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Molecular and functional characterization of the chemotactic genes in the PCBs-degrader *Pseudomonas pseudoalcaligenes* KF707

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Preface

Bacteria may encounter a large spectrum of different environments during their life cycles. Indeed, the capacity to adapt and survive in changing environments is a fundamental property of living cells and bacteria have developed effective mechanisms to regulate their behaviour accordingly. Chemotaxis, i.e. the migration of microorganisms under the influence of a chemical gradient, allows bacteria to approach chemically favorable niches for their growth and survival avoiding unfavourable ones. Since the most of microorganisms inhabiting heterogeneous environments are motile, the chemotactic behavior is achieved by integrating signals received from receptors that sense the environment. Apparently, the main reason for which environmental bacteria have retained during the "evolution" a large number of genes involved in motility and chemotaxis (Macnab, 1996), is because they provide a selective advantage and play a significant role in the dynamics of microbial populations (Pilgram and Williams, 1976; Freter *et al*., 1978; Kennedy and Lawless, 1985; Kennedy, 1987; Kelly *et al*., 1988; Lauffenburger, 1991).

Bacterial chemotaxis can be therefore considered the *prerequisite* for population survival, metabolism and interactions within ecological niches (Chet and Mitchell, 1979). In line with this, it has been reported that chemotaxis has important roles in colonization of plant roots by plant growth-promoting *Pseudomonas fluorescens* (De Weger *et al.,*1987; de Weer *at al*., 2002), infections of plants by *Pseudomonas syringae* and *Ralstonia solanacearum* (Yao and Allen, 2006), and animal infections by *Pseudomonas aeruginosa* (Drake and Montie, 1998). Notably, chemotaxis is also a selective advantage to degradative bacteria which colonize contaminated sites as microorganisms, with a

chemotactic ability toward xenobiotic compounds in polluted niches, have been isolated and characterized (Harwood *et al*., 1990; Grimm and Harwood, 1997; Bhushan *et a*l., 2000a; Bhushan *et al*., 2000b; Parales and Harwood, 2002).

The soil bacterium *Pseudomonas pseudoalcaligenes* KF707 is know for its ability to degrade biphenyl and polychlorinated biphenyls (PCBs) (Furukawa *et al*, 1986), to which the strain is chemically attracted. PCBs are toxic compounds of great concern since they have been recognized as important harmful environmental contaminants in the EPA (Environment Protection Agency) priority list of pollutants.

The understanding of bacterial chemotaxis toward pollutants is a topic of particular interest, so that strategies for bioremediation by means of strains with degradative abilities, have been developed. However, the low bioavailability of organic contaminants is a limitation for the microbial remediation of contaminated sites, as toxic hydrophobic chemicals are often adsorbed to a non-aqueous-phase-liquid (NAPL) (Stelmack *et al*., 1999). In bioremediation processes, target compounds can be easily accessible to bacteria by dissolution in the aqueous phase; alternatively microorganisms might have access to a polluted surface through biofilm formation. In this respect, chemotaxis is a key factor in biofilm formation (Pratt and Kolter, 1998; O'Toole and Kolter, 1998; , Prigent-Combaret *et al*, 1999; Watnick and Kolter, 1999) and flagella are required for attachment to solid surfaces and the initiation of biofilm formation (Pratt and Kolter, 1998; Stelmack *et al*., 1999). In addition, motility and chemotaxis are required for biofilm growing bacteria to move along the surface, facilitating the spread of the biofilm (Stelmack *et al*., 1999).

Recent findings have shown that a *Pseudomonas pseudoalcaligenes* KF707 chemotactic mutant in a *che*A gene (*che* stands for chemotaxis) is impaired in motility and chemotaxis as well as in biofilm development (Tremaroli *et al*, 2011). However,

recent studies on sequencing, assembly and annotation of *Pseudomonas pseudoalcaligenes* KF707 genome (Triscari *et al*., 2012; see also this Thesis work), have clearly demonstrated that the KF707 genome contains multiple putative operons encoding for different chemotaxis pathways and therefore multiple *che*A genes are present. This finding was not surprising since genome analyses have revealed that a large number of environmental motile bacteria, such as *Pseudomonas spp*., *Vibrio spp.*, *Rhodobacter spp.*, own several gene clusters involved in chemosensing and chemotactic signal transduction, which may work in parallel or be expressed under different environmental conditions.

The goals of this present study were to investigate the role in motility, chemotaxis as well as in biofilm formation, of the various *che*A genes we found by sequencing analysis of KF707 genome and to compare their functions with those previously attributed to a *che*A gene in a KF707 mutant strain constructed by a mini-Tn5 transposon insertion (Tremaroli *et al*., 2011). Further, since it has been reported that communication *via* quorum sensing (QS) is involved in organizing group motility and biofilm formation, the ability to produce signal molecules by KF707 was also investigated.

CHAPTER A - General introduction

Motility and chemotaxis are peculiar traits common to many bacterial species. In the microbial world different kinds of motility can be observed. In addition, microorganisms can be sensitive to different stimuli and responde to them with variable taxis strategies.

A-1 Motility systems

Swimming is the most common strategy for motility in fluid environments and is the outcome of the flagellar rotation, which exert a pushing force that drives bacteria at a speed up to 20–60 nm/sec. Several types of flagellar motility have been found and they depend on the number and the position of flagella and on the species. In Table A-1.1, various types of flagellar motility are listed.

Table A-1.1: Variety of flagellar motility in bacteria. This table was taken and modified from Eisenbach (2001).

Flagellation	Examples of species	Description of motility
A single flagellum at one of the cell poles	*Pseudomonas spp*	The flagellum, depending on its direction of rotation, pushes or pulls the cell.
A single flagellum in the middle between the poles	*Rhodobacter sphaeroides*	The flagellum either rotates clockwise or pauses. Consequently the cell swims in a rather straight line and occasionally stops for reorientation.
A bundle of flagella at one of the poles	*Chromatium okenii, Halobacterium salinarium*	The bundle, depending on its direction of rotation, pushes or pulls the cell.

Table A-1.1 *continued*		
A bundle of flagella at each of the two poles	Some cells of *H. salinarium*	The bundles, depending on their direction of rotation, push or pull the cell. Consequently, the cell goes back and forth or stops
5–10 flagella randomly distributed around the cell	*Escherichia coli, Salmonella typhimurium, Bacillus subtilis*	Most of the time the flagella rotate counterclockwise and the cell swims in a rather straight line (a run). Intermittently, the flagella rotate clockwise or pause, as a result of which the cell undergoes a vigorous angular motion (a tumble)
A polar tuft of 2 flagella + 2–4 lateral flagella	*Agrobacterium tumefaciens*	Flagella rotate clockwise or pause; consequently the cell swims in a rather straight line or turns
One flagellum at one end, one or more Flagella subterminally at each end. All the flagella are contained within the periplasmic space	*Spirochaetes*	The cells exhibit smooth swimming, reversals, flexing and pausing. When the flagellar bundles at both cell poles rotate in opposite directions (one pulls and one pushes), the cell swims in a rather straight line. When both bundles switch synchronously, the cell reverses. When both bundles rotate in the same direction, the cell flexes

Swarming is an organized translocation of differentiated cells on a solid surface generally due to type IV pili and cell-to-cell communication appears to be essential for this motile behaviour. Swarming bacteria located in the outer layer of a colony, expand outwardly and the evacuated space is filled with new growing cells. Irregular branches can appear at the periphery of the colony, forming a dendritic pattern on the surface.

Gliding is a particular kind of movement characterized by bacterial migration on a solid surface covered with a liquid film, without the formation of external structures and no cellular differentation.

Twitching motility is a form of translocation on solid surfaces which is dependent on pili-assisted motility (Henrichsen 1972;1983).

Propulsion by actin filaments is a peculiar mode of motility to pathogens such as *Listeria*, *Shigella* and *Rickettsia* in host eukaryotic cells. The bacteria assemble actin filaments for propulsion in the cytoplasm of the infected host cell.

