Responses of KT2440 Pseudomonas putida to mild water stress

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Department of Environmental Engineering

PhD Thesis
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Preface

This thesis is based on the PhD project carried out at the Department of Environmental Engineering, Technical University of Denmark. The project was supervised by Professor Barth F. Smets, and Dr. Arnaud Dechesne from Department of Environmental Engineering (DTU).

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This thesis is based on 3 journal articles (I, II, and III) and 1 report (IV).

I. Dechesne A., G. Wang, G. Gulez, D. Or, and B.F. Smets. 2010. Hydration controlled bacterial motility and surface dispersal. Proc. Natl. Acad. Sci. USA. 107:14396-14372

II. Gulez, G., A. Dechesne, and B.F. Smets. 2010. The Pressurized Porous Surface Model: An improved tool to study bacterial behavior under a wide range of environmentally relevant matric potentials. J. Microbiol. Methods. 82: 324-236

III. Gulez, G., A. Dechesne, C. Workman, and B.F. Smets (2011, in revision for Appl. Env. Microbiol.) Whole genome expression dynamics of *Pseudomonas putida* KT2440 under water stress on porous surfaces

IV. Role of EPS components on the surface colonization of *Pseudomonas putida* KT2440 under unsaturated conditions

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment:
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……
Living like a tree,

Lone and free;

And like a forest,

Brotherly;

The longing is ours…

Nazim Hikmet Ran

Abstract

Water deprivation is a major potential stressor in many natural or engineered microbial habitats. In unsaturated soils, water availability is controlled by the matric potential (Ψ_m). The Ψ_m, together with pore geometry, controls the thickness of thin liquid films in the soil pores. A key factor to pollutant biodegradation in soil is that bacteria and pollutant molecules should be in contact. When the conditions become more unsaturated (i.e., more negative Ψ_m), the liquid films become so thin that water pathways become disconnected; limiting bacterial motility and pollutant diffusion in the soil pores. To cope with water deprivation, bacteria exhibit adaptive response like extracellular polymeric substance (EPS) production. The EPS it has been suggested to allow a micro-colony to retain its hydrated state for a longer time upon soil drying. This effect could be beneficial for pollutant removal, as it would ensure ongoing removal under unsaturated conditions and rapid recovery upon rewetting.

In this thesis I investigated the role of flagellar motility and EPS production under matric stress using the recently developed Porous Surface Model (PSM), which creates the thin liquid film effects by controlling the Ψ_m. We demonstrated that flagellar motility was limited under matric stress; *Pseudomonas putida* KT2440 had significantly higher colony expansion rate at near saturation condition (-0.5 kPa Ψ_m) compared to colonies grown under matric stress (at -3.6 kPa Ψ_m). Moreover, 1:1 competition experiments with both phenotypes showed that under wet conditions (-0.5 kPa), wild type KT2440 outcompete its non-flagellated mutant in terms of surface colonization, whereas both phenotypes coexist under matric stress (-3.5 kPa). This thesis also showed that bacteria could benefit from sudden wetting events (5 minutes, twice a day) by exhibiting flagellar motility, suggesting that even rare wet events could offset the cost associated with flagellar synthesis and explain the sustained presence of flagellated cells in soil habitats.

We also investigated colony expansion rates and colony morphologies of diverse EPS deficient mutants of *P. putida* KT2440. The preliminary results with mutant showed that alginate (a major EPS) deficient mutant and wild type did not exhibit significant difference in colony expansion rates and morphology under wet (-0.5 kPa) and dry (-0.4 MPa) conditions. This suggests that alginate does not have an effect on surface colonization under matric stress.

One major goal of this thesis was to improve the PSM to extend the range of matric potentials (i.e down to -1.5 MPa, the vegetal wilting point) as the range of matric potentials that can be created with the PSM is narrow and practically limited to maximum 1 m H_2O (10 kPa). Wit that aim, we modified the PSM so that we can set the desired Ψ_m as the negative of an applied pneumatic pressure. Hence we named the new system the Pressurized Porous Surface Model (PPSM). We showed the PPSM is suitable platform to study bacterial behavior under a wide range of matric potentials by validating bacterial growth and detectable gene expression levels.

Finally, we determined the whole genome expression dynamics of *Pseudomonas putida* KT2440 under matric stress. We identified the differentially expressed genes at 4, 24, and 72 hr of -0.4 MPa Ψ_m relative to near saturation condition (-0.5 kPa). Although our hypothesis was that flagellar genes would be down-regulated under matric stress, we detected significant up-regulation of flagellar genes, suggesting that matric stress plays an important role in the expression of flagellar. This thesis also showed significant up-regulation of alginate synthesis genes, supporting our hypothesis and the findings of the previous studies regarding the importance of alginate under unsaturated conditions. In addition, we evaluated whether polyethylene glycol, with a molecular weight of 8000 Da, (PEG-8000) simulated water potential effects provide good proxies of direct matric potential in terms of whole genome expression of KT2440. We showed that PEG-8000 simulated water stress resulted in a different gene expression profile than in the case of directly applied matric stress.

Overall this thesis showed the importance of flagellar motiliy and EPS with respect to matric stress. The results of this thesis would contribute significantly to our understanding of pollutant-microbe interactions in the vadose zone.

Dansk Resume

Vandmangel er en vigtig potentiel stressfaktor i mange naturlige eller konstruerede mikrobielle miljøer. I umættede jorde er tilgængeligheden af vand kontrolleret af matric potential (Ψ_m). Sammen med poregeometrien styrer Ψ_m tykkelsen af de tynde væske-film, der dannes i jordens porer. En vigtig faktor for bionedbrydningen af forurenende stoffer i jord er, at bakterier og forurenende molekyler er i kontakt med hinanden. Når betingelserne bliver mere umættede (dvs. mere negativ Ψ_m), bliver væske-filmen så tynd, at vandets veje bliver afbrudt, hvilket fører til begrænsning af bakteriel motilitet og dermed begrænsning af diffusion af de forurenende stoffer i jordens porer. Som reaktion på vandmanglen udviser bakterier forskellige former for adaptiv respons såsom produktion af ekstracellulært polymert stof (EPS). Tidligere undersøgelser indikerer, at EPS gør mikro-kolonier i stand til at bevare deres hydrerede tilstand i længere tid efter udtørring af jorden. Denne effekt kan være gavnlig for fjernelse af forurenende stoffer, da det ville sikre en løbende fjernelse under umættede forhold samt hurtig genopretning efter tilbagevenden til mættede betingelser.

I denne afhandling blev betydningen af flagellær motilitet og produktion af EPS under matric stess undersøgt ved brug af den såkaldte Porous Surface Model (PSM), som genererer væske-filmens effekter ved kontrol af Ψ_m. Det blev vist, at flagellær motilitet var begrænset under matric stress; *Pseudomonas putida* KT2440 kolonier udviste væsentlig højere vækstrater tæt ved mætningsbetingelser (-0.5 kPa Ψ_m) end kolonier dyrket under matric stress (ved -3.6 kPa Ψ_m). Desuden viste 1:1 kompettitive eksperimenter med begge phenotyper ved mættede betingelser, at den naturligt forekomne KT2440 udkonkurrerer dens ikke-flagellare mutant med hensyn til overflade-kolonivækst, hvorimod begge phenotyper sameksisterer under matric stress (-3.6 kPa). Denne afhandling har endvidere vist at bakterierne kunne drage fordel af pludseligt forekomne mættede betingelser (5 minutter, to gange dagligt) idet flagellær motilitet blev observeret. Dette resultat indikerer, at selv sjældent forekomne mættede betingelser kan mindske omkostningerne forbundet med flagellær syntese og dermed forklare den vedvarende tilstedeværelse af flagelceller i jordmiljøer.

Vækstrater og morfologi blev undersøgt for forskellige EPS-manglende mutanter af bakterien *P. putida* KT2440. De indledende resultater med mutanterne viste ingen signifikante forskelle i vækstrater og morfologi for hhv. naturligt forekomne KT2440 og alginat-manglende (et væsentligt EPS) mutanter under

våde (-0.5 kPa) og tørre (-0.4 MPa) betingelser. Dette indikerer, at alginat ikke har nogen effekt på overflade-kolonidannelse under matric stress.

Et af hovedformålene med denne afhandling var at forbedre PSM-metoden, således at rækken af matric potentials udvides (dvs. ned til -1.5 MPa, vegetations-visnings-punktet), idet det nuværende spektrum af matric potentials, der kan genereres med PSM-metoden, er smalt og nærmest begrænset til 1 m H_2O (10 kPa). Med dette formål modificerede vi PSM-metoden, sådan at den ønskede Ψ_m kan fastsættes svarende til den negative værdi af det givne pneumatiske tryk. Denne modificerede model har vi kaldt Pressurized Porous Surface Model (PPSM). Gennem validering af bakterievækst og målbare genudtryksniveauer har vi vist, at PPSM kan anvendes til at studere bakteriers adfærd under et bredt spektrum af matric potentials.

Endelig blev den fuldstændige genudtryknings-dynamik for *Pseudomonas putida* KT2440 under matric stress klarlagt. Vi identificerede de forskelligt udtrykte gener efter 4, 24 og 72 timer ved -0.4 MPa Ψ_m relativt til nær-mætnings-betingelser (-0.5 kPa). Selvom vores hypotese var at flagellære gener ville nedreguleres under matric stress, detekterede vi signifikant opregulering af alginat-syntese-gener. Dette indikerer, at matric stress spiller en afgørende rolle i udtrykningen af flageller. Den observerede opregulering af alginat-syntese-gener understøtter vores hypotese om betydningen af alginat under umættede betingelser og er desuden i overensstemmelse med tidligere undersøgelsers resultater. Desuden evaluerede vi hvorvidt vandpotentialeeffekter simuleret via polyethylen-glycol (PEG-8000), med en molekylevægt på 8000 Da, kan udgøre gode estimeringer af direkte matric potential mht. fuldstændig genudtrykning af KT2440. Vores undersøgelser viste, at vandtryk simuleret via PEG-8000 resulterede i en anderledes genudtryksprofil end for direkte anvendt matric stress.

Overordnet har denne afhandling vist betydningen af flagellær motilitet og EPS med hensyn til matric stress. Disse resultater bidrager væsentligt til forståelsen af interaktionerne mellem mikroorganismer og forurenende stoffer i den umættede zone.

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Glossary

Adaptive Response: responses that bacteria exhibit under stress that resulting in adaptation to certain environmental conditions

Desiccation: lack of water, extreme water unsaturation in soil,

Down-regulation: the reduction in the expression of a gene relative to a gene at the reference condition.

Fitness: the properties that can add to survival and spreading of bacteria

Induction: up-regulation

Isopiestic: isobaric, having equal pressure.

Matric Stress: the stress due to the matric potential component of the soil water potential. When matric potential decreases (becomes more negative), the matric stress increases.

Microbial Stress: a physical, chemical, environmental condition that impairs cellular activities.

Saturated Soil: a soil, pores of which are filled with water/bulk solution. In this case the matric potential equals zero, and there is no matric stress.

Stress Tolerance: the degree to which bacteria can cope with stress

Suppression: down-regulation

Transcription: the process of copying a DNA sequence to produce a RNA transcript. This is the first step in the expression of a gene.

Transcriptome: the set of RNA transcripts produced by the cells.

Unsaturated Soil: a soil, pores of which are partially filled water and air. In this case, the matric potential is negative and the degree of matric stress increases as the conditions become more unsaturated.

Up-regulation: the increase in the expression of a gene relative to a gene at the reference condition.

Vadose Zone: unsaturated zone. The region below the soil surface where all solid, liquid and gas interface exist.

Water Stress: the stress related to any reduction of the total water potential.

Xenobiotics: chemical compounds (like pesticides, drugs, carcinogens, etc.) that are foreign to the organisms

1. Introduction

Agricultural soils are the intentional and unintentional recipients of a wide range of xenobiotic organic chemicals, the presence of which threatens the health of the ecosystem. Some soil bacteria have the ability to degrade xenobiotics, and using these microorganisms for soil remediation seems to be a promising technology. In agricultural soils, bacteria live attached to soil grains in pore spaces that are rarely fully water-saturated. Nevertheless, most of our detailed knowledge on bacteria-pollutant interactions has been obtained under well-mixed and water-saturated conditions, ignoring the particularities of bacterial adaption to unsaturated conditions.

