavad, et Fis mõjutab biofilmi moodustamist *lapA* ekspressiooni soodustades.

- Fis-i üleekspresseeriva tüve biofilm on valgurikas ja moodustub vaid LapA olemasolul.
- Fis-i üleekspressioon suurendab LapA hulka statsionaarse faasi rakkudes.
- Fis aktiveerib *lapA* transkriptsiooni.
- Fis seondub *lapA* promootoralale *in vitro* kuude positsiooni.
- Kaks seondumiskohta, Fis-A5 ja Fis-A7, on olulised Fis-i soodustavaks mõjuks *lapA* transkriptsioonile. Fis seondumine Fis-A7-le soodustab allavoolu jääva promootori transkriptsiooni ja Fis-A5-le seondumine mõjutab arvatavasti promootorala topoloogiat.

Töö teise osana kirjeldasime *lapA* promootorala ehitust. Kuigi *lapA* transkriptsiooni dünaamika oli selleks hetkeks juba kirjeldatud, siis promootorite arv ja asukoht oli teadmata. Teadmised *lapA* promootorite kohta võib kokku võtta järgnevalt:

- Kirjeldasime 6 *lapA* promootorit.
- Geenile lähim promootor on arvatavasti ka kõige tugevam.
- Kolm geenist kaugeimat promootorit on osaliselt σ^S-sõltuvad.

P. putida biofilmi ja Fis-i hulk paistab korreleeruvat: eksponentsiaalses kasvufaasis on palju Fis-i ja tugev biofilm ning statsionaarseks faasiks on mõlema hulk oluliselt langenud. Kui me kiire kasvu faktori, Fis-i üleekspresseerime, tõlgendavad rakud seda kui märki headest tingimustest ja moodustavad palju biofilmi. Kooskõlas sellega ei suuda täiendav Fis eksponentsiaalselt kasvavates rakkudes indutseerida tavapärasest kõrgemat biofilmi taset. See on arvatavasti tingitud sellest, et kiirelt kasvavates rakkudes on looduslik Fis-i tase juba niivõrd kõrge, et Fis-i biofilmi reguleerivad seondumiskohad on küllastunud. Kui aga looduslik Fis-i tase langeb, hoiab kunstlikult üleekspresseeritud Fis biofilmi kõrgel tasemel sõltumata sellest, et toitained on otsa lõppemas ja kasv aeglustub. Seega on *P. putida* biofilm pigem ikkagi heaolufenotüüp, mis kaasneb toitainete rikkuse ja kiire kasvuga.

Kokkuvõttes lõi see töö uusi teadmisi *P. putida* biofilmi regulatsioonist ja aitab seega paremini mõista selle kasuliku mullabakteri elu väga olulist etappi.

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PUBLICATIONS

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2. Lahesaare, A., Ainelo, H., Teppo, A., Kivisaar, M., Heipieper, H.J. and Teras, R., 2016. LapF and its regulation by Fis affect the cell surface hydrophobicity of *Pseudomonas putida*. Plos one, 11(11): p.e0166078.
3. Moor, H., Teppo, A., Lahesaare, A., Kivisaar, M. and Teras, R., 2014. Fis overexpression enhances *Pseudomonas putida* biofilm formation by regulating the ratio of LapA and LapF. Microbiology, 160(12): 2681–2693.
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