A-2 The link between flagellar rotation and the bacterial swimming behaviour

Flagella are specialized structures (see Fig. A-2.1) which enable bacteria to swim in an aqueous solution.

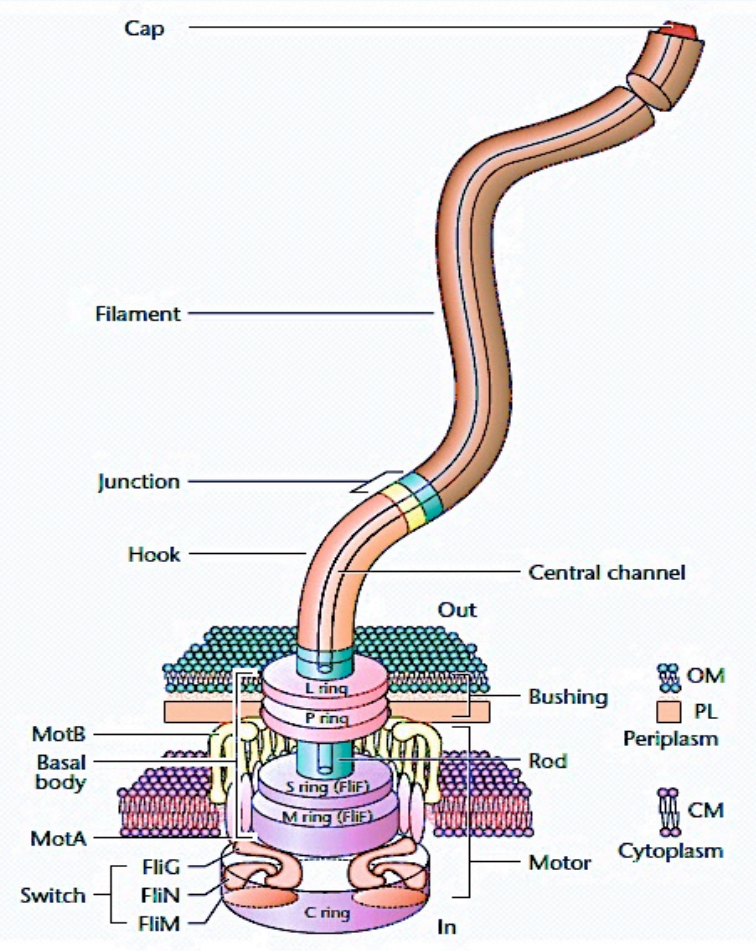


Fig. A-2.1: Structural organization of a bacterial flagellum**.** It consists of three major parts: a basal body, a hook and a filament. Bacterial flagella may vary between species and families, but the main structural aspects are common to all. This picture was taken and modified from Eisenbach (2001).

Bacteria such as *E. coli* have two main swimming patterns: smooth swimming in a straight direction (run) and an overturning motion (tumble). In absence of stimuli, when the concentration of nutritional compounds in the enviroment is uniform, cells run for about a second, then tumble for about a tenth of a second, changing orientation and, as a consequence, running in a new direction. Consequently, the bacterial cells walk randomly, with no net vectorial movement (Fig. A-2.2a). Specifically, the run is the consequence of a counterclockwise rotation while the tumble is the consequence of a clockwise rotation of the flagella.

When a cell detects increasing concentrations of attractants or decreasing concentrations of repellents, tumbles occur less frequently, and there is a net movement towards attractants and away from repellents (Fig. A-2.2b). Cells make temporal comparisons of chemo-effectors concentrations during a run and they decide, second by second, the movement direction, suppressing tumbles if the level of chemo-attractant increases. On the other hand, negative stimuli increase the probability of clockwise rotation (Tsang *et al*., 1973) and cells tumble more frequently.

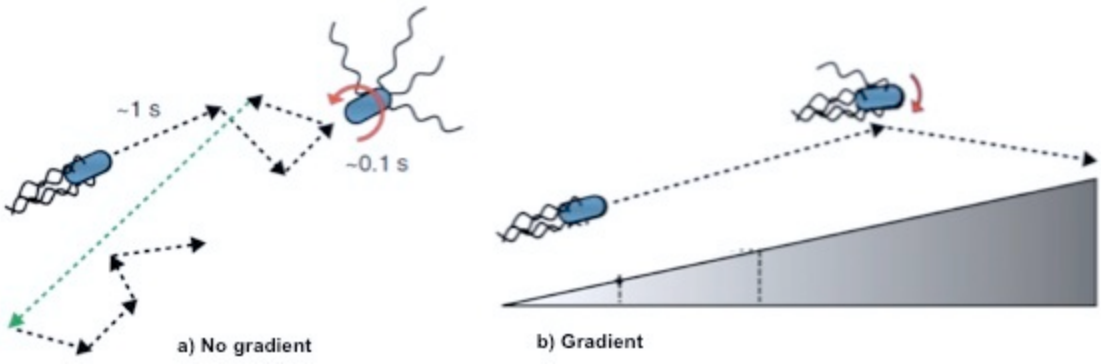


Fig. A-2.1.: Bacterial biased random walk in absence of stimuli (a) and movement under attractant gradient (b). This picture was taken and modified from Sourjik and Wingreen (2012).

A-3 Chemotaxis network in *Escherichia coli*

Prokaryotic chemosensory pathways are depending on two-component signal transduction system with conserved components regulating flagellar activity (Stock *et al*., 2000; Wolanin *et al*., 2002). Generally, a two-component system includes a histidine protein kinase (HPK) that catalyzes the transfer of a phosphoryl group from ATP to an aspartate residue on the response regulator (Borkovich *et al*., 1989).

The *E. coli* chemosensory network is considered as the simplest model to describe bacterial chemotaxis although much more complex as compared to a basic two-components system. Indeed, the six Che proteins, CheA, CheW, CheY, CheZ, CheR and CheB and the five chemoreceptors, Tsr, Tar, Tap, Trg and Aer, constitute the *E. coli* chemotaxis system.

The CheA protein, unlike orthodox membrane-bound histidine kinases, does not interact directly with chemo-effectors, because it lacks of a sensory domain. It is in fact connected to the transmembrane receptor proteins (chemoreceptors) via the 'adapter' protein CheW. Together, chemoreceptors - CheA - CheW, form large complexes that integrate enviromental informations to control CheA kinase activity in phosphorylating the response regulator CheY. CheR and CheB are, respectively, involved in methylation and demethylation of the chemoreceptors cytoplasmic domain (West *et al*., 1995; Djordjevic and Stock, 1997). CheR is an S-adenosyl-methionine-dependent methyl-transferase that methylates specific glutamate residues ; CheB is an esterase with an opposite function as it hydrolyzes the methyl esters formed by CheR. These antagonist activities play a critical role in adaptation (Okumura *et al*., 1998; Levit and Stock 2002; Sourjik and Berg, 2002), conferring

also a memory mechanism. The stochastic nature of these modifying activities ensures a variety of receptor sensitivity and capacity of response in the different cells of a bacterial population.

A-3.1 Signal transduction in response to a negative stimulus

When chemo-repellents bind to the receptors, they switch to an active form and together with CheW, stimulate CheA autophosphorylation. The histidine kinase, in turn phosphorylates CheY. Phosphorylated CheY (CheY~P) diffuses to flagellar motors (Li *et al*., 1995; Sourjik and Berg, 2002), where it acts as an allosteric regulator on the flagellar proteins FliM, changing the sense of rotation from counterclockwise to clockwise and, consequently, tumble occurs (Alon *et al*., 1998). The response is termined by the CheZ phosphatase, by enhancing CheY~P dephosphorylation (Stock, A.M. and Stock, J.B., 1987; Wang and Matsumura, 1996).

A-3.2 Signal transduction in response to a positive stimulus

When chemo-attractants bind to the receptors, they do not undergo to conformational changes, so CheA autophosphorylation is inhibited, causing reduced levels of CheY~P and promoting smooth swimming as the probability of clockwise rotation decreases. The results are prolonged runs alterned to rare tumbles.