A key factor to pollutant biodegradation is that bacteria and pollutant molecules should be in contact. However the likelihood of achieving such contact is relatively low, considering that bacteria occupy only a very small portion of total surface area available in soil (assuming 10^{10} bacteria per gram of soil, a bacterial population has a total surface area of 0.0157 m^2 per gram of soil, while sand and clay have a total surface area of 3 and 208 m^2 per gram, respectively) (Chenu and Stotzky, 2002). Although bacterial motility and diffusion of pollutants aid to achieve contact, this can be limited depending on the soil spatial structure and water availability, as both motility and diffusion require hydrated pathways in the porous network.

Bacteria in soil are most often distributed as micro-colonies embedded in an extra-cellular polymeric substance (EPS) matrix. The EPS constitutes the interface between bacterial cells and their environment. It has been suggested to allow a micro-colony to retain its hydrated state and potentially water-soluble pollutants for a longer time upon soil drying. This effect could be beneficial for pollutant removal, as it would ensure ongoing removal under unsaturated conditions and rapid recovery upon rewetting.

For an effective bioremediation, understanding the limitations towards pollutant biodegradation and bacterial adaptive traits under unsaturated conditions is crucial and is the focus of this thesis. Before proceeding further, I introduce some key concepts with respect to unsaturated soil, followed by the study approach and objectives. Then, I provide a brief review of methodologies used to study bacterial behavior under unsaturated conditions. In the subsequent chapters I review the ecophysiological and transcriptomic responses under unsaturation, highlighting the contributions I have made in this thesis. In the final chapter, I present all the publications and reports that constitute this thesis.

1.1. Soil Water Potential and Water Stress

Soil water potential is defined as the work required to move a unit quantity of pure water from a standard state to a certain state. The water potential in a soil system is the summation of processes and can be expressed by Eq.1 (Or et al., 2005).

$$\Psi_w = \Psi_P + \Psi_s + \Psi_m + \Psi_z \qquad \text{(Eq.1)}$$

Ψ_P, the pressure potential, is due to the hydrostatic pressure applied by water. Ψ_z, the gravitational potential, is due to the gravitational force acting on the water and responsible for the downward flow of water. Ψ_s, the solute or osmotic potential, is due to the presence of solutes in the pore water. Ψ_m, the matric potential, is the result of adsorptive and capillary forces acting upon water held in the soil pores (Figure 1.1). In saturated soil, Ψ_m is equal to zero and in unsaturated soils Ψ_m is negative. Some researchers include Ψ_m in Ψ_P because Ψ_m is a negative pressure potential and it unifies the pressure potential concept in saturated and unsaturated environments. Others separate them, as these two components cannot exist simultaneously, thus leaving the Ψ_m as the only pressure component in unsaturated soils (Hillel, 1998).

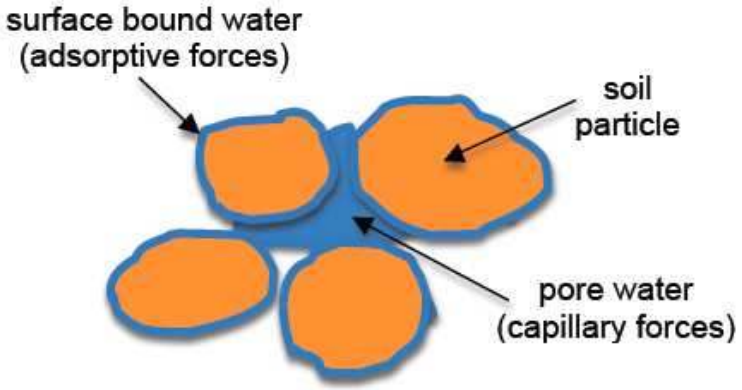

Figure 1.1. Capillary and adsorptive forces in soil pores.

In non-saline environments, the effect of Ψ_s is negligible and Ψ_m is the dominant component of water potential in unsaturated soils (Holden, 2001). Ψ_m, together with pore geometry, controls the thickness of thin liquid films in the pores (Tuller *et al.*, 1999). Figure 1.2 shows how different geometries can affect the amount of water retained in the pores. In this case a perfectly circular pore may not maintain water, while a triangular pore can maintain water at the edges at the same matric potential because of its geometry. As the conditions get more

unsaturated (i.e., more negative Ψ_m) the liquid films become so thin that water pathways become disconnected; limiting bacterial motility and pollutant diffusion in the soil pores (Griffin and Quail, 1968; Or *et al*., 2007b).

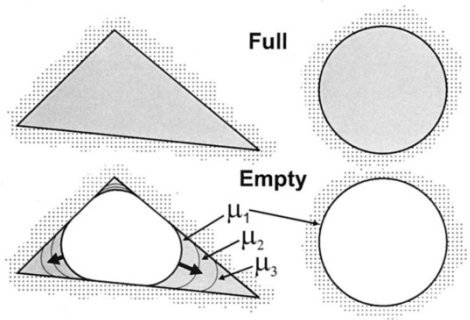

Figure 1.2. Liquid retention in triangular and cylindrical pores; μ= matric potential with $\mu_3 < \mu_2 < \mu_1$, (Tuller *et al*., 1999).

Any change in the water potential is experienced as water stress by the bacteria. When the Ψ_s is negative, bacteria experience osmotic stress. Similarly, when the Ψ_m is negative, bacteria experience matric stress. Depending on the value of Ψ_m, bacteria face mild to extreme (desiccation) water stress and their growth and physiological activities are reduced (Potts, 1994). The ability of bacteria to sense and respond to such stress is important for their survival.

1.2. Study Approach and Objectives

To progress in our knowledge of bacterial behavior under unsaturated conditions, first, it is necessary to grow microbes in observable experimental systems that recreate realistically thin liquid films. Although a range of methods exist where the matric potential (Ψ_m) is simulated with non-penetrating solutes such as polyethylene glycol MW 8000 (PEG-8000) (Holden, 2001, Chang and Halverson, 2003), none of them result in the creation of controlled thin liquid films. The recently developed Porous Surface Model (PSM) overcomes the limitations of the previous methods (Dechesne *et al*., 2008) and has also been used in this thesis. However the range of matric potentials that can be created with the PSM is narrow and practically limited to maximum 1 m H_2O (10 kPa) (Dechesne *et al*., 2008). Therefore one major goal of this thesis was to improve the PSM to extend the range of matric potentials that can be created.

Progress in our knowledge not only requires the right experimental platform, but also an integrative approach that seeks answers at different scales of microbial ecology and combines them. In this thesis I followed such an approach by conducting experiments at the population, ecophysiological and molecular scales

to construct a holistic view of bacterial responses to matric stress (Figure 1.3). Each response at a lower scale has an influence on the responses at a higher scale. However, the degree of such influence may not necessarily be significant at the higher level. For that reason it is important to conduct studies at multiple scales so we can identify the governing mechanisms of higher scale events and use this knowledge to develop and optimize bioremediation technologies.

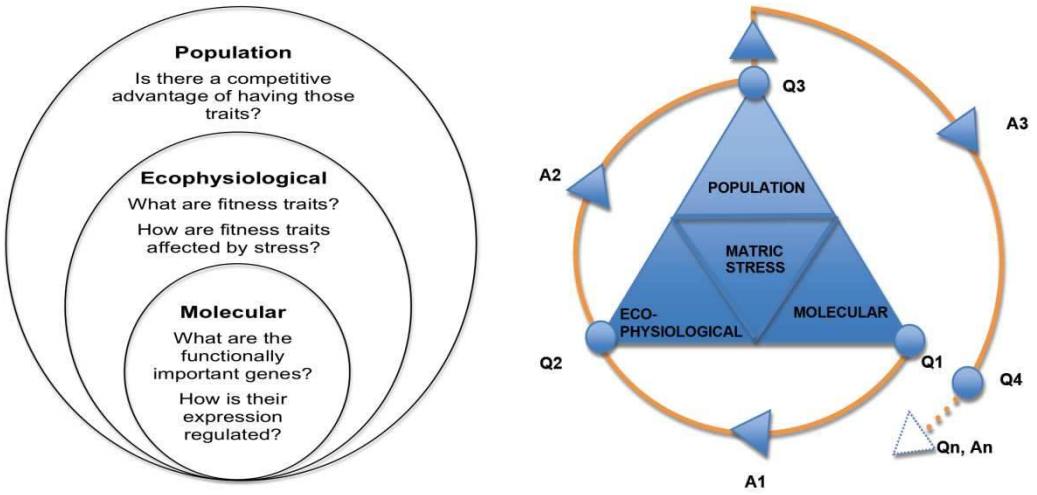

Figure 1.3. (Left) Ecological scales; (Right) Study approach: Q= Question, A= Action. I call this "helix of knowledge". It represents how I get information at one scale and use that to drive my quest to the next scale. At the same time, it represents an extended and higher level of knowledge as it extends and goes higher up while moving around each scale.

This thesis investigates the relationship between mild Ψ_m (i.e., down to -1.5 MPa vegetal wilting point) and bacterial adaptive traits, with an emphasis on flagellar motility and EPS production. Flagellar motility is an important trait, which allows bacteria to colonize new surfaces when their environment becomes inhabitable (e.g., lack of nutrients, temperature change, etc.). When the conditions become more unsaturated (i.e., more negative Ψ_m) in heterogeneous environments like soil, the liquid films become so thin that water pathways become disconnected; limiting bacterial motility and pollutant diffusion in the soil pores (Griffin and Quail, 1968; Or *et al*., 2007b). EPS is equally important; it has been suggested to allow a micro-colony to retain its hydrated state for a longer time upon soil drying. This effect could be beneficial for pollutant removal, as it would ensure ongoing removal under unsaturated conditions and rapid recovery upon rewetting. Given that, the central hypothesis of this thesis is that flagellar motility and EPS production would be significantly affected by the matric stress. We hypothesized that flagellar motility would be limited and no

longer be a fitness determinant under increasing matric stress. The effect of this would be observed at the lower scale, as the suppression of flagellar synthesis genes. On the other hand, EPS production would aid mobilizing the bacteria and be a fitness determinant in surface colonization under matric stress. At a lower scale, induction of EPS synthesis genes would be observed. In addition, we hypothesized that polyethylene glycol (PEG-8000) simulated water stress would cause a different gene expression profile than the directly applied matric stress.

To test our hypotheses, *Pseudomonas putida* strain KT2440 was used as model organism. It is a common pollutant degrading strain and an effective plant root colonizer in soil (Espinosa-Urgel *et al*., 2002). KT2440 is a Gram-negative bacterium, which belongs to gamma Proteobacteria. It is the plasmid-free derivate of the toluene degrading *P. putida* strain mt-2 (Williams and Murray, 1974). The KT2440 genome is 6.18 Mb long with an average G+C content of 61.6% (Nelson *et al*., 2002). It has 5420 open reading frames (ORFs), 85% of which are homologous to genes found in *P. aeruginosa* PAO1. The metabolic versatility, the fully sequenced genome and having many genes involving in sensing and generating adaptive responses makes KT2440 a suitable model organism to reach the research aims. The objectives of this thesis are as follows:

1. Investigate the role of flagella under matric stress resulting in controlled thin liquid films **(Article 1)**
    a. Determine the colonization kinetics of KT2440 and a Δ*fliM* mutant defective in flagella synthesis on porous surfaces
    b. Determine the relative fitness of wild type KT2440 over the Δ*fliM* mutant under various degrees of matric stress in competition experiments
    c. Determine how KT2440 responds to short time wetting events when grown under water-deprived conditions

2. Investigate the role of EPS under matric stress using PSM **(Report 1)**
    a. Determine the colonization kinetics of KT2440 and several EPS deficient mutants
    b. Determine the relative fitness in competition experiments
    c. Determine the growth rate and biofilm formation capacity of KT2440 and EPS deficient mutants
    d. Determine the survival of KT2440 and EPS deficient mutants after desiccation

3. Design a system that can be operated to grow bacteria under a wide range of matric potentials (i.e., down to -1.5 MPa, the vegetal wilting point). Validate bacterial growth and verify differential gene expression at different matric potentials **(Article 2)**

4. Determine genome-wide transcriptome dynamics of *Pseudomonas putida* KT2440 at matric potentials of -0.4 MPa and -0.5 kPa. Evaluate whether PEG-8000 simulated water potential effects provide good proxies of direct matric potential in terms of whole genome expression of KT2440 **(Article 3)**

2. Methods of Culturing Bacteria under Unsaturated Conditions

Thanks to Holden (2001), a thorough review of commonly used methods to culture bacteria under unsaturated conditions is already available (Figure 2.1). Here I provide an overview of those methods based on Holden's review and our previous study (Dechesne *et al*., 2008).