A-3.3 Signal transduction in response to multiple stimuli

Generally, cells are exposed to multiple positive and negative stimuli. Bacteria are able to integrate all these inputs and to show a unique behavioural response. Thus, movement towards attractants and away from repellents is determined by the efficiency of temporary response and the memory of past informations, properties that allow bacteria to make second-to-second decisions to continue swimming or tumbling and change direction (Berg, 2000; Bourret and Stock, 2002; Wadhams and Armitage, 2008).

A-4 Components of the "receptor-signalling complexes"

Receptors-signalling complexes can be viewed as ternary complexes resulting from the interactions between the membrane receptors and the chemotaxis proteins CheW and CheA. Receptors act anchoring the chemotactic proteins in the inner membrane and are necessary for signal transmission from the periplasmic domain (which binds the ligand), through the membrane, to the cytoplasmic complex.

In the following paragraphs the single components of receptors-signalling complexes are described.

A-4.1 Chemoreceptors: structure and classification

Chemoreceptors are transmembrane proteins with variable periplasmic sensing domains – which are able to bind specific ligands - and a conserved cytoplasmic domain – which acts as a scaffold for the anchoring of the histidine kinase CheA and the adpter CheW (Le Moual and Koshland, 1996; Zhulin, 2001). Binding of a ligand to the sensing domain causes a conformational change,

inducing a “piston-like movement" which in turn causes the transmission of signals across the cell membrane for the control of CheA kinase activity in the cytoplasm (Mowbray and Sandgren, 1998), (Otteman *et al*., 1998; 1999). The function of the chemoreceptors is strictly related to their structure. The cytoplasmic part of chemoreceptors can be divided into four subdomains: (i) the histidine kinase, adenylyl cyclase, methyl-binding proteins and phosphatases domain (HAMP); (ii) methylated helix 1 (MH1); (iii) signaling domain; (iv) methylated helix 2 (MH2). Together the methylated helixes (MH1 and MH2) contain four or more glutamate residues that are substrates for CheR and CheB modification (Terwilliger *et al*., 1983; 1984). Since chemoreceptors are substrates for methylation and demethylation, they are also known as methyl-accepting chemotaxis proteins (MCPs).

MCPs are classified on the basis of different properties: cellular localization (membrane-bound or cytoplasmatic), abundance, size (cluster I receptors with a ligand-binding region between 120 and 210 amino acids whereas cluster II receptors have larger ligand-binding regions of 220–299 amino acids), the ligand-binding region (extra-cellular space or cytosol). Notably, MCPs are different with respect to the sequence of their periplasmic part and the presence of the binding site for CheR in their cytoplasmic side. Thus, receptors possessing this CheR-binding site are known as “major receptors” and can function independently. The other MCPs, without this binding site, have an adaptation mechanism depending on the presence of the first type of receptors: this may explain a possible reason for receptors organization in clusters. Moreover, several MCPs are able to respond to different compounds at the same time: for example the *E. coli* Tar receptor, sense

aspartate and maltose. Aspartate binds directly to the periplasmic ligand binding domain (Yen *et al*., 1996) whereas maltose binds to the periplasmic maltose binding protein (MBP) associated to the MCP.

A-4.2 The histidine kinase CheA and the adapter protein CheW

CheA and CheW chemotactic proteins play an important role in the organization of clusters of receptors. On the other hand, MCPs represent anchors for the assembly of chemotactic proteins. Recent studies have shown that deleted mutants in *che*A or *che*W genes are impaired in receptor arrays formation. In order to understand the interaction between of both CheA and CheW, it is fundamental to know the tridimensional structures of these proteins and if particular conserved domains are involved in the interaction with the cytoplasmic receptor domain.

CheA is divided into five domains with specific and distint structure and function: the histidine phosphotransfer domain (P1), the response regulator binding domain (P2), the dimerization domain (P3), the histidine protein kinase catalytic domain (P4), and the regulatory domain (P5). The P1 domain belongs to the histidine phosphotransfer (HPT) family of proteins that transfer the phosphoryl groups between ATP and the phospho-accepting aspartate of the response regulators. The response regulator binding domain, P2, is flanked by two flexible linker sequences connecting it to P1 and P3 (Zhou *et al*., 1996). When P2 is in complex with CheY, the CheY active site undergoes a conformational change that increases the accessibility of the phospho-acceptor aspartate, Asp57. More importantly, P2 binds CheY in close proximity to the phospho-P1 domain and increases its effective concentration (Stewart, 1997; 2000). P3 and P4 domains

constitute the histidine protein kinase (HPK) catalytic core. P5 is homologous to CheW (Bilwes *et al*., 1999) and mediates binding to the chemoreceptor signaling domains (Levit *et al*., 2002).

CheW is a monomeric soluble protein, know as adpter and its role is anchoring the histidine Kinase CheA to the chemoreceptors arrays (Surette and Stock, 1996; Griswold and Dhalquist, 2002; Griswold *et al*., 2002).

A-4.3 Organization of the receptors-signal complexes in clusters

Generally, bacterial chemoreceptors are organized in clusters located at one or both the cell poles. The chemoreceptors are organized into units of 'trimers of dimers', which form ternary signalling complexes with the chemotaxis histidine protein kinase CheA and the linker protein CheW. In these clusters, receptors with different ligand specificities are uniformly mixed and arranged in hexagonal arrays. Receptor arrays are not perfectly regular structures: the hexagonal order appears to be distorted (Khursigara *et al*., 2008) with a variable stoichiometry of the receptors to CheW and CheA (Levit *et al*, 2002; Sourjik and Berg, 2004). All the other chemotaxis proteins localize to the clusters by interaction with either receptors or CheA and CheW. CheR and CheB both bind to the NWETF pentapeptide sequence at the C-terminus of the major receptors.

A-5 Homologies among bacterial chemotactic pathways

Unlike *E. coli*, the most of known bacterial species show more complex chemotactic pathways as they possess multiple chemotaxis proteins and cytoplasmic chemoreceptors, alternative adaptation and signal termination strategies (Rao *et al*., 2008; Schweinitzer and Josenhans, 2010; Silversmith, 2010)

(see Table A-5.1). Many species possess homologues of the CheA, CheB, CheR, CheW and CheY chemotaxis proteins. Studies on *Rhodobacter sphaeroides* (Porter *et al*., 2008) have provided proof for the existence of multiple signalling cascades. This bacterium has three major operons (*che*Op1-3) encoding homologues of signalling proteins and two different flagellar systems, named *fla*1 and *fla*2 (del Campo *et al*., 2007). Experimental observations have shown that genes encoded by *che*Op1 control the activity of the *fla*2 system whereas proteins of *che*Op2 and *che*Op3 regulate *fla*1 activity. The transmembrane chemoreceptors localized at the cell poles were found to interact with proteins encoded by *che*Op2 whereas the cytoplasmic chemoreceptors cluster with proteins encoded by *che*Op3 (Wadhams *et al*., 2003). Therefore, cytoplasmic and membrane chemoreceptors form two separate signalling complexes, enabling *Rhodobacter sphaeroides* to sense cytoplasmic and extracellular signals independently. However, it was observed that there are interactions between the two signalling pathways as the loss of either *che*Op2 or *che*Op3 signalling proteins causes lack of chemotaxis, hence both signalling pathways are necessary to generate a chemotactic response (Porter *et al*., 2002). As annotation of putative chemotaxis genes is based on nucleotidic sequence similarity, there is evidence that not all annotated chemotaxis gene clusters are involved in taxis. For example, *Myxococcus xanthus* was found to have eight gene clusters containing proteins typically associated with taxis. Some of these clusters are involved in taxis whereas others can be associated with developmental processes leading to the formation of fruiting bodies (Zusman *et al*., 2007).

Table A-5.1: Some example of homologues and alternative chemosensory-like pathways in bacteria (reviewed in Porter *et al*., 2011).

	E.coli	*R.sphaeroides*	*P.aeruginosa*	*M.xanthus*
Number of MCP	5	13	26	21
Chemoreceptor types	Transmembrane	Transmembrane Cytoplasmic	Transmembrane Cytoplasmic	Transmembrane Cytoplasmic
Chemotaxis pathways	1	3	4	8
Gene sets encoding flagella	1	2	1	0
Signal termination	cheZ	cheA3	cheZ	cheC homologue
Role of *che*-like pathways	chemotaxis	chemotaxis	c-diGMP biofilm	EPS production

A-6. Regulation of the signal termination

The chemotactic signalling cascade is characterized by a specific lifetime, which guarantees an effective response and allows to recover the pre-stimulus steady-state. The control of the lifetime of the cellular response has a crucial role in signal transduction systems and is depending on CheY~P dephosphorylation. The response regulator can catalyze its self-dephosphorylation, but this occurs slowly. Generally, CheY~P dephosphorylation is due to other phosphatases. Some kinases are able to dephosphorylate their response regulator (Zhu *et al*., 2000; Gao and Stock, 2009; Kenney, 2010), but the most of the times dephosphorylation is catalyzed by the phosphatase CheZ. This enzyme consists of two symmetric monomers, each containing a binding site for CheY~P. It has been reported that the binding between CheZ and CheY~P shows a positive cooperativity (Blat et al., 1998; Silversmith *et al*., 2008). Therefore, CheZ activity is suppressed at low CheY~P concentration, thus ensuring that CheY~P levels do not get too low and maintaining a steady-state condition.

Many species have multiple homologues of the *E. coli* chemosensory system (Silversmith *et al*., 2005) and some of them are involved in CheY~P dephosphorylation mechanism. For example, *Sinorhizobium melioti* owns two CheY homologues (Guhaniyogi *et al*., 2008), one of which is able to interact with the flagellar motor and the other one is involved in signal termination as it acts as a phosphate sink (Lukat *et al*., 1991). Another example is that in *B. subtilis*, where in lack of CheZ, CheY~P dephosphorylation is due to FliY enzyme, homologue with the CheX-like phosphatase proteins found in other species (Park *et al*., 2004).

A-7. Memory in the chemotactic response

The chemosensory pathway in *E. coli* is maintained at a steady-state of the histidine kinase CheA activity and, as a consequence, of the CheY~P levels. The system is set up for an optimal response to both positive and negative stimuli. Adaptation, due to different mechanisms of feedback, works in order to guarantee this balanced state, enabling a bacterial population to sense a temporal gradient of attractant and/or repellent. One example of feedback mechanism is the modification, by methylation/demethylation, of the MCPs cytoplasmic signalling and adaptation domain, containing the NWETF peptide and specific glutamate residues. The chemotactic protein CheR shows high affinity towards this pentapeptide and, constitutively, adds methyl groups to glutamate. This works antagonistically to CheB, a methylesterase that - when phosphorylated by CheA - removes methyl groups from glutamate residues. The methylation/demethylation of MCPs is depending on their own state, associated to the binding of the ligand. Active MCPs (bound to the ligand) are demethylated by CheB and inactive MCPs

(without ligand) are methylated by CheR (Alon *et al*., 1999; Boldog *et al*., 2006). Moreover, even though the CheB methylesterase and the response regulator CheY are both activated by the histidine kinase CheA, CheB is phosphorylated with a slight delay as compared to CheY: this mechanism ensures that the switch of the flagellar motor can occur before adaptation.

This simple system is common to many bacterial species (Marchant *et al*., 2002). Other species have CheV, CheC and CheD enzymes, as additional proteins involved in adaptation feedback loops (Szurmant and Ordal, 2004; Rao *et al*., 2008). As it has been reported in *Bacillus subtilis*, CheV acts by modulating CheA activity, *via* a CheW-like domain. CheC acts as a CheY phosphatase. CheD is a MCPs deamidase, which converts glutamine residues in glutamate, which can be modified by CheB or CheR. All these mechanisms have the important role to reset the chemosensory system at the pre-stimuls steady-state condition.

The time-lag between the chemotactic response and the adaptation is known as “memory” lenght (Macnab and Koshland, 1972), and it depends on the stimulus strength, the gradient stepness and also on the bacterial lifestyle. An optimal memory lenght must allow bacteria to “remember” still relevant past conditions in order to compare them to present ones and choose the swimming direction (Macnab and Koshland, 1972).

It has been reported that bacteria in a population show variability in adaptation time, memory lenght (Vladimivor *et al*., 2008; Meir et al., 2010), number of CheR and CheB units (Li and Hazelbauer, 2004) and MCPs abundance and distribution in the membrane (Thiem and Sourjik, 2008; Greenfield *et al*., 2009). The fact that all the cells in a population, do not have the same behaviour in

unpredictable and variable environmental conditions, can be seen as an evolutionary advantage which guarantees the survival of the polulation as a whole.

A-8. Role of both motility and chemotaxis in biofilm formation

Commonly, in natural environments, bacteria grow as biofilms, i.e. organized mixed cells communities adhering to biotic or abiotic surface and packaged in an extracellular polysaccharide matrix known as exopolysaccharide (EPS) (Costerton *et al*., 1995). Interestingly, bacteria can switch between the planktonic and the biofilm lifestyles in response to nutritional cues.

Biofilm formation occurs accordingly to a gradual and well regulated process, namely:

- the adhesion to a surface *via* the cells pole, this step being known as "reversible attachment" (O'Toole and Kolter 1998a-b; Hinsa *et al.,* 2003);
- "irreversible attachment" *via* the long cell axis (Marshall *et al.,* 1971; Fletcher 1996);
- micro-colonies formation *via* the recruitment of planktonic cells from the medium or migration of attached cells on the surface by twitching motility (O'Toole *et al.,* 2000a; O'Toole and Kolter, 1998b);
- cells maturation with the formation of the EPS matrix (Danese *et al.,* 2000; Hellmann *et al.,* 1996; Watnick and Kolter 1999; Yildiz and Schoolnik, 1999);
- dispersal of cells due to starvation (Gjermansen *et al.,* 2005).

Klausen *et al*., (2003), have reported that flagella and type IV pili are important factor in *P. aeruginosa* biofilm development, as they mediate attachment

to solid surfaces. Other studies have suggested that swarming motility - depending on quorum sensing (QS, see § F), rhamnolipid production, type IV pili and the presence of flagellum - can also contribute to early stages of *P. aeruginosa* biofilm formation (Köhler *et al*., 2000). Moreover, it is likely that motility and chemotaxis are required to swim towards nutrients associated with a surface.

CHAPTER B - General Materials and Methods Common to Chapters D, E, F.

B-1 Bacterial strains, media and growth conditions

All strains and plasmids used in this study are listed in Table B-1.1.

Table B-1.1: Bacterial strains and plasmids.