One widely used method is to grow bacteria on PEG-8000 amended solid media. Being a high molecular weight (8000 Da) compound, PEG-8000 cannot enter the cell membrane and creates conditions similar to Ψ_m. Alternatively, the PEG-8000 amended solid medium can be located on the lid of the Petri dish and the bacteria are grown in the petri dish such that the Ψ_m is controlled isopiestically. Although these are the simplest methods of growing bacteria in a controlled water potential environment, they are unable to create thin liquid films that would be the case under Ψ_m. Another alternative involves culturing biofilms on a semipermeable membrane, which is positioned on a liquid medium. The water potential is controlled by adding permeating (NaCl) or nonpermeating (PEG-8000) solutes into the liquid medium. The membrane separates the bacterial colony from the large osmolytes but allows nutrients to pass through. However, this way of simulating Ψ_m cannot create the thin film effect on the bacterial colony. Moreover, this method has not been applied in the recent microbial ecology literature. Other alternatives involve culturing the bacterial colonies in a porous medium with a prescribed quantity of water. The major drawback of these systems is that they do not allow in-situ monitoring of the bacterial colonies.

Recently, Dechesne *et al*. (2008) developed an alternative method, called the Porous Surface Model (PSM) (Figure 2.2), to grow bacteria under controlled matric potentials. The PSM consists of a filter holder in which a ceramic plate, serving as the porous surface, is located. The filter holder is connected to a reservoir containing the growth medium. In this system, microbes inoculated onto a porous ceramic surface grow in thin liquid films because the medium that wets the ceramic plate is under mild suction; which is the Ψ_m experienced by the microbes. This system can practically be operated in a range from 0 to -100 cm (0 to -10 kPa). The major advantages of the PSM are that it can create thin liquid films by controlling the Ψ_m. It also allows in-situ and real-time monitoring of bacterial colonies on porous surfaces.

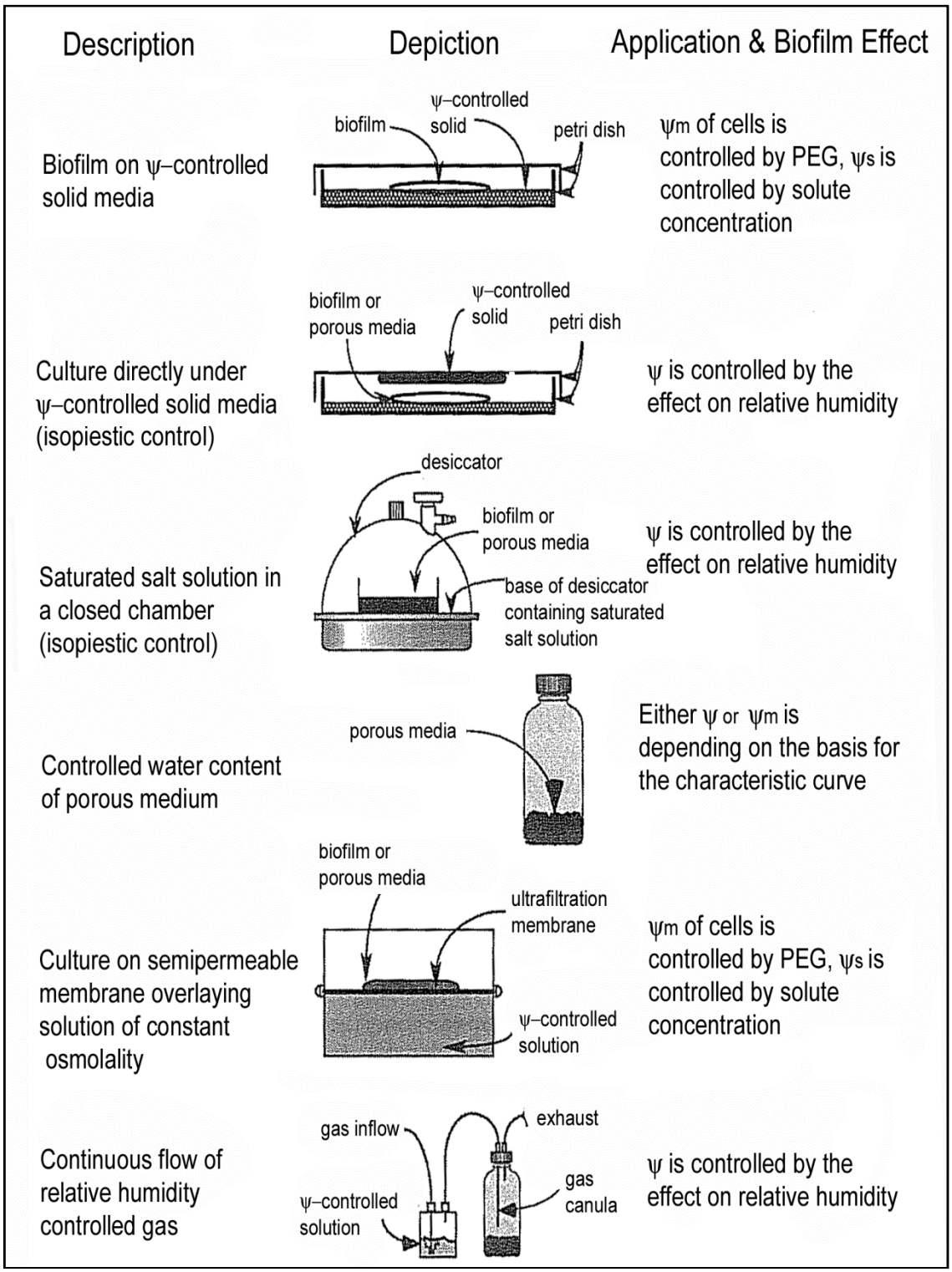

Figure 2.1. Methods of bacterial cultivation under unsaturated conditions (reproduced from Holden, 2001).

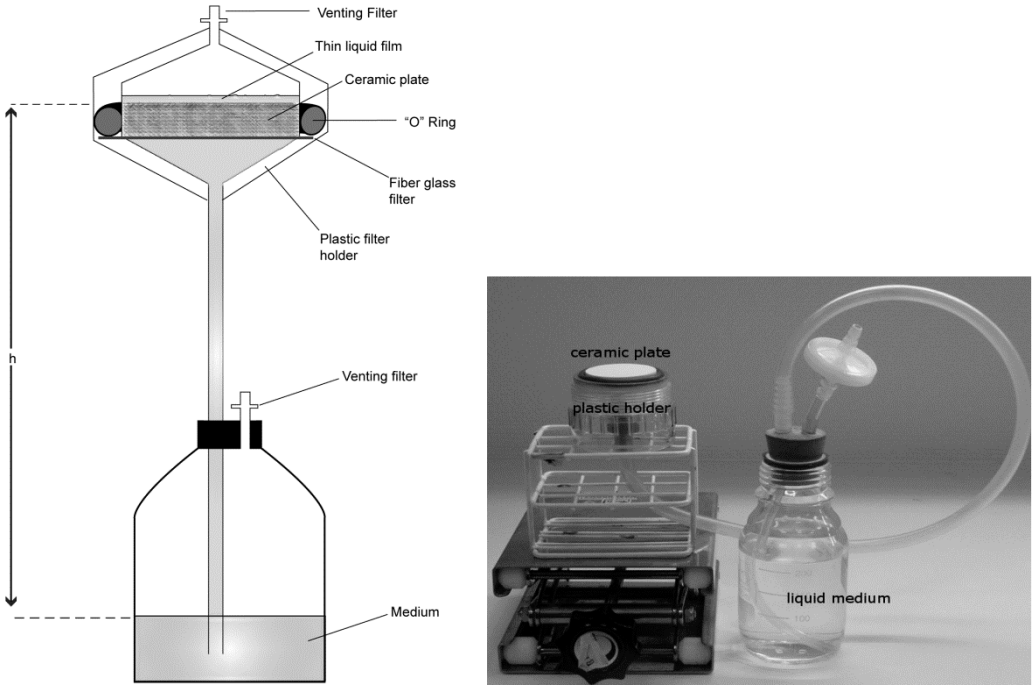

Figure 2.2 (Left) Sketch of the PSM. The liquid film thickness at the surface of the ceramic plate is determined by *h*, the height of the fluid column connecting the medium reservoir to the ceramic plate. (Right) Photo of a PSM with its lid removed to expose the ceramic surface (Dechesne *et al*., 2008).

As mentioned before, one of the objectives of this thesis was to design a new system that could be operated under a wide range of matric potentials. In the PSM, the Ψ_m is controlled by changing the height of the water column (h in Figure 2.2) connecting the medium reservoir and ceramic plate (Dechesne et al., 2008). However this way is practically infeasible if we want to create matric potentials down to -1.5 MPa, as it would require a water column at a height of 150 m! Therefore we modified the PSM so that we can set the desired Ψ_m as the negative of an applied pneumatic pressure. Hence we named the new system the Pressurized Porous Surface Model (PPSM) (Figure 2.3). Like the PSM, the PPSM has the advantage of creating thin liquid film effects. It also allows the monitoring of bacterial cells after each operation although real time monitoring is not possible. In **Article 2**, we discussed further details of the PPSM and presented the evidence of bacterial growth and detectable changes in gene expression levels under matric stress.

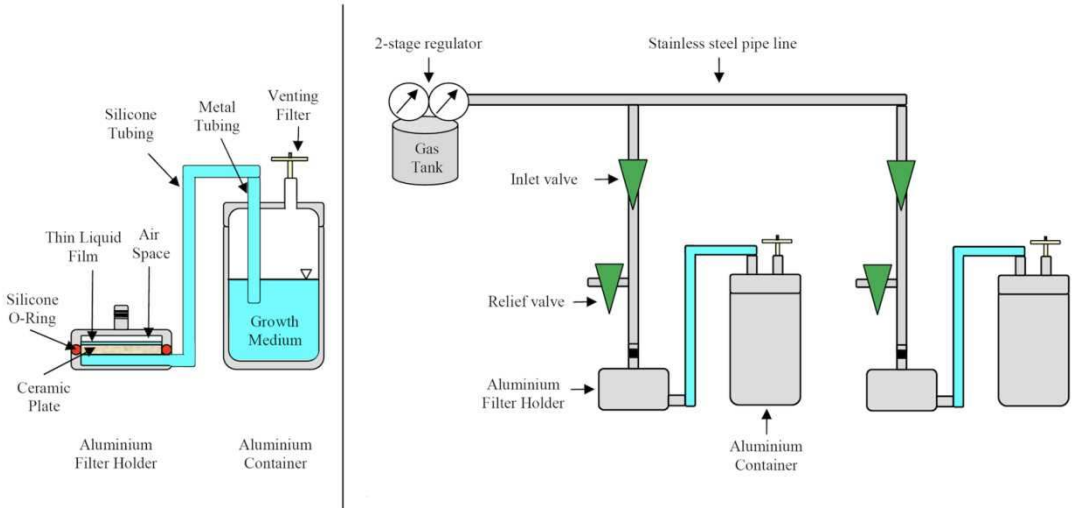

Figure 2.3. (Left) Schematic representation of the cross-section of a PPSM. (Right) system in operation (drawing not in scale).

3. Ecophysiological Changes and Limitations under Water Stress

Bacteria are affected in many ways when they are under water stress. These range from damage to proteins and nucleic acids, to reduced or ceased growth rate, death as well as limitations in preventing them escape from a stressed environment. To cope with such damages and limitations, bacteria can undergo physiological, morphological and behavioral changes. Some of the changes may increase their immediate fitness, while other changes can bring about competitive advantages or can even aid cooperation and benefit the other in the surroundings. Figure 3.1 summarizes the effects of major water stress and adaptive responses of bacterial populations. Many of the listed responses are not unique to water stress but are also associated with diverse environmental stresses. As flagellar motility and EPS are the focus of my thesis, I discuss these topics separately.

3.1. Limitation of Flagellar Motility under Water Stress

Henrichsen (1972) listed six distinct types of bacterial surface motility: swimming, swarming, twitching, gliding, sliding and darting. Among these, swimming and swarming are flagella dependent (Harshey, 2003). Non-flagella dependent motility is out of scope of this thesis, and curious minds are invited to read the extensive reviews by Henrichsen (1972) and Harshey (2003).

Figure 3.2 shows the schematic representation of a typical flagellar assembly. The main parts of a flagellum are: the basal body, motor, switch, hook, filament, caps, junctions and export apparatus. The MS ring, P ring and L ring together form the basal body. The flagellar motor is around 50 nm (Berg, 2003). The filament is usually around 5-10 μm long and 20 nm in diameter (Harshey, 2003). The motor consists of a stator and rotor. MotA and MotB proteins form the stators. The rotor is made up of FliG proteins and generates the torque necessary for movement. The switch functions to change direction of the rotation. The hook is considered as a universal joint. The filament is the long cylindrical part which is turned by the motor.