Bacterial strains	Relevant genotype	Reference
***Pseudomonas pseudoalcaligenes* KF707**		
Wild type	Amp^R	Furukawa *et al*., 1986
*che*A1::*Km*	*che*A::*Km*, Km^R, Amp^R	Tremaroli *et al*., 2007
Δ*che*A2	Δ*che*A2, Amp^R	This study
Δ*che*A3	Δ*che*A3, Amp^R	This study
Δ*che*A2cheA1::*Km*	Δ*che*A2*che*A1::*Km* Km^R, Amp^R	This study
Δ*che*A3*che*A1:: *Km*	Δ*che*A3*che*A1::*Km* Km^R, Amp^R	This study
Δ*che*Y	Δ*che*Y, Amp^R	This study
Δ*che*Z	Δ*che*Z, Amp^R	This study
Escherichia coli		
S17λpir	TpSm *recA thi pro hsdR* RP4:2-Tc:Mu:Km *λpir*	Simon *et al.,* 1983
HB101	Sm, *rec*A *thi pro leu hsd*R	Boyer and Rolland-Dussoix, 1969
DH5α	*supE*44*hsdR*17*recA*1*endA*1 *gyrA*96*thi*1 *relA*1	Hanahan, 1983

Table B-1.1: *continued*

Top10F'	F´{*lac*Iq, Tn*10*(TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen™
pSB401	harbouring the *lux*CDABE plasmid construct	Winson *et al*., 1998
JM109	*end*A1,*gln*V44,*thi*-1,*rel*A1 *gyr*A96,*rec*A1,*mcr*B$^+$,Δ(*lacpro*AB) e14-[F'*tra*D36*pro*AB$^+$*lac*I^q *lac*ZΔM15] *hsd*R17($r_K^-m_K^+$)	Yanish-Perron *et al*., 1985
Agrobacterium tumefaciens		
NTL4	/	Farrand *et al*., 2002
WCF47	/	Zhu *et al*., 1998
Chromobacterium violaceum	/	
CV026	Km^R	McClean *et al*., 1997
Plasmids	**Relevant genotype or characteristics**	**Reference**
pUC19	Amp^R, cloning vector	Sambrook *et al*., 1989
pUT mini-Tn5 Km	Amp^R Km^R, delivery plasmid for mini -Tn5 Km	de Lorenzo *et al*., 1990
pRK2013	Km^R ori ColE1 RK2-Mob+ RK2-Tra+	Figursky *et al*., 1979
pG19II	Gm^R *sac*B *lac*Z, cloning vector conjugative plasmid	Maseda *et al*., 2003
pSB401	*lux*CDABE reporter fusion	Winson *et al*., 1998
pZLR4	*lac*Z reporter fusion	Farrand *et al*., 2002
pCF218	codifing for *tra*R	Zhu *et al*., 1998
pCF372	*tra*I promoter-*lac*Z phusion	Zhu *et al*., 1998

Liquid cultures of all bacterial strains were grown in agitation at 150 rpm at the optimal temperature (*Escherichia coli* at 37°C; *Pseudomonas pseudoalcaligenes*,

Agrobacterium tumefaciens and *Chromobacterium violaceum* at 30°C). The compositions of the media used in this study are reported in Table B-1.2.

Table B-1.2: Media composition.

Rich media		
Luria-Bertani (LB) pH 7	Trypton	10g/l
	Yeast extract	5g/l
	NaCl	10g/l
Defined media		
Sucrose-Asparagine (SA) pH 7	Sucrose	20g/l
	Asparagine	2g/l
	K_2HPO_4	1g/l
	$MgSO_4$ 10% (w/v)*	5ml/l
Minimal salt medium (MSM) pH 7	K_2HPO_4	4,4g/l
	KH_2PO4	1,7g/l
	$(NH_4)_2SO_4$	2,6g/l
	$MgSO_4$*	0,4g/l
	$CaSO_4$*	0,0031g/l
	$MnSO_4$*	0,05g/l
	$FeSO_4$*	0,01g/l
	Succinate*or byphenil crystals	5mM
AB glucose pH 7	20X buffer solution*:	
	K_2HPO_4	60g/l
	$NaH_2PO_4xH_2O$	23g/l
	20X salts solution*:	
	NH_4Cl	20g/l
	$MgSO_4$	2,9g/l
	KCl	3g/l
	$CaCl_2$	0,2g/l
	$FeSO_4x7H_2O$	0,05g/l
	Carbon source: glucose	5g/l

The asterix (*) indicates medium components which were prepared as concentrated stock solutions, autoclaved separately and added at the medium at the final concentration of 1X.

For growth on solid media, agar was added at the final concentration of 15 g/l. X-Gal stock solution was prepared at a concentration of 50 mg/ml and stored in 1 ml aliquotes, protected from light, at -20°C. Antibiotics stock solutions were prepared as reported in Table B-1.3 and stored at -20°C in 1 ml aliquotes until use.

Table B-1.3: Antibiotics stock solutions and concentrations used for selective growth.

Stock solution	**Final Concentration in μg/ml**			
	P.ps.alcaligenes - *E.coli* - *A.tumefaciens* – CV026			
Ampicillin, 100 mg/ml, water solution	100	50	/	/
Kanamycin, 100 mg/ml, water solution	50	50	/	50
Gentamycin, 30 mg/ml, water solution	20	20	30	/
Tetracycline, 20 mg/ml, 70% ethanol solution	20	20	20	/
Spectinomycin, 50 mg/ml, water solution	/	/	50	/

B-2 Extraction of genomic DNA from *Pseudomonas pseudoalcaligenes KF707*

Genomic DNA from *Pseudomonas pseudoalcaligenes* KF707 was extracted with the following protocol. A 10 ml of an over-night grown culture was centrifuged at 5000 rpm at 4°C for 15 minutes and washed with 10 ml of TES solution (50 mM TrisHCl, 20 mM EDTA, 50 mM NaCl pH 8.0). The cell pellet was resuspended in 5 ml of TE buffer (50 mM TrisHCl, 20 mM EDTA pH 8.0). Lysozyme solution, prepared in the same buffer, was added at the final concentration of 20 mg/ml; the solution was incubated at 37°C for 30 minutes and mixed by inversion every 10 minutes. At the end of incubation, 500 μl of a 10%

SDS solution and Proteinase K at the final concentration of 10 mg/ml were added, followed by 1 h incubation at 37°C; the reaction was stopped by adding a solution of 10 mM EDTA and 3 mM sodium acetate. The lysate was incubated with RNase at 37°C for 1h after which an iso-volume of a phenol-chlorophorm-isoamyl alcohol 25:24:1 v/v mixture was added and the sample was mixed by inversion at room temperature for 15 minutes. The water phase containing the genomic DNA was separeted from the organic phase and cell debris by centrifugation at 5000 rpm at 4°C for 15 minutes. The extraction was repeated three times and phenol traces were removed by adding an iso-volume of a 24:1 v/v mixture of chlorophorm-isoamyl alcohol. The water phase was recovered after centrigugation at 5000 rpm at 4°C for 15 minutes in a clean beacker. 1.5 volumes of cold absolute ethanol were added to the extracted water phase and the genomic DNA was collected using a clean glass stick. The DNA was washed by immersing the glass stick in a cold 70% ethanol solution and then air dried. After this, the stick with DNA was immersed in a small volume of sterile nuclease free water and left at 4°C over-night to allow the DNA to suspend. The resuspended genomic DNA preparation was stored at the temperature of -20°C.

B-3 DNA manipulations and genetic techniques

All restriction digests, ligations, cloning and DNA electrophoresis, were performed using standard techniques (Sambrook *et al*, 1989). Taq polymerase, the Klenow fragment of DNA polymerase, alkaline phosphatase, restriction endonucleases and T4 DNA ligase were used as specified by the vendors (Roche, Fermentas, Invitrogen, Sigma-Aldrich, NEB Biolabs). The plasmid pUC19 was

routinely used as the cloning vector and recombinant plasmids were introduced into *E. coli* host by transformation of chemically competent cells, prepared according to the CaCl2 method (Sambrook et al, 1989). To detect the presence of insert DNA, X-Gal was added to agar media at a final concentration of 50 µg/ml. X-Gal stock solutions were prepared at a final concentration of 50mg/ml in N-N-dimethylformamide and stored as 1 ml aliquots at - 20 °C protected from light. Kits for plasmid mini- midi- and maxi-preps, PCR purification and DNA gel extraction were obtained from QIAGEN (Milan, Italy) and used according to the manifacturer's instructions.

B-4 DNA sequencing and sequence analysis

Genomic DNA fragments of interest were cloned in the pUC19 cloning vector and positive plasmids were sent for sequencing to the BMR-genomics service of the University of Padova (Padova, Italy). Samples were prepared according to the recommended procedures (www.bmr-genomics.it). M13 Forward and Reverse primers were used for sequencing the extremities of DNA fragments cloned into the pUC19 vector from the M13 promoter. Sequence identities were determined by DNA homology searches using the BLAST program to search both NCBI and TIGR databases.