Individual cells swim by flagellar rotation and the number of flagella may vary depending on the species. For example *E. coli* and *S. typhimurium* can have up to 10 peritrichous flagella (Harshey, 2003), while *P. aeruginosa* has a single polar flagellum (Dasgupta *et al.*, 2003) and *P. putida* has multiple polar flagella (Harwood *et al.*, 1989). When flagella rotate in a counter clockwise (CCW

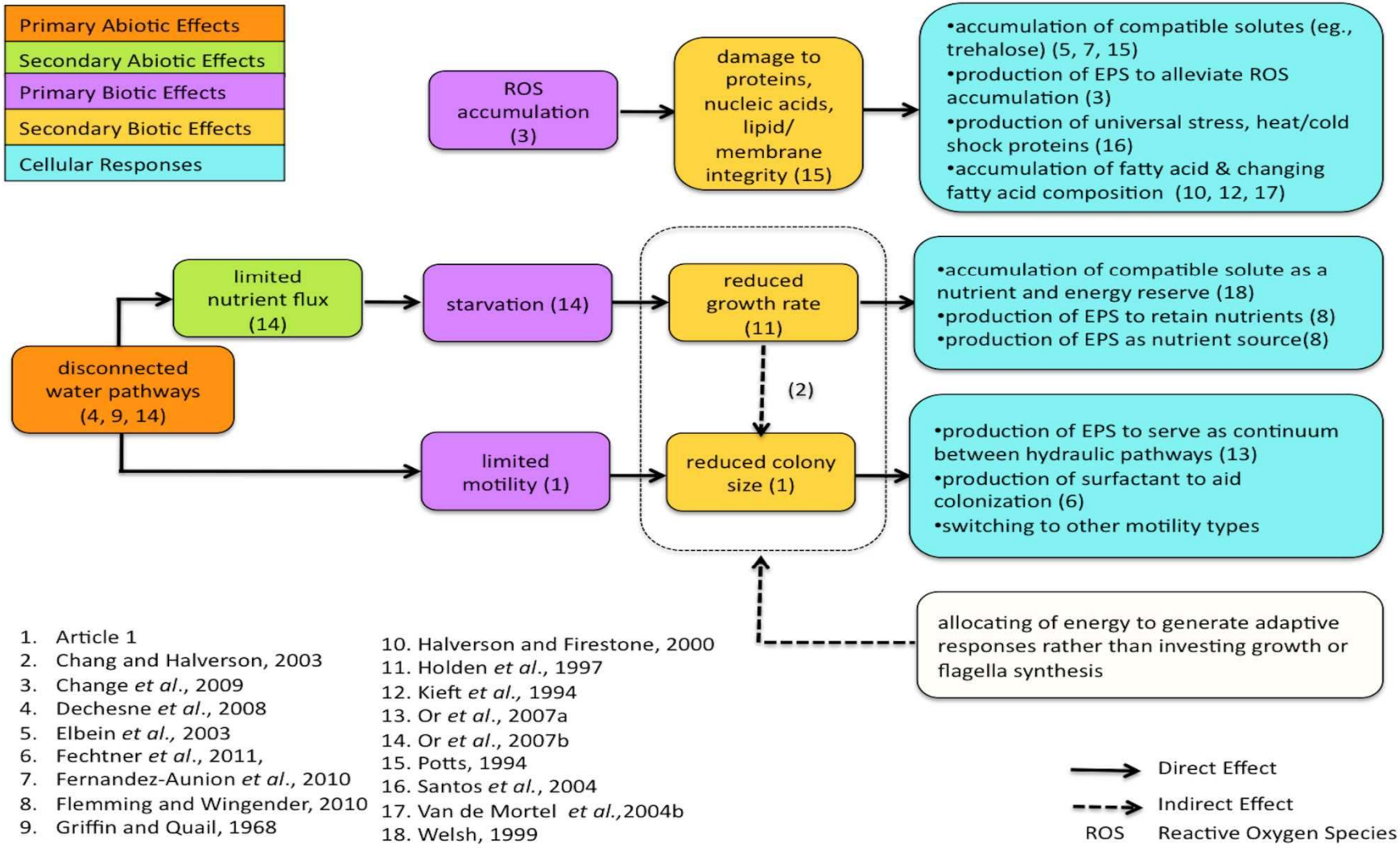

Figure 3.1. Water stress effects and cellular responses

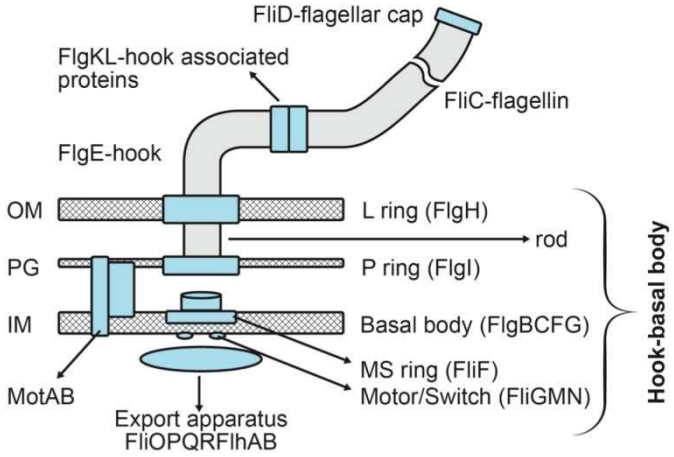

Figure 3.2. Flagellar assembly for *P. aeurigosa* (reproduced from Jyot and Ramphal, 2008).

direction, they push the cell forward and the cell exhibits unidirectional swim called "run". On the other hand, when some of the flagella rotate clockwise (CW) and others rotate CCW; cells start to "tumble" (Berg, 2003). Cells move in a way by alternating between run and tumble and the alternation between run and tumble is believed to be random (Berg, 2000). However, when they exhibit chemotactic behavior, i.e., sensing the gradients of an attractant or repellent substrate, they change the frequency of tumble and run. For instance, when the cells sense increasing concentrations of attractants they tumble less frequently and swim longer times, whereas when they detect decreasing concentrations of attractants they tumble more and decrease run times (Berg, 2000). Unlike swimming, swarming involves collective movement of the cells (Harshey, 2003). To swarm, cells become hyperflagellated and elongated (Kaiser, 2007). Swarming motility has been observed for some *Pseudomonas* species like *P. aeruginosa, P. syringae* (Shrout *et al*., 2006; Quinones *et al*., 2005) but has not been observed for *P. putida*. Although *P. putida* exhibited swarming-like motility assisted by pyoverdine iron acquisition (Matilla *et al*., 2007), since it was not flagella dependent motility, it is out of scope of this thesis.

Surface wetness plays a key role in swimming and swarming (Harsey, 2003; Berg, 2005). Therefore in surface colonization, surface wetness is an important fitness determinant. Our previous study showed decreased surface colonization rates of *P. putida* KT2440 with decreasing Ψ_m; suggesting that liquid film thickness, controlled by the Ψ_m, is an important parameter affecting flagellar motility in PSM (Dechesne *et al*., 2008). In our follow-up study **(Article 1),** we compared the colony expansion rates of non-flagellated mutant to wild type

KT2440. We found that both non-flagellated mutant and wild type had decreased colony expansion rates with decreasing Ψ_m. However, the colony expansion rates of wild type KT2440 at -0.5 kPa and -1.2 kPa were significantly higher than its non-flagellated mutant (Figure 3.3). We also showed that under low matric potential flagellar motility was no longer a competitive advantage for surface dispersal of KT2440, by resulting in the same colonization pattern as its non-flagellated mutant in 1:1 competition experiments (Figure 3.4). These suggest that more than bacterial intrinsic-growth kinetic parameters, surface microtopography, hydration status and bacterial flagellation are essential parameters in bacterial surface colonization. We also showed that bacteria were able to take advantage of very short wetting events to disperse quickly by flagellar motility. In this work, wild type exposed to 5 minutes wetting events (twice a day) had significantly higher colony expansion rate than the one that was not exposed, while non-flagellated mutant was not affected by the wetting events (Figure 3.5). This suggests that even rare wet events could offset the cost associated with flagellar synthesis and explain the sustained presence of flagellated cells in soil habitats.

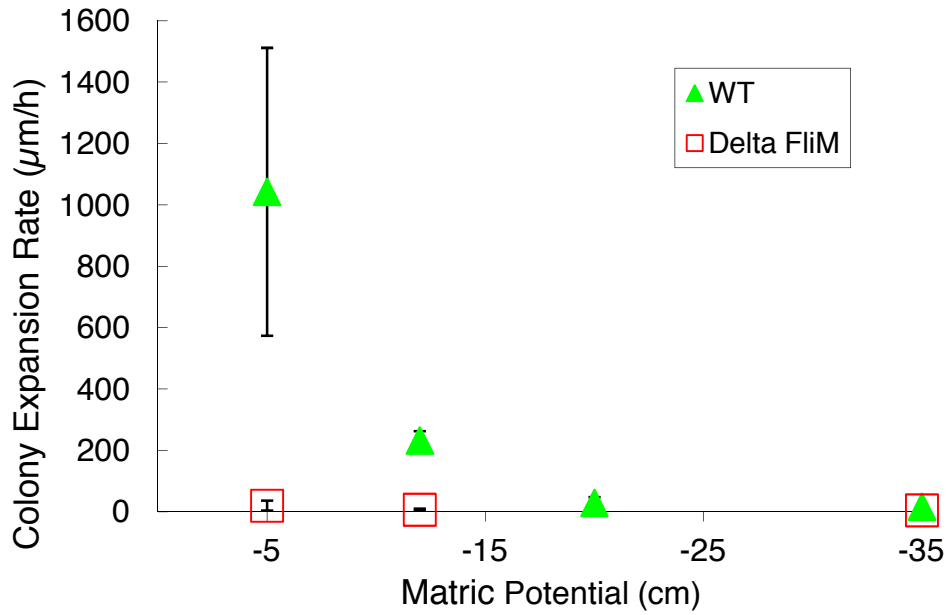

Figure 3.3. Average surface expansion rates for wild type KT2440 and Δ*fliM* mutant defective in flagella synthesis. Error bars indicate standard deviations.

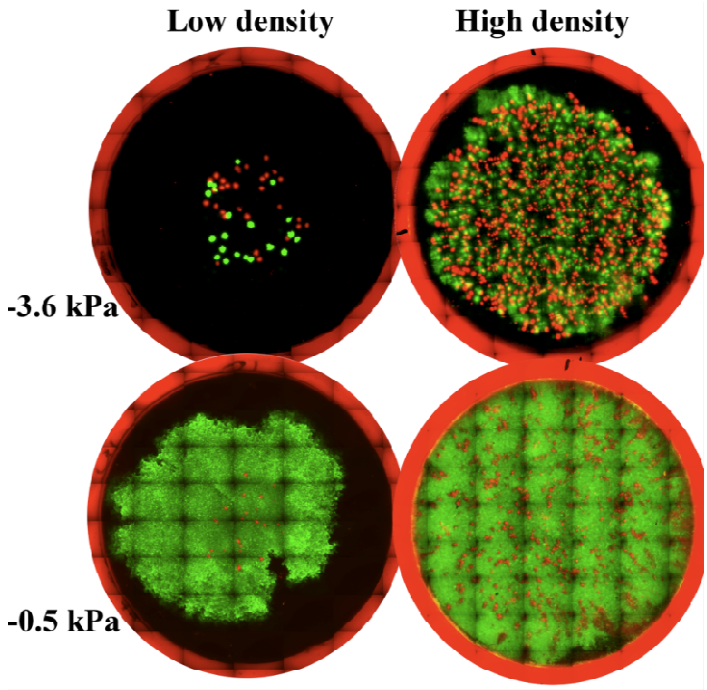

Figure 3.4. The competition between *P. putida* KT2440 wild type (flagellated; green) and its Δ*fliM* isogenic mutant (nonflagellated; red). (Article 1)