B-5 Conjugation

Day I. Donor, receiver and helper strains were streaked out on LB agar plates with the appropriate antibiotics. LB plates were incubated over-night at 37°C and 30°C for *E. coli* and *P. pseudoalcaligenes* KF707 optimal growth temperature

respectively. *E. coli* HB101 strain carrying the mobilization plasmid pRK2013 was commonly used as helper strain for tri-parental mating (see Table 1 for strain and plasmid features).

Day II. Donor, receiver and helper strains were inoculated in LB broth from single colonies grown on the agar plates. The appropriate antibiotics were added to LB medium in order to maintain selection. LB liquid cultures were grown over-night at the appropriate temperature under agitation at 150 rpm.

Day III. Donor, receiver and helper strains were inoculated with a 1% inoculum in liquid LB medium without antibiotics from over-night grown liquid cultures. Cells were grown at the appropriate temperature and under shaking for 2 – 3 h, in order to obtain early exponential phase cultures (OD660 ~ 0.2 – 0.3). 1 ml aliquot from each culture was collected in a sterile tube, spun down at room temperature and washed twice with 1 ml LB medium. Cells were suspended in 1 ml of fresh LB and then used for the preparation of conjugation mix by adding equal volumes (100 µl) of donor, receiver and helper suspensions to a sterile tube. The conjugation mix was incubated at 30 °C for 30 min and spots were plated onto well dry LB agar plates without selection. Controls for each conjugation were carried out with 100 µl of the receiver, donor or helper cell suspensions alone added to sterile tubes and processed in the same way as conjugation mix. LB plates were incubated for 24 h at 30°C.

Day IV. The bacterial biomass was collected from each plate with a sterile loop and suspended in 1 ml of fresh LB with 20 % glycerol. 10 fold serial dilutions of cell suspensions of conjugation mix and controls were carried out in 0.9 % saline. The remaining part of the conjugation mix suspended in LB with 20 %

glycerol was stored at – 80 °C. Appropriate dilutions were plated on agar plates containing the antibiotics for transconjugants selection. For the selection of KF707 transconjugants, cells were plated on SA or AB glucose medium in the presence of appropriate antibiotics. The two media were used to counter-select *E. coli* donor and helper strains, given that these medium do not support *E. coli* growth, thus resulting selective for *P. pseudoalcaligenes* KF707. Plates were incubated at the appropriate temperature until transconjugants growth was clearly visible (i.e. 24 h for *E. coli* transconjugants growing on LB and at least 36 h for KF707 growing on SA or AB glucose).

Day V. Transconjugants were streaked out on the appropriate agar media in the presence of antibiotic selection and incubated at the optimal temperature until growth was clearly visible. The selection was repeated at least twice, in order to obtain a pure culture and remove both donor and helper strain backgrounds.

B-6 Electroporation of *Pseudomonas pseudoalcaligenes KF707*

Pseudomonas pseudoalcaligenes KF707 was inoculated over-night in 10 ml of Luria-Bertani broth without NaCl. 1 mL of the overnight culture was transferred to 100 ml of the same media in a 500 ml flask. Cells were grown at the appropriate temperature (30°C) and under shaking (150 rpm) until the culture reached the exponential phase (OD600 ~ 0.5-0.6). Cells were collected by centrifugation at 5000 rpm at 4°C for 15 minutes and then washed three times with ice-cold 300 mM sucrose solution (the first two times with 100 ml and the last one with 50 ml of the washing solution). The cells were harvested by spin at 5000 rpm for 15 minutes at 4ºC and after discarding the supernatant, they were resuspended in 1 ml of sucrose

300mM. 100 µl aliquots from the suspension were transferred into microcentrifuge tubes on ice and immediately used for the electroporation. 1 µg of DNA of interest was added to the 100 µl aliquot to be electroporated. The mix was incubated on ice 5-10 minutes before being transferred in 0.2 cm-cuvettes (Biorad) and being subjected to electroporation with the following parameters: 2.5 kV, 25 µF and 400 Ω. After incubation on ice for 1 minute, 500 µl of SOC recovery medium was added to each electroporated suspension. The cells were then recovered for 2 hours under shaking at 150 rpm at 30ºC before being spread onto LB plates supplemented with the appropriate antibiotic.

B-7 Construction of *Pseudomonas pseudoalcaligenes KF707* miniTn*5* transposon mutant library

Random mutagenesis was performed by inserting miniTn5 Km transposon into the chromosome of *P. pseudoalcaligenes* KF707 using bi-parental conjugation with E. coli S17-λpir/mini-Tn5 Km (donor strain) and *P. pseudoalcaligenes* KF707 (receiver strain) as previously described (de Lorenzo *et al*., 1990). Kanamycin resistant exconjugants were selected on SA plates supplemented with Km (50 mg/ml.

CHAPTER C

The Genome Project of the polychlorinated-biphenyl degrader *Pseudomonas pseudoalcaligenes* KF707

C-1 Introduction

Pseudomonas pseudoalcaligenes KF707 is a soil biphenyl and PCBs (polychlorinated biphenyls) degrader (Furukawa *et al.*, 1986), able to grow both planktonically as well as biofilm (Tremaroli *et al.*, 2008) even in the presence of various toxic metals and metalloids (Di Tomaso *et al.,* 2002; Zanaroli *et al.,* 2002, Tremaroli *et al.,* 2007). KF707 shows also chemotactic response towards biphenyl and PCBs (Tremaroli *et al.*, 2010), physiolgical traits that enable KF707 to survive in hostile environments and also to be employed in bioremediation procedures in polluted sites.

In order to obtain more information about the genetic bases of the peculiar physiological aspects and environmental behaviour of KF707 strain, such as chemotaxis, biofilm formation and metabolic degradation properties, we recently started the ”Genome project of *Pseudomonas pseudoalcaligenes* KF707” in collaboration with Prof. R.J.Turner (University of Calgary, Calgary, Ca) and Prof. M.Attimonelli (University of Bari, Bari I).

Next - generation - sequencing (NGS) technologies as 454 Life Sciences pyrosequencing (Genome Sequencer FLX System, Roche Applied Science) and Illumina (HiSeq2000, Solexa), were performed. Output data were statistically analyzed, validated and subsequently assembled using the Newbler software based

on the OLC (overlap-layout-consensus) approach and the AbySS software based on the Brujin-graph approach (Pevzner *et al*., 2001). Optical Mapping technology (Samad *et al.*, 1995) was also performed with the aim to complete the sequence assembly of the whole genome. The RAST (Rapid Annotations using Subsystems Technology) Prokaryotic Genome Annotation server (Aziz RK *et al*., 2008) was used for genes annotation.

C-1.1 Prokaryotic genome projects pipeline

C-1.1.1 Second generation sequencing technologies

Next generation sequencing technologies had have a big impact on genomics. They are know as massively parallel systems, since they ground on the use of plataforms which deliver several Gbp (Giga base pair) of DNA sequences per week, with a dramatic drop in cost as compared to shotgun sequencing based on the Sanger method (www.genome.gov/sequencingcosts). Moreover, they allow to bypass library construction and to avoid bias generated during the sub-cloning process.

Four second generation platforms are available (the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, the Applied Biosystems (ABI) SOLiD Analyzer and the Polonator G.007), although, currently, they have already been supplanted by third generation sequencing technologies. *P. pseudoalcaligenes* KF707 genome sequencing have been performed by means of 454 FLX and Illumina platforms.