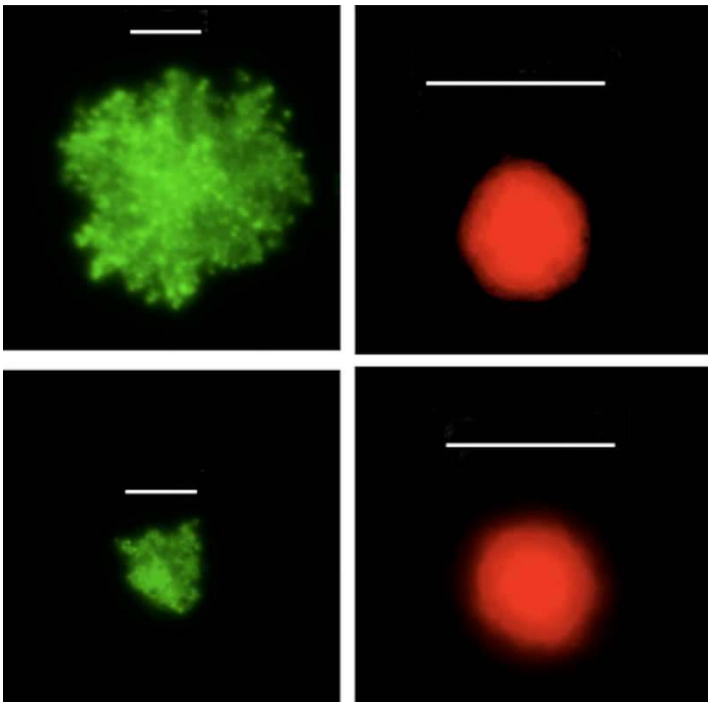

Figure 3.5. (Upper) Response of *P. putida* KT2440 wild type (green) and its Δ*fliM* isogenic mutant (red) to 5 minutes wetting events. (Lower) *P. putida* KT2440 wild type (green) and its Δ*fliM* isogenic mutant (red) on control plates which were kept at dry conditions at all times. The images are captured after 3 days of incubation. The bars indicate 1 mm. (Article 1)

3.2. Role of EPS under Water Stress

Bacterial cells can excrete polysaccharides such as alginate, cellulose, xantan, dextran, etc. (Rehm, 2010). These polymers accumulate around the cells, forming a slime layer or capsule. Biosynthesis of these polymers is controlled by diverse mechanisms ranging from quorum sensing (QS) to two-component signal transduction systems, over sigma factors to cyclic-di-GMP dependent controls (Rehm, 2010), some of which are discussed in the next chapter. EPS forces cells to aggregate by immobilizing them and adhering them to surfaces, which is considered the initial step of biofilm formation. Together with polysaccharides, eDNA, proteins and glycolipids (Sutherland, 2001b; Flemming and Wingender, 2001) form the extracellular polymeric matrix in which the cells are embedded. This complex EPS matrix can provide many advantages: it can act as a protective barrier from toxins to predators, accumulate nutrients, pollutants, heavy metals, and enzymes, be a direct nutrient source and be asink for excess carbon and waste products. (Flemming, 2011)

EPS production has also long been suspected as a major response to desiccation (Wilkinson, 1958). Studies have reported increased EPS production with increased desiccation stress (Roberson and Firestone, 1992) and have described how EPS acts as water binding agent (Sutherland, 2001a). The EPS matrix can hold approximately 10 times its weight in water (Roberson and Firestone, 1992, Chenu and Roberson, 1996), and owes this magnificent water retention capacity to surface, capillary and osmotic forces (Or *et al*., 2007a). The polymer composition and structure influence the ion binding and gelation ability of EPS, and depending on the type of polysaccharide, the water retention capacity also change. For instance, xantan can retain more water than dextran at the same water potential (Chenu, 1993). Since xantan has a network structure as opposed to a coiled structure without networks for dextran, in porous systems xantan can retain more water due to capillary forces where dextran only relies on adsorptive forces (Chenu, 1993). Although the importance of EPS with respect to water retention has been described earlier, it has recently been shown that EPS protects cells from air drying (Tamaru *et al*., 2005): all cells coated with EPS were active whereas only 30 % of the cells without EPS were active after overnight drying. This suggested that EPS production is an important fitness trait under water stress and can provide a competitive advantage to the bacteria. However, it has also been argued that once produced, EPS becomes a common good and can be considered as a cooperative trait, benefiting all the cells in the vicinity (Brockhurst et al., 2008). Most research concerning the role of EPS in

cooperation/competition is still at a theoretical level (Xavier and Foster, 2007) and would definitely benefit from experimental studies.

We have already shown how flagellation, hydration status and surface topography can affect colonization patterns and hinder spatial expansion of the cells under matric stress (**Article 1)**. However we do not know how EPS may affect the spreading of the cells under matric stress. It has been suggested that under unsaturated conditions, EPS can act as a continuum agent between the soil particles and re-establish hydraulic pathways (Or *et al*., 2007a). This is due to the swelling of the EPS matrix by retaining water and becoming a porous network itself. The higher diffusion rate of glucose under unsaturated conditions in the presence of EPS (Chenu and Roberson, 1996) is attributed to this continuum effect of EPS matrix. Maybe the same structure can aid in mobilizing bacteria. Thus, in surface colonization, EPS could be an asset under unsaturated conditions. However, the current state of knowledge suffers from many gaps in this aspect and further investigations are needed to determine the role of EPS as a colonization aid under unsaturated conditions. Such investigations should not only focus on the physical aspects of EPS but should also be extended to evaluate how EPS' ability to change the physicochemical characteristics of surface (Neu and Marshall, 1990) can influence spreading of the cells under unsaturated porous surfaces. As part of this thesis, I have initiated an investigation on how different EPS polymers affect surface colonization and colony morphologies under unsaturated conditions (**Report 1**) and discuss the preliminary results below.

Nearly all *Pseudomonas* species, including *P. putida,* have the ability to produce alginate, a major EPS polysaccharide (Halverson, 2009). Alginate is a negatively charged polymer and is composed of ß-1,4 D-mannuronic and L-guluronic acids linked via ß-1,4-glycosidic bonds (Remminghorst and Rehm, 2006). Alginate plays a role in creating hydrated environments (Chang et al., 2007) and helps to alleviate the effect of oxidative stress by controlling the amount of reactive oxygen species (ROS) under water stress (Chang et al., 2009) in *P. putida* mt-2. Alginate is not the only EPS component that can be produced by *Pseudomonas putida*. *Pseudomonas putida* KT2440 has the genetic components for cellulose production (Nelson et al., 2002). Moreover, recent studies (Nielsen et al, 2011, Nilsson et al., 2011) also indicated the presence of two other EPS gene clusters, putida exopolysaccharide A (*pea)* and putida exopolysaccharide B (*peb*) besides alginate (*alg)* and cellulose (*bcs*) in *P. putida* KT2440. Nilsson *et al*. (2011) evaluated the role of *alg, bcs, pea* and *peb* clusters in saturated KT2440 biofilms.

They found that although *alg, bcs, pea* and *peb* mutants could form biofilms, the stability the biofilms formed by the *pea* and *peb* mutants were low, suggesting that the products of *alg* and *bcs* gene clusters were not contributing to biofilm formation as significantly as the products of *pea* and *peb* gene clusters. They also used multiple mutants resulting in the production of only one EPS component (Alg^+, Bcs^+, Pea^+, Peb^+) and similar results as in the single mutants were obtained except that the stability of Bcs^+ was higher than the *pea* mutant. This is explained due to increased expression of one gene cluster in the absence of other three gene clusters, suggesting one EPS gene cluster compensates the absence of other EPS gene clusters. Nielsen *et al*. (2011) investigated the role of *alg, bcs* and *pea* gene clusters of *P. putida* mt-2 in maintaining hydration under water limiting conditions (-1.5 MPa water potential on PEG 8000 amended solid medium) in addition to biofilm stability and rhizosphere colonization. Based on their gene expression study, they suggested that under water limiting conditions the products of *pea* and *bcs* genes may contribute hydration, but not to the extent of alginate's. Their experiments with multiple mutants also suggested a compensation-like mechanism; in the absence of alginate, Bcs and Pea production can increase and contribute to hydration. Furthermore, they also found that Bcs and alginate, but not Pea, contribute to rhizosphere colonization. Our desiccation tolerance assay also indicated that alginate has an important role under water stress. We found that alginate deficient mutant could not tolerate 24 hr desiccation as much as the wild type KT2440 could. However, we also found that Pea and Peb were as important as alginate in desiccation tolerance. On the other hand, the assay did not indicate an importance for the Bcs, as both *bcs* mutant and wild type tolerated the 24 hr desiccation event equally well **(Report 1)**. Despite its significance in desiccation tolerance, the alginate did not contribute surface colonization on the PSM at -0.4 MPa matric potential **(Report 1)**. Our results did not indicate a significant difference between alginate deficient mutant and WT in terms of final colony areas and morphological parameters (Figure 3.6) (experiments with *bcs, pea* and *peb* mutants are not completed). This suggests that alginate's primary role is to protect the cells from water stress by maintaining hydrated conditions rather than aiding in surface colonization. However, it is possible that the products of *bcs, pea* and *peb* gene clusters compensated the absence of alginate in terms of surface colonization. Therefore, further investigations to elucidate the role of different EPS components under water stress using multiple EPS mutants are necessary and recommended as a follow-up study of this thesis. [At the time of our study with EPS mutants, multiple EPS mutants were not available].

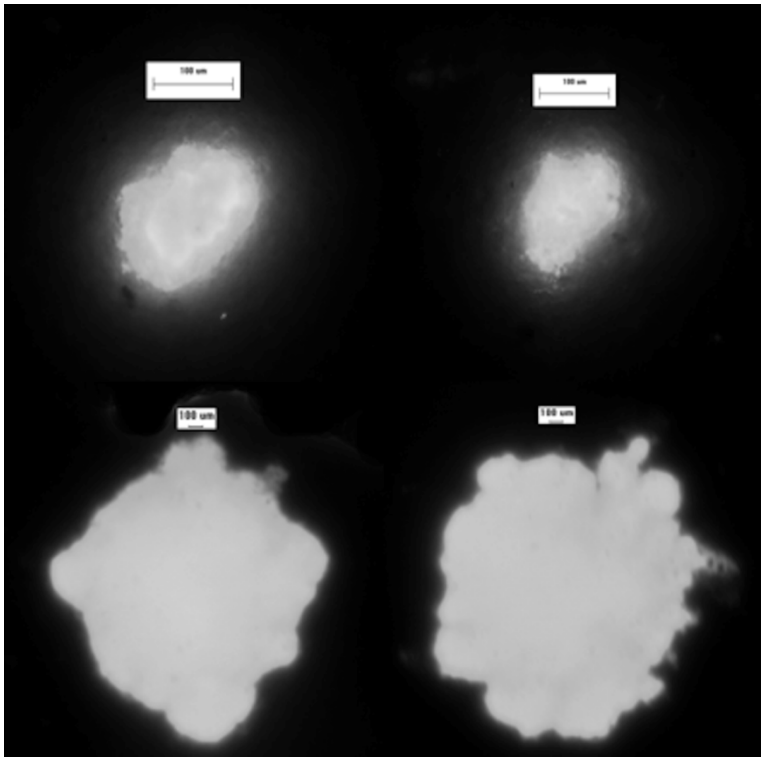

Figure 3.6. *Pseudomonas putida* KT2440 wild type (left) and its alginate deficient mutant (right). Alginate mutant at -0.4 MPa (upper) and -0.5 kPa (lower) after 5 days of incubation. The bars indicate 100 μm. (Report 1)

4. Molecular Basis of Water Stress Adaptation

4.1. Identification, Expression and Regulation of Genes

In order to make sense of the phenotypic responses, it is necessary to study the transcriptomic responses, as the phenotypes are the ultimate manifestation of gene expression and transcription is the first step in gene expression. It is through their regulation of gene expression that bacteria adapt to changing conditions even though there are also post transcriptional and post translational regulation. Hence, identification of potentially important genes, understanding how they function and how their expressions are regulated at transcriptomic level will contribute significantly to our knowledge of bacterial stress adaptation mechanisms.

There are many approaches to identify the genes that are responsible for distinct phenotypes with competitive advantages. One way is experimenting with mutants and checking if they lead to different phenotypes under the test conditions. Mutants can be generated either through random mutagenesis or through targeted gene disruption (Pucci, 2006). In this thesis, I used the latter approach to start the investigation with flagellar (**Article -1**) and EPS (**Report 1**) synthesis defective mutants. This approach is especially useful when performing experiments at the phenotypic level. However it does not provide any information about how a gene is expressed and regulated and interacts with other genes.

Regulation of gene expression is complex. It is a hierarchical network where genes are organized into operons, operons into regulons and regulons into modules. Each unit regulates itself but is also under the control of the one at the higher hierarchy. Although it is a hierarchical network, interactions happen at and between each level, making it a complex network with many feedback loops. Such complexity requires suitable methods that can provide information at the whole genome level expression and regulation. Previously developed methods like reporter gene, northern blot and qRT-PCR are low to medium output methods which also cannot provide an overall view of gene expression and regulation (Richards, 2005). Some of those methods were used to study gene expression of *P. putida* under mild water deprivation corresponding to -1.5 MPa. These studies identified a number of water deprivation related genes in *P. putida* (Van de Mortel et al., 2004a) and elucidated the spatio-temporal dynamics of alginate gene expression under water deprivation using reporter gene technology (Li et al., 2010). Nonetheless, these studies did not provide genome-wide

expression profiles, which require high throughput technologies like DNA microarray (Richards, 2005). The major advantage of using such a high throughput technology is that one can study the whole genome expression of an organism at once and use the acquired information to visualize the interactions of genes and regulatory networks. However, data analysis can be cumbersome and results are often hard to interpret. Many resources regarding the microarray fundamentals and data analysis (Knudsen, 2004, Leung and Cavalieri, 2003, Dharmadi and Gonzalez, 2004) are already available for the interested reader. Given the advantages, we used a microarray technology to determine expression dynamics of the whole genome of *P. putida* KT2440 at -0.4 MPa matric stress **(Article 3)**. In this study, we identified the differentially expressed genes at 4, 24 and 72 hours of matric stress. The major response was observed at 4 hr, while the number of differentially expressed genes decreased dramatically at 24 and 72 hrs (Figure 4.1), suggesting that there is shift towards a non-stressed behavior from 4 to 72 hrs. As expected, alginate synthesis genes were up-regulated but only detected at 4 hr and not at later times (Table 4.1). Interestingly, flagella synthesis genes were also up-regulated, and both their number and expression level increased from 4 to 72 hrs (Table 4.1). In addition, we showed that directly controlled matric stress and PEG-8000 simulated water stress resulted in different gene expression profiles **(Article 3),** and there was not even a single alginate or flagella synthesis gene expressed under PEG-8000 simulated water stress.

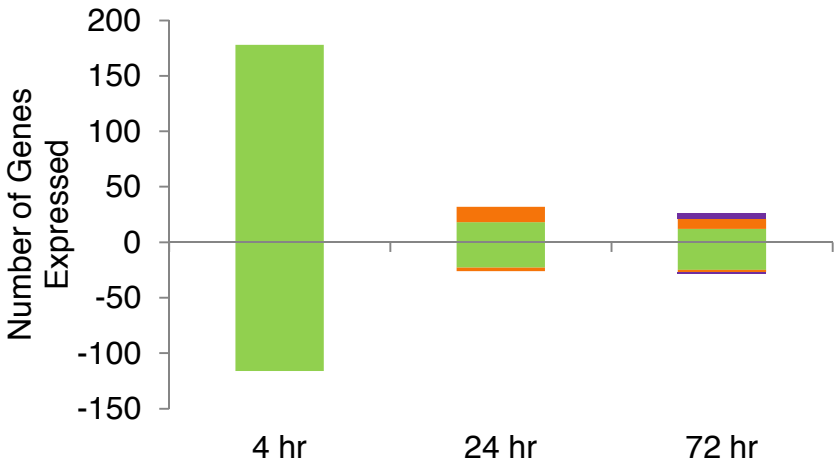

Figure 4.1. Number of up- and down-regulated genes under matric stress. Positive and negative numbers correspond to up-and down-regulated genes, respectively. Green: Genes expressed at 4 hr, some of which also expressed at 24 and 72 hrs. Orange: Genes expressed at 24 hr, some of which also expressed at 72 hr. Purple: Genes only expressed at 72 hr.

Table 4.1. Log_2 -fold changes for flagella and alginate synthesis genes and sigma factors (positive and negative values correspond up- and down-regulation, respectively).

Gene	Annotation	4 hr	24 hr	72 hr
fliM	flagellar motor switch protein FliM			1,63
fliL	flagellar protein FliL	1,62	2,42	2,85
fliE	flagellar hook-basal body complex protein FliE	1,84	2,45	3,01
flgG	flagellar basal-body rod protein FlgG		1,50	1,77
flgF	flagellar basal-body rod protein FlgF	1,75	1,83	2,42
flgC	flagellar basal-body rod protein FlgC		1,56	2,05
flgB	flagellar basal-body rod protein FlgB		1,74	2,25
flgM	negative regulator of flagellin synthesis FlgM	1,79		
algB	alginate biosynthesis transcriptional regulatory protein	2,10		
algA	mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase	1,51		
algF	alginate O-acetyltransferase	1,90		
algJ	alginate O-acetylation protein AlgJ	1,66		
algI	alginate O-acetylation protein AlgI	1,72		
alg8	alginate biosynthesis protein Alg8	1,58		
algD	GDP-mannose 6-dehydrogenase	1,79		
mucA	sigma factor algU negative regulatory protein MucA	2,08		
mucB	sigma factor algU regulatory protein MucB	2,06		
algT	RNA polymerase sigma-H factor AlgT	1,89		
rpoS	RNA polymerase sigma factor RpoS	-1,98		
rpoH	RNA polymerase sigma-32 factor	2,17		

4.2. Mechanisms of Gene Regulation under Water Stress

Bacteria sense their environment through signaling proteins like histidine kinases and response regulators, which form the two-component system (TCS) and constitutes the major signal transduction system in bacteria (Alm *et al.,* 2006). When environmental conditions change, the sensor histidine kinase is activated and causes the response regulator to be phosphorylated. The phosphorylated response regulator controls the expression of target genes. Other proteins like methyl-accepting chemotaxis proteins, diguanylate and adenylate cyclases, c-di-GMP phosphodiesterases, serine/threonine/ tyrosine protein kinases and extracytoplasmic function (ECF) sigma factors can also be involved in signal transduction (Galperin and Gomelsky, 2005, Helmann, 2002). Although once believed to be independently acting mechanisms, the signaling mechanisms are now seen as interactive and complex networks (Galperin, 2005). For example, regulation of alginate synthesis (discussed in the next section) involves diverse mechanisms including ECF sigma factors, TCSs and c-di-GMP (Wozniak and Ohman, 1994, Yu *et al*., 1997, Merighi *et al*., 2007), while the same signaling

mechanism may play roles in many regulatory pathways, for example, the AlgZ-AlgR TCS is involved in alginate synthesis but also in twitching motility and cyanide production (Yu *et al*., 1997, Whitchurch *et al*., 2002, Cody *et al*., 2009). This suggests that signal transduction and transcription regulation are complex, and detailed investigations are often needed to reveal the mechanisms and interactions of sensing and signaling. It is beyond the scope of this thesis to elucidate these mechanisms. However in the following sections I provide a brief introduction on how some of these mechanisms take part in the regulation of flagella and alginate synthesis. Before proceeding with flagella and alginate synthesis regulation, I will review sigma factors separately as many of them are associated with the transcription of genes linked to stress tolerance.

4.2.2. Sigma Factors

Transcription in bacteria is mediated by RNA polymerases (RNAP). To initiate transcription, RNAP needs proteins called sigma factors to form the RNAP-holoenzyme (initiation specific enzyme). The RNAP holoenzyme detects and binds to the specific promoter region and RNAP can initiate transcription (Ishihama, 2000). Classification of sigma factors seems to differ depending on the source of the information as well as the species. To alleviate confusion throughout the thesis, here I use a classification (Figure 4.2) of sigma factors based on the literature regarding *P. aeruginoasa* (Potvin et al., 2008), *P. putida* KT2440 (Martinez-Bueno et al., 2002) and eubacteria in general (Woesten, 1998).

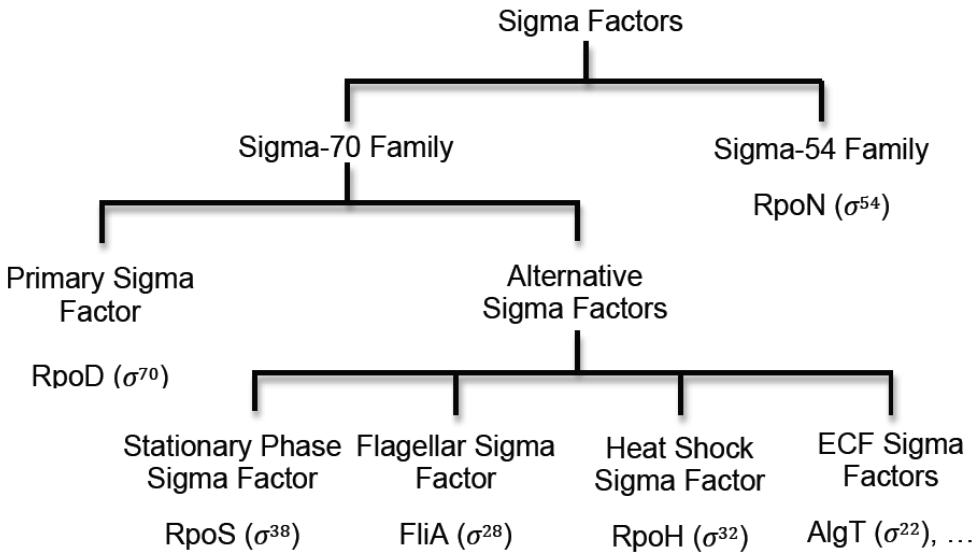

Figure 4.2. Sigma Factors.

Based on the DNA sequence similarities, sigma factors can be divided in two main protein families: sigma 70 and sigma 54 (Woesten, 1998; Oguiza *et al*., 2005). The sigma 70 family has 2 subcategories: i) the primary sigma factor RpoD (σ^{70}) which is involved in the transcription of housekeeping genes and exponential phase transcription and ii) the alternative sigma factors (ASF) which play important roles in transcription of stress related genes.

RpoS (σ^{38}) is the stationary phase sigma factor, a subcategory of ASF. It is associated with environmental fitness of *P. fluorescens* (Stockwell and Loper, 2005). Stockwell and Loper (2005) reported that an *rpoS* mutant of *P. fluorescens* was more sensitive to several stresses including desiccation. Van de Mortel and Halverson (2004a) also reported that 14 of their 35 water deprivation related genes could be under the control of RpoS. In *E. coli,* RpoS controls trehalose synthesis genes (Welsh, 2000). Its involvement under diverse stress conditions and in different bacterial species suggests a global stress regulator role for RpoS. Considering that RpoS is associated with general stress response in many species, it was surprising that *rpoS* was down-regulated at 4 hr of matric stress in our study (Table 4.1). It is, however, speculated that there could be competition/antagonism between RpoS and AlgT (AlgU) in *P. aeruginosa* (Behrends *et al*., 2010). AlgT is a major ECF sigma factor (discussed below) and we detected *algT* up-regulation at 4 hr (Table 4.1); suggesting that AlgT, rather than RpoS, is the controlling factor for water stress adaptation in our settings.

Extracytoplasmic function (ECF) sigma factors are another subfamily of the sigma-70 family and they are especially known in generating adaptive stress responses (Martinez-Bueno *et al*., 2002). Although *P. putida* KT2440 is reported to have 19 ECF sigma factors (Martinez-Bueno *et al*., 2002), we only detected *algT* up-regulation in our study. AlgT (AlgU, σ^{22}) is a major ECF sigma factor and plays a critical role under various environmental stresses. It is found to be important in osmotic, oxidative stress and heat shock tolerance in *P. syringae* (Keith and Bender, 1999), desiccation and osmotic tolerance in *P. fluorescens* (Schnider-Keel *et al*., 2001) and osmotic tolerance in *P. aeruginosa* (Aspedon *et al*., 2006). It controls the expression of the alginate operon in *P. aeruginosa* (Wozniak and Ohman, 1994), which is discussed in the next section. Although the role of AlgT in *P. putida* is not fully elucidated, the high genome similarity between *P. putida* and *P. aeruginosa* and its documented up-regulation under water stress (Li *et al*., 2010, **Article 3**) suggest a similar role for AlgT in *P. putida* as in *P. aeruginosa*. In addition to AlgT, PvdS is also a well-characterized ECF in *P. aeruginosa* (Potvin *et al*., 2008). PvdS plays a role in pyoverdine

synthesis and, therefore, is involved in iron regulation. In *P. aeruginosa*, the expression of *pvdS* is controlled by Fur (Ferric uptake regulator). When there is enough iron in the environment, Fur suppresses the transcription of *pvdS*. The same regulator (Fur) controls PfrI (ECF sigma factor) in *P. putida* (Potvin *et al*., 2008). Interestingly, our experiments with PEG-8000 simulated water stress (-0.5 and -1.0 MPa) revealed down–regulation of a few genes involved in iron uptake including *pfrI* (**Article 3**). However, in the case of directly applied matric stress, we did not detect any of those genes (**Article 3).** This suggests that there might be a connection between iron imbalance and PEG-8000 simulated water stress. Although a previous study with *B. subtilis* revealed that increased osmotic stress (from 0.7 M to 1.2 M, equivalent to -3.1 to – 5.3 MPa water potential) caused the cells to experience iron starvation (Hoffmann *et al*., 2002), a recent study (Argandona *et al*., 2010) suggested that the iron demand of *Chromohalobacter salexigens* decreased under osmotic stress (from 0.75 M to 2.5 M NaCl, equivalent to -3.2 to – 11 MPa water potential). The latter also suggested a double role for Fur; while repressing iron uptake genes, it was also involved in the expression of ectoine, which is an osmoprotectant. In our microarray with PEG-8000 we detected down-regulation of ectoine related genes. This indicates that PEG-8000 induced stress activates different mechanisms than osmotic stress exerted by NaCl. We should note however that in both studies the severity of osmotic stress and the model organisms were different. Further studies are definitely needed to elucidate how PEG-8000 simulated water stress affects the cells, especially focusing on iron uptake.