The sequencing *via* the GS FLX (454 – pyrosequencing) involves four main steps, from purified DNA to analyzed results (Margulies *et al.*, 2005). The

first step consists in the library preparation: a low amount of DNA (few μg) is fragmented by nebulization into 300-800 bp fragments, purified, blunted and phosphorylated. Adapters (A and B) are added to each end and used for both amplification and sequencing. The B adapters contain 5' biotin tags, which allow the fragments to remain immobilized on streptavidin-coated magnetic beads during the denaturation, whereas not-biotinylated strands are released. In the second step amplification starts after the beads are dropped off into independent microreactors and emulsified with a mixture containing PCR reaction components. In the third step pyrosequencing is performed: nucleotides are flowed across a Pico-Titer-Plate device in a fixed order. During the extension step by means of a DNA polymerase, released pyrophosphate (PPi) is converted by the sulfurylase enzyme in ATP, which is subsequently used by luciferase enzyme to emit photons (pyrosequencing). This chemioluminescent signal is recorded by a CCD camera. The combination of signal intensity and positional information generated across the Pico-Titer-Plate device, allows the software to determine the sequence of more than 1.000.000 individual reads of about 500 bp in length. The output is provided in a *.sff (standard flowgram format) file, which contains the sequences and the corresponding quality scores for all the high-quality reads (filtered reads).

Illumina technology is a platform based on a sequencing-by-synthesis (SBS) approach and gives as output paired-end reads of about 150 bp in lenght. Genomic DNA is randomly fragmented, adapters are ligated to both ends of the fragments, which subsequently are immibilized on the surface of a flow ell channels. Unlabeled nucleotides and enzyme are added to initiate solid-phase

bridge amplification. The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate and million of clusters of double-stranded DNA are generated: this represents the library used for the subsequent sequencing. The first sequencing cycle begins by adding a PCR reaction mixture with labeled dNTPs. Indeed, these modified dNTPs have their 3'-OH chemically inactivated, ensuring the incorporation of only one base per cycle. When the first dNTP is incorporated, emission of fluorescence occurs, the signal is captured and the first base is identified. The sequencing cycles are repeated to determine the sequence of all the fragments in the library, one base at a time (Mardis, 2008). The standard sequencing output files of the HiSeq 2000 consist of a ∗.bcl (base call) files, containing the "bases calls" and quality scores relative to each cycle. Subsequently they can be converted into ∗qseq.txt files by BCL Converter (www.illumina.com).

C-1.1.2 Overview of computational workflow for prokaryotic assembly and annotation of sequenced prokaryotic genomes

C-1.1.2.1 Reads quality control

Although new generation technologies have reduced the time and the cost of whole-genome sequencing, reads are more error-prone than those obtained by performing Sanger sequencing approach. Moreover, NGS data need to be clipped to remove low-quality regions and adapter sequences. Therefore, a quality check is necessary before starting the assembly. Several softwares have been developed to overcome these problems. In order to remove contaminations (low quality regions and adapters), all sequences must be collected and

processed by using open source softwares such as FastQC, FastX, Trimmomatic and HtSeq. FastQC is usually used for quality check; FastX and Trimmomatic are employed for Illumina paired-ends clipping. Furthermore, assembly softwares (Newbler and AbySS), provided by the sequencing companies, include alghorithms for quality assessment and clipping.

C-1.1.2.2 Genome assembly

Assembly is a hierarchical procedure which allows the contruction of the original DNA sequence by align and joining groups of reads into contigs and contigs into scaffolds. The scaffolds, also called supercontigs or metacontigs, define the contig order and orientation and the sizes of the gaps between them (Miller *et al*., 2010). Two assembly strategies can be adopted: assembly using reference genomes or *de novo* assembly. Several softwares have been developed for both assembly approaches.

The Newbler software (Margulies *et al*, 2005), based on an "overlap–layout–consensus" (OLC) approach, allows to obtain a consensus alignment of all the reads, genereting step by step longer contigs (Pevzner *et al*., 2001; Miller *et al*., 2010).

The AbySS software (Simpson *et al*., 2009), is based on the "de Brujin graph approach" (DBG) and it works by breaking up the reads in oligomers of *k* length. The de Bruijn graph is constructed on the resulting k-mers groups. The graph contains nodes of (k−1) in length (Pop, 2009); two nodes are linked by an edge if the adjacent (k−1)-mers have an exact overlap of length (k−2). Euler, Velvet, AllPaths, SOAP-denovo are assembly softwares all based on the "de Brujin

graph" approach. (reviewed by Miller *et al.*, 2010). MOSAIK assembler (see McKernan *et al.*, 2009) is suggested for short-reads data and for cross-species comparison and can be used in assemblies using reference genomes (§ C-2.3).

C-1.1.2.3 Genome scaffolding

Newbler and AbySS assembly algorithms, as well as others based on both OLC and DBG, increase reads length, however they do not give as outup the complete closed genome. Consequently, the assembled genome is only a draft version (Nielsen *et al*., 2009). Generally, the complete map of a genome, could be closed by re-sequencing the genome and with high probability the assembly of new data may give in output contigs that overlap with those of the previous assembly; eventually, gaps may be closed by performing chromososme walking by PCR. In addition, the latter strategy is quite expensive and represents also a waste of time. Therefore, other computational approaches for scaffolding are suggested.

Physical and genetic maps may be helpful for scaffolding (Beyer *et al*., 2007). Physical maps are obtained by means of genome restriction and electrophoretic separation of the fragments; moreover, the migration pattern is useful for clones overlapping (Nathans and Smith, 1975). *Long Read DNA Extension Methods* were developed on the basis of restriction mapping.

Optical Mapping System (Samad *et al.*, 1995) is the most common "*Long Read DNA Extension Methods"* technology which gives whole genome analysis (Lin JY, 1999). Maps are constructed by restriction analysis (~ 500 Kb in size fragments are obtained) and directly visualized by

fluorescence microscopy. Resultig restriction maps are used as scaffolds to assemble contigs and orienting them in the right directions. Moreover, they give informations about the size of the gaps, the size of the genome and reveal assembly errors. OpGen (www.opgen.com) has developed an advanced technology to construct optical maps. The first step consists in the genome extraction by Adapted Agencourt Genfind V2 bead or agarose plug extractions, procedures that both allow to obtain an as much as possible intact genome. Sample is then electrostatically fixed on the surface of the MapCard and processed by adding a mixture containing restriction enzyme, reaction buffer, stain. After processing, the instrument scans the lanes of the MapCard surface, measuring each fragments and collecting data to assemble the genome. The MapSolver software has been developed to manipulate data from Optical Mapping: it is useful to perform comparison with other optical solved genomes; contigs can be aligned and correctly oriented on the Optical Map covering up to 80-90% of the genome (Nagarajan *et al*., 2008), allowing to validate assemblies and identify probes to close gaps for whole genome finishing.

C-1.1.2.4 Genome annotation

The genome annotation (structural and functional) consists on the identification of elements on the genome and assigning to them a biological information. Structural annotation identifies ORFs and their localization, gene structure, coding regions and location of regulatory motifs. Functional

annotation consists in the assignment of biological information to genomic elements such as biochemical and biological functions, regulation and expression. Several algorithms have been developed for gene prediction and annotation.

GeneMark (http://exon.gatech.edu/) supplies a group of gene prediction softwares GeneMark-P, GeneMark.hmm-P, GeneMarkS) for prokaryotic gene annotation (Borodovsky and McIninch, 1993). They allow online access and sequences in multiple formats (FASTA, EMBL, GenBank, PIR, or Phylip) can be processed. The sequences are analyzed by carrying on the genetic code in one of six possible frames (including three frames in complementary DNA strand). In addition to the basic GeneMark, the GeneMark.hmm algorithm allows to find exact gene starts.

RAST (Rapid Annotations using Subsystems Technology) is an automated annotation service for gene prediction and metabolic reconstruction (Aziz RK et al., 2008). The prokaryotic genome of interest, in the form of a set of contigs in FASTA format, is uploaded to start the computational process. Contigs are scanned and genes are identified and assigned to subsystems of *FGIfam* protein families collection. To identify the tRNA, tRNAscan-Se is used (Lowe and Eddy, 1997) while rRNA encoding genes are identified by the "search-for-RNAs" (Overbeek *et al*., 2005) tool.