Another sigma factor, RpoH (σ^{32}), controls the heat shock regulon in *E. coli* (Grossman *et al*., 1987). The role of RpoH in *P. putida* is not fully understood; there is evidence that it is activated under heat shock (Aramaki *et al*., 2001), although other studies could not identify this function (Manzanera *et al*., 2001). In *P. aeruginosa* AlgT causes *rpoH* to be expressed (Schurr and Deretic, 1997). Our results indicated up-regulation of *rpoH* after 4 hr matric stress (Table 4.1), suggesting that RpoH plays a role in matric stress tolerance.

Sigma 28 (RpoF, FliA) controls flagellin synthesis in *P. aeruginosa*. The mechanism of *fliA* transcription is still unknown but is suggested to be constitutive (Dasgupta *et al*., 2003). This may make sense considering the advantage of having flagella to disperse and colonize new areas. However, the activity of FliA is inhibited by its anti-sigma-factor, FlgM (Frisk *et al*., 2002). In our study we did not detect *fliA* expression, but *flgM* was up-regulated at 4hr of stress (Table 4.1).

The other major sigma factor family is the sigma-54 protein. Sigma-54 (RpoN, σ^{54}) is reported to be involved in nitrogen assimilation, zinc tolerance, phage-shock response and flagella biosynthesis (Totten *et al*., 1990, Arora *et al*., 1997, Potvin et al., 2008), and absence of it inhibited flagellar synthesis in KT2440 (Cases *et al*., 2003). Cytryn *et al*. (2007) has also reported that under desiccation stress, σ^{54} and related genes of *B. japonicum* were up-regulated and the level of regulation increased with time. However, we did not detect differential expression of *rpoN* in our study.

4.3. Regulation of Flagellar and EPS Synthesis Genes

4.3.1. Flagellar Synthesis Genes

Flagellar synthesis is energetically costly and if the environmental conditions are not in favor of flagellar motility, suppression of flagella synthesis is well expected. Flagella synthesis is suppressed in *Salmonella typhimurium* at decreased water levels, suggesting that flagella can regulate their own biosynthesis by sensing the water level in the environments (Wang *et al*., 2005). van de Mortel and Halverson (2004a) detected down-regulation of flagellar genes in *P. putida* mt-2 even under mild water stress (-1.5 MPa). Nevertheless, up-regulation of flagellar genes has been also reported; Cytryn *et al*. (2007) detected transient expression of flagellar genes: down regulation with short desiccation time but up-regulation with prolonged desiccation. Our microarray study also showed up-regulation of many flagellar genes, and their number also increased with prolonged stress (Table 4.1). We do not know if the flagellar gene expression is related to motility or flagella's ability to sense the environments. There might be different explanations. It could be that cells, which attempt to migrate to favorable environments, could for example be a starvation response. It has been already reported that despite decreased motility, *Rhizobium meliloti* cells were still maintaining their chemotactic responses under starvation in an attempt to reach nutrients (Wei and Bauer, 1998). It is also possible that flagellar export apparatus is involved in exporting other proteins unrelated to flagellar assembly under water stress. In *P. putida* DOT-T1E flagellar gene expression has been suggested to contribute to organic solvent tolerance (Segura *et al*., 2004). Maybe the flagella of *P. putida* can also act like water level sensor as suggested in *Salmonella typhimirium* (Wang et al., 2005). All of these possibilities and previously observed roles suggest that flagella may have many more roles than we are aware of. This also makes sense evolutionarily; as a complex structure starts to emerge, it may have a different function than its final form (Pallen and Matzke, 2006) leading into different roles. Therefore, it would be worthwhile to

direct our efforts to elucidate the different roles of flagella and flagellar operons.

Based on the synthesis of literature, Dasgupta *et al*. (2003) suggested a model of flagellar gene regulation in *P. aeruginosa* (Figure 4.3). As *P. aeruginosa* and *P. putida* are 85 % similar in their genomic sequence, it is likely that mechanisms are similar for both species (Nelson *et al*., 2002). Unlike peritrichously flagellated bacteria, polarly flagellated bacteria have four classes of genes involved in the regulation of flagellar synthesis. *fleQ* and *fliA* are class I genes. FleQ is the master regulator of flagellar genes, and its regulation is controlled by sigma-70 and Vfr (cyclic nucleotide-binding transcriptional regulator). Class II genes require FleQ and RpoN to be transcribed. Class II operons include *fleSR, fliEFGHIJ, flhA, flhFfleN, flgA, fliLMNOPQRflhB* and *fliDSSfleP*. The products of class II genes form the basal body, MS ring, P ring, motor switch, flagellar export apparatus and filament cap. Among class II proteins, FlhF, FleN, FleS and FleR are the regulatory proteins. FlhF determines the location of polar flagella in *P. putida* and a similar role is suggested for *P. aeruginosa*. FleN is the antiactivator of FleQ, and by inhibiting FleQ activity it is responsible for controlling the number of flagella. FleS and FleR form a two-component system, acting sensor kinase and response regulator, respectively. What FleS senses is still unknown. FleQ, FleR and RpoN are responsible for the transcription of class III operons, which are *flgBCDE, flgFGHIJKL* and *fliK*. Class IV operons are *fliCfleL* and *flgMN*, transcriptions of which is controlled by FliA. It has been suggested that FlgM, the negative regulator of flagella synthesis, has a dual role. As a class II protein, FlgM inhibits the activity of FliA and negatively regulates the *fliC* transcription, and as a class IV protein its secretion frees the FliA. FliA then activates the transcription of class IV genes and flagellar synthesis is completed.

All of the flagellar genes that we detected as differentially expressed (Table 4.1) belong to first and/or second gene in the 5 of the 17 flagellar operons as known in *P. aeruginosa:* ***fliE****FGHIJ* (class-2), ***fliLM****NOPQRflhB* (class-2), ***flgBC****DE* (class-3), ***flgFG****HIJKL* (class-3) and ***flgM****N* (class-2 & 4) (Dasgupta *et al*., 2003). Many expressed genes encode proteins of the basal body: FliE, FlgB and FlgC form the proximal rod, FlgF and FlgG form the distal rod and FliM is a motor/switch component. *flgM*, on the other hand, is the negative regulator of flagellin synthesis (*fliC*). With the exception of *fliM*, the class-2 genes showing differential expression were up-regulated to high levels earlier than the class-3 genes, consistent with the hierarchical expression of flagellar genes shown in Figure 4.3. *flgM* is expressed as a class-2 gene and FlgM, as an anti-sigma factor,

prevents expression of the FliA sigma factor- dependent class-4 genes such as *fliC*. No class-4 gene, other than *flgM,* which is considered both a class-2 and class-4 gene, was differentially expressed at any of the sampling times. Transcripts for the first gene(s) in an operon are often more abundant than for the downstream genes (Ullmann *et al*., 1979), and therefore the inability to detect differential expression of downstream genes likely reflects a lack of statistical power to demonstrate differences. Moreover, observing only class 2- and class-3 genes but not class-4 strongly suggests that neither flagellation nor hyperflagellation of cells took place during the stress, indicating a different role for the devoted expression of some flagella synthesis operons.

Non-flagellar specific regulators can also affect flagella synthesis genes. In *P. aeruginosa* AlgT negatively regulates flagellar synthesis by suppressing *fleQ* transcription. Recently, it has been found that MorA, a membrane-localized regulator, is involved in the timing of flagellar synthesis in *P. putida* (Jyot and Ramphal, 2008). However, how these mechanisms, which regulate flagellar synthesis, have not been fully understood and further exploration of these mechanisms in *P. putida* would contribute to our understanding.

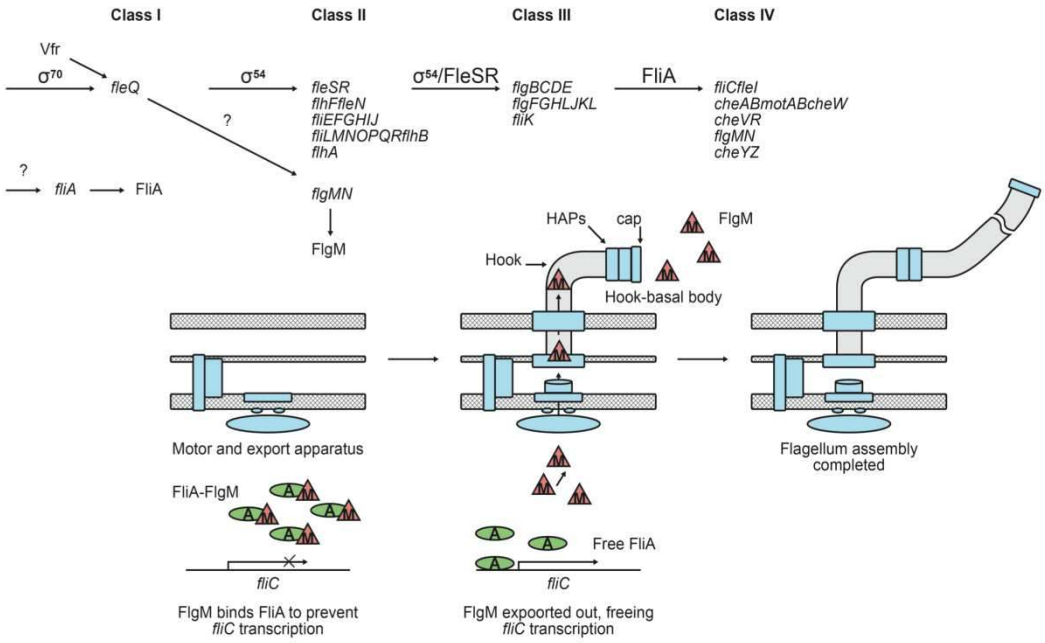

Figure 4.3. Model for flagellum synthesis in *P. aeruginosa* (reproduced from Jyot and Ramphal, 2008).

4.3.2. EPS Synthesis Genes

Although the importance of EPS for maintaining hydration under unsaturated conditions is established, the regulation of EPS gene expression under unsaturated conditions is still to be discovered. Studies have reported both up- and down regulation of EPS related genes, which seems to be dependent on the duration and severity of the water stress and the bacterial species (Cyrtyn *et al*., 2007, Katoh *et al*., 2004, Van de Mortel and Halverson 2004a, Li *et al*., 2010). As mentioned in the previous chapter, in *P. putida* KT2440 alginate is the major EPS polysaccharide, but it has genes for the synthesis of cellulose (*bcs)*, putative exopolysaccharide a (*pea*) and putative exopolysaccharide b (*peb*) (Nelson *et al*., 2002, Nielsen *et al*., 2011, Nilsson *et al*., 2011).

Gene expression studies with *Pseudomonas putida* mt-2 (Van de Mortel and Halverson, 2004a) under water deprivation (-1.5 MPa) have revealed up-regulation of *algA*. AlgA functions as a catalyzer of the first step in alginate synthesis (Gacesa, 1998). The same gene was also up-regulated in our study (Table 4.1). Recently Li *et al*. (2010) investigated the temporal dynamics of alginate induction under water deprivation (down to -1.5 MPa) in *P. putida* mt-2 using an alginate bioreporter. They reported that *algD* promoter activity increased with decreasing water potentials and the extent of alginate induction changed with respect to biofilm age. More specifically they found that the number of cells expressing alginate decreased after 12 hours and their qRT-PCR showed that the level of up-regulation for *alg8* and *algT* was higher at 6hr of stress but decreased at 24hr, indicating a transient expression of alginate genes under water stress. Our microarray results showed a similar transient expression of alginate synthesis genes of KT2440 under -0.4 MPa matric stress (Table 4.1.); we found that alginate synthesis genes were up-regulated at 4 hr but not detected at 24 or 72 hrs stress. In a recent study with strain mt-2, Nielsen *et al*. (2011) detected *alg8, bcsA* and *peaI* expression under water stress, suggesting that *bcs* and *pea* may contribute to maintaining hydration although not to the extent of alginate. Although our study showed significant expression of alginate genes, expression of *bcs, pea* and *peb* was not detected. This suggests that alginate plays the major role under matric stress.

Regulation of alginate synthesis is complex, involving many mechanisms. Figure 4.4 shows the hierarchical regulatory network of alginate operons. AlgT (AlgU) is associated with the mucoid phenotype in *P. aeruginosa* (De Vries and Ohman, 1994). In *P. aeruginosa*, AlgT negatively controls flagellum synthesis (Garret *et al*., 1999). According to the hierarchical model proposed by Wozniak and

Ohman (1994) *algT*, *mucA* and *mucB* are the top genes responsible to determine the alginate producing phenotype. Xie *et al*. (1996) reported that MucA inhibits AlgT activity, thereby inhibiting alginate synthesis in *P. aeruginosa*. They further speculated that MucB might have a role in orchestrating the balance between AlgT and MucA. We found that *algT, mucA* and *mucB* were up-regulated, suggesting that alginate synthesis might be initiated and stopped when it is no longer needed. In *P. aeruginosa*, AlgT is required for *algB* transcription (Wozniak and Ohman, 1994), and AlgB is required for *algD* transcription, which is the first gene in the alginate operon. *algFJI* are alginate O-acetylation genes (Franklin and Ohman, 1993, 1996) and O-acetylation affects physico-chemical structure of alginate and increases its water binding capacity (Skjåk-Bræk *et al*., 1989, Halverson, 2009). We also detected that *algB* and half of the genes in the *algD* operon, including *algFJI*, were up-regulated (Table 4.1) strengthening alginate's presumed role of creating a hydrated environment under water derivation. It is likely that this coordinated expression of many alginate genes, being part of the major alginate operons, may bring about alginate production. It is known that alginate creates a hydrated environment under water deprivation in *P. putida* (Chang *et al*., 2007). This may explain the transient gene expression that we observed (Table 4.1) as the creation of a hydrated environment might alleviate perceived water stress and stop over-expression of the alginate operons at later times.

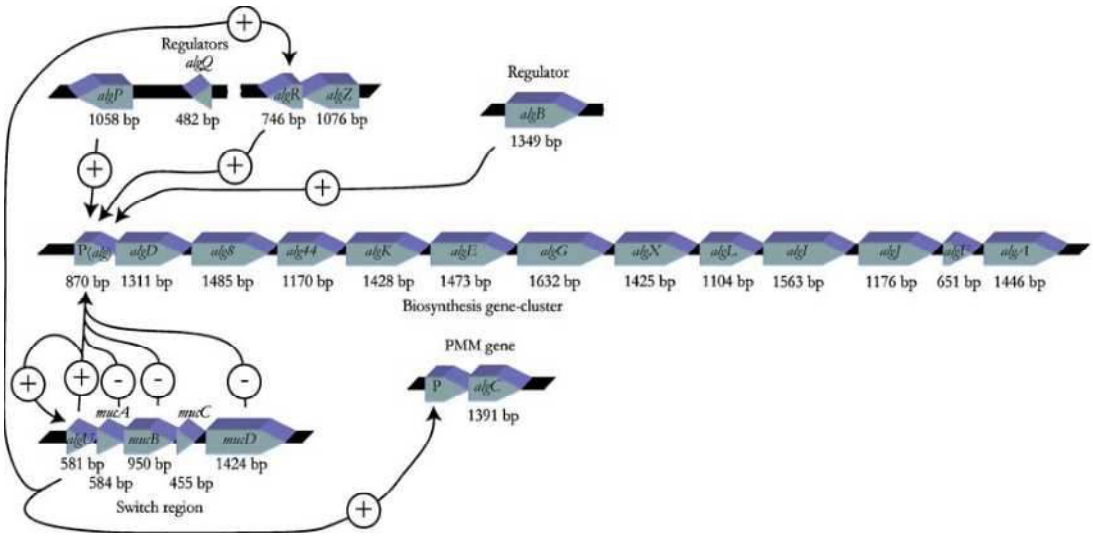

Figure 4.4. Alginate Gene Regulation (Remminghorst and Rehm, 2006).

Previous research has suggested that in *P. aeruginosa*, alginate synthesis is controlled by the KinB-AlgB TCS (Ma *et al*., 1997). KinB is the inner membrane protein, which has histidine protein kinase activity which phosphorylates the

response regulator AlgB (Ma *et al*., 1997). Although KinB and AlgB form a TCS (Ma *et al*., 1997), in a subsequent study Ma *et al*. (1998) showed that KinB was not necessarily needed to phophorylate AlgB for alginate production. In a recent study, Leech *et al*. (2008) reported that AlgB is directly interacting with the promoter of *algD* operon and the way KinB activates AlgB is probably related to another regulatory mechanism than alginate synthesis. What environmental factors activate KinB and why it forms a TCS with AlgB is still to be discovered. Although our microarray study indicated *algB* up-regulation, we did not detect any *kinB* activity (**Article 3**), suggesting that *algB* expression was independent of TCS. Yu *et al*. (1997) reported another TCS, AlgZ-AlgR, as controlling alginate production in *P. aeruginosa*. Whitchurch *et al*. (2002) have reported that AlgZ-AlgR was involved in type 4 pili mediated twitching motility in *P. aeruginosa*. The same TCS was also reported as involved in cyanide production in *P. aeruginosa* (Cody *et al*., 2009). These suggest that AlgZ and AlgR control more than just alginate synthesis. Having the homologes of AlgT, AlgB, AlgZ and AlgR with a high sequence similarity suggests that *P. putida* may have similar mechanisms of alginate synthesis regulation (Li *et al*., 2010). In *P. aeruginosa*, alginate production is also regulated by bis-(3'-5')-cyclic –GMP (c-di-GMP), the bacterial second messenger. It has been reported that Alg44, in the *algD* operon, has a PilZ domain which acts as a c-di-GMP receptor and increased level of c-di-GMP resulted in increased alginate production (Merighi *et al*., 2007). It has been reported that in *P. putida* c-di-GMP controls the expression of LapA, outer membrane-associated protein, which is associated with a cellulase-degradable exopolysachharide (Gjermansen *et al*., 2010). Further investigation regarding the role of c-di-GMP in the production of alginate or other exopolymers in *P. putida* is needed. Quinones *et al*. (2005) has suggested that alginate production in *P. syringae* may be regulated by quorum sensing. In quorum sensing, the expression of certain traits is controlled by cell-density. Bacteria sense the cell density by detecting the concentration of N-Acyl homoserine lactone (AHL) signaling molecules produced by the cells. Although *P. putida* KT2440 does not exhibit a traditional QS, Espinosa-Urgel *et al*. (2004) reported cell-density dependent seed colonization of *P. putida* KT2440, and the RoxX/RoxR TCS is suggested to play a role in generating this response (Fernandez-Pinar *et al*., 2008). Our study did not indicate activation of any these systems.

5. Conclusions and Perspectives

In this thesis, I investigated how *P. putida* KT2440 responds to water stress by conducting experiments at different scales of microbial ecology. One of the major hypotheses of this thesis was that flagellar motility, and thereby colony expansion, would be limited under matric stress. I clearly showed how flagellar motility was limited, resulting in smaller colony areas under matric stress compared to the saturation reference condition. I also showed that KT2440 can benefit from sudden wetting events by exhibiting flagellar motility, suggesting that even rare wet events can offset the cost associated with flagellar synthesis and explain the sustained presence of flagellated bacterial cells in soil habitats. Although at the ecophysiological and population levels our hypotheses were satisfied, at lower scales it was not. In contrast to our hypothesis, we detected that many flagellar genes were up-regulated. The fact that all the expressed genes belong to class-2 and class-3 but not class-4 suggests that this up-regulation is not related to flagellation or hyperflagellation to attempt dispersal but probably due to another reason (i.e., a stress response, sensing role, etc.), which needs to be investigated. As Howard Berg states, "Perhaps it is time to learn what the flagella are actually doing"(Berg, 2005).

We also showed that EPS was affected by the matric stress. We observed significant up-regulation of alginate, a major EPS, synthesis genes, supporting the findings of the previous studies regarding the importance of alginate under unsaturated conditions. On the other hand, the colony morphology experiments performed with alginate deficient mutant did not indicate a difference compared to the wild type. However this does not eliminate the importance of alginate in colonization on porous surfaces, and further experiments with mutants deficient in multiple EPS components have to be performed to assess the absolute role of alginate and other polymers under unsaturated conditions.

We also showed that PEG-8000 simulated water stress resulted in a different gene expression profiles than the directly applied matric stress **(Article 3)**. Both this study and our previous work with PEG-8000 (Dechesne *et a*l., 2008) criticize the common notion of PEG-8000 simulating matric stress and indicate a need of different terminology to denote the stress caused by PEG-8000. Further exploration at a more fundamental level is needed to elucidate the mechanisms of how bacteria sense osmotic, matric and PEG-8000 simulated water stress.

I also demonstrated that the newly designed PPSM is a suitable platform to study bacterial behavior under a wide range of matric potentials. More importantly, with PSM **(Article 1)** and PPSM **(Article 3)** we were able to explore the importance of bacterial traits (flagellar motility) that could not studied by previous methods. Although the (P)PSM has many advantages, it can benefit from improvements. Since one of the ultimate goal of these studies would be to examine microbe-pollutant interactions in unsaturated conditions, coupling the system with a component allowing the measurement of degradation kinetics would be a good investment. Also, extending the 2-D (P)PSM into a 3-D Porous Network Model, while still maintaining the simplicity and advantages of the (P)PSM, presents itself as a worthwhile challenge. Such improvements in experimental models would provide stronger insights in the soil-microorganism-pollutant interactions.

It is true that laboratory scale experiments cannot fully reflect the complexity and reality of the natural environments. However it is actually this simplicity and level of abstraction, which is necessary to unravel the governing mechanisms in soil-microbe-pollutant interactions. By conducting pure culture experiments under controlled environments, we acquire quantitative information on the key processes. The quantitative results can further be used to that to develop and/or validate models. Indeed, our results regarding the colony expansion rates of flagellated bacteria under diverse matric potentials were already used to validate an individual based computational model, simulating bacterial dispersal on rough unsaturated surfaces (**Article -1**). As a next step, effect of EPS production on bacterial dispersal can be implemented on such individual based models.

It is not only the changes in water levels that affect pollutant-microorganism interactions in the vadose zone. Other abiotic factors like temperature, pH, oxygen levels, etc are also important for the microbial activities. Extending our experiments to incorporate those factors and investigating their combined effect on the microbial activities would definitely advance our understating of the biodegradation mechanisms. Now that we showed the importance of EPS and flagellar motility, we can investigate how integrating other factors and their interaction would influence these traits. With the PSM, it should be possible to study the combined effect temperature, pH and matric potential and quantify the effect on the surface colony expansion. Once the results are obtained, they can be used to complicate the simulation models.

Bacteria in soil are not found as pure cultures, but coexist as mixtures with many other organisms, some of which may be playing key roles in pollutant degradation. Therefore, it would be also worthwhile to extend our efforts to understand the interactions between different types of microorganisms in soil. Fungal networks, for example, have been suggested to provide continuous liquid films for bacteria to move in the vadose zone (Wick *et al*., 2010). Studying such interactions in a liquid film controlled system like the PSM would definitely contribute to our understanding of different mechanisms of bacterial dispersal in the vadose zone.

By selecting the potentially important genes based on the microarray results, we can also deepen our understanding of the function and activity of those genes and assess their effect on bacterial dispersal under unsaturated conditions. One way of doing this is to investigate the spatio-temporal expression of those genes using bioreporter strains. Knowing when and where in a colony a gene is activated may be an indicator of when and where to the product of that gene is manifested. For example, we can explore if EPS genes are more active at the colony edges and how would this affect colony dispersal at that time.

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7. Papers

I. Dechesne A., G. Wang, G. Gulez, D. Or, and B.F. Smets. 2010. Hydration controlled bacterial motility and surface dispersal. Proc. Natl. Acad. Sci. USA. 107:14396-14372

II. Gulez, G., A. Dechesne, and B.F. Smets. 2010. The Pressurized Porous Surface Model: An improved tool to study bacterial behavior under a wide range of environmentally relevant matric potentials. J. Microbiol. Methods. 82: 324-236

III. Gulez, G., A. Dechesne, C.T. Workman, and B.F. Smets (2011, in revision for Appl. Env. Microbiol.) Whole genome expression dynamics of *Pseudomonas putida* KT2440 under water stress on porous surfaces

IV. Role of EPS components on the surface colonization of *Pseudomonas putida* KT2440 under unsaturated conditions

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment:
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