C-2 Materials and Methods

C-2.1 *Pseudomonas pseudoalcaligenes* KF707 genome sequencing and preliminary analyses

Next–generation sequencing technology 454 Life Sciences pyrosequencing (§ C-1.1.1) was performed at the NRC Plant Biotechnology Institute (Saskatoon, Canada), using the Genome Sequencer FLX System (Roche Applied Science), in a quarter of a PicoTiterPlate.

Illumina (Solexa) sequencing (§ C-1.1.1) was performed at the IGA (Institute of Applied Genomics, Udine, Italy) on 1/3 of an Illumina HiSeq2000 platform.

C-2.2 Next generation sequencing data quality analysis

454 reads were filtered by the GS FLX platform and checked by the in-built tools of the Newbler assembly software (§ C-1.1.2.1). FASTQC software (§ C-1.1.2.1) was used to perform quality control of the Illumina reads dataset, whereas Trimmomatic software (§ C-1.1.2.1) was employed for clipping. Bases at the extremities of each Illumina read -i.e. the adpter oligomers used during the sequencing run - were cut.

C-2.3 Genome assembly

Assembly was performed adopting two different approaches: use of reference genomes of two *Pseudomonas* strains (*P. mendocina ymp and P. aeruginosa PAO1*, phylogenetically related to KF707) and de-novo assembly (§ C-1.1.2.2).

MosaikAssembler was employed for assembly with reference genomes. The software consists of four modular programs: Build, Aligner, Sort and Assembler.

Mosaik Build translates external read formats to a format that the aligner can use. In addition, to processing reads, the program also converts reference sequences from a FASTA file to an efficient binary format. Mosaic Aligner performs pairwise alignment between reads of the read dataset and the set of reference sequences. The maximum mismatch percent threshold was set at a value of 0.2. In this way all sequences with a mismatch equal or bigger than 20% were excluded. MosaikSort takes the alignment output and prepares it for multiple sequence alignment. MosaikAssembler takes the sorted alignment file and produces a multiple sequence alignment which is saved in an assembly file format.

With regard to the *de novo* assembly, 454 reads dataset was assembled with the Newbler software (v.2.3), with default parameters for single-end libraries. Illumina paired end reads were processed with the AbySS software, only after trimming was performed to improve the reads quality assessed by FastQC.

C-2.4 Optical map and contigs scaffolding

The *P. pseudoalcaligenes* KF707 optical map was constructed at the Canadian Food Inspection Agency, following the protocol supplied by OpGen (http://www.opgen.com). Genome was extracted following the Adapted Agencourt Genfind V2 bead or agarose plug extraction protocols and digested withBamHI. Optical map data were provided in a *.xml file, compatible with the MapSolver software (§ C-1.1.2.2).

C-2.5 Gene prediction

GeneMark software and Rast server were used for gene prediction and annotation (§ C-1.1.2.4).

C-3 Results

C-3.1 454 and Illumina reads datasets

454 pyrosequencing yielded 213.206 single-end reads (Fig. C-3.1.1).

GACT (Library)		Region 1	2	3	4	Total
	Raw Wells			342,386		342,386
	Key Pass Wells			322,171		322,171
Failed	Dot			3,155		3,155
	Mixed			53,994		53,994
	Short Quality			51,285		51,285
	Short Primer			273		273
	Passed Filter Wells			213,464		213,464
	% Dot + Mixed			17.74		17.74
	% Short			16.00		16.00
	% Passed Filter			66.26		66.26

Fig. C-3.1.1: Statistical information about 454 reads quality observed during the sequencing run. Percentage of failed sequences (dot and mixed) is below 20%, thus indicating a good quality of the sequencing run. Reads pre-processing by Newbler retained all 213.206 reads for the assembly stage; 0.16% of bases at 5' and 3' read ends were trimmed by the default settings.

Reads length ranged from 60 bp to 540 bp, with a modal value of 370 bp (Fig C - 3.1.2).

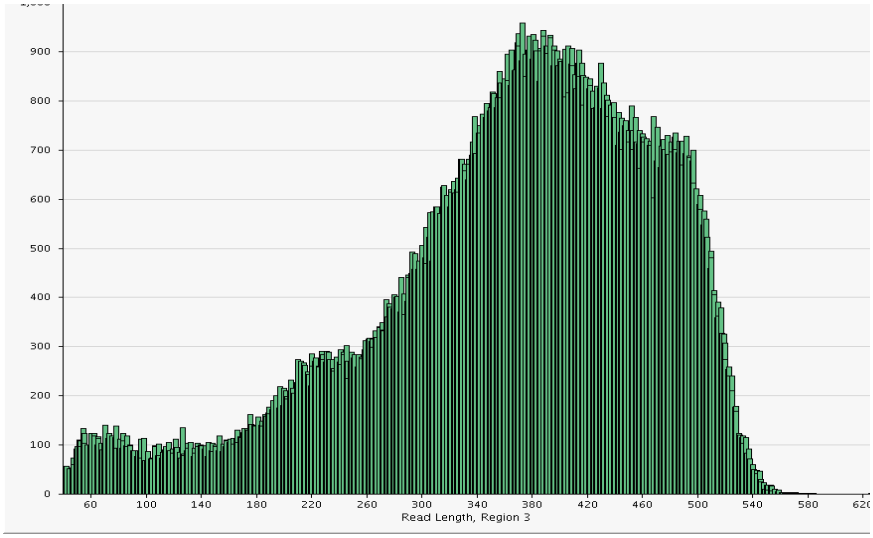


Fig. C-3.1.2: 454 reads length distribution. X axis: reads length in base pair; Y axis: number of reads.

Illumina sequencing, performed on a third of a HiSeq2000 plate, yielded ~110.000.000 paired-end reads, each of 101 bp in lenght (Fig. C-3.1.3).

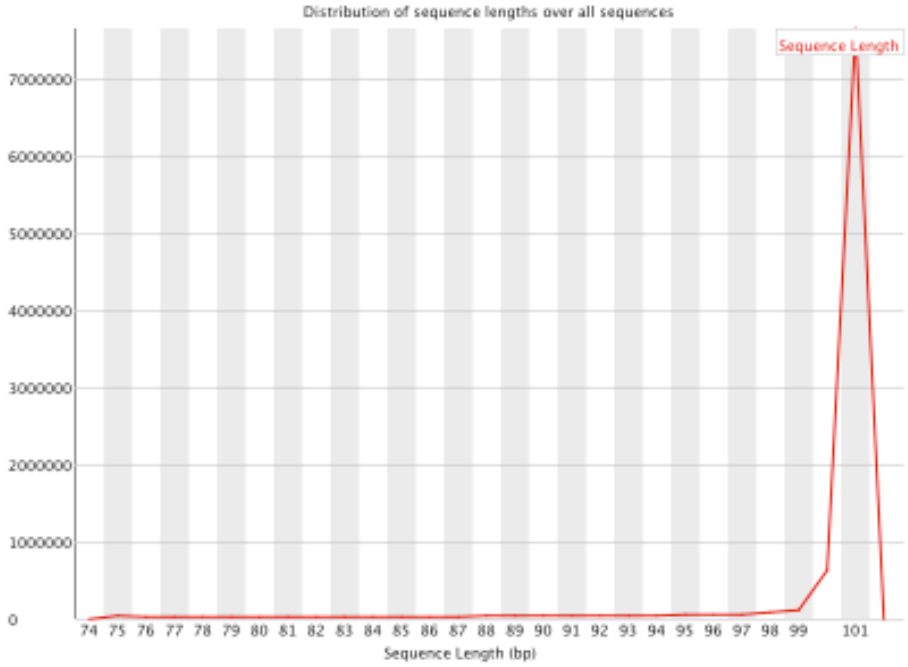


Fig. C-3.1.3: Illumina reads length distribution. X axis: reads length in base pair; Y axis: number of reads.

Quality check of Illumina paired-end datasets (both forward and reverse reads) was performed with the open-source software FastQC.

Subsequently, paired-end reads were trimmed. Reads clipping was performed by using Trimmomatic software which gave as output a sequence dataset with high-quality reads (Fig. C-3.1.4 a; C-3.1.4 b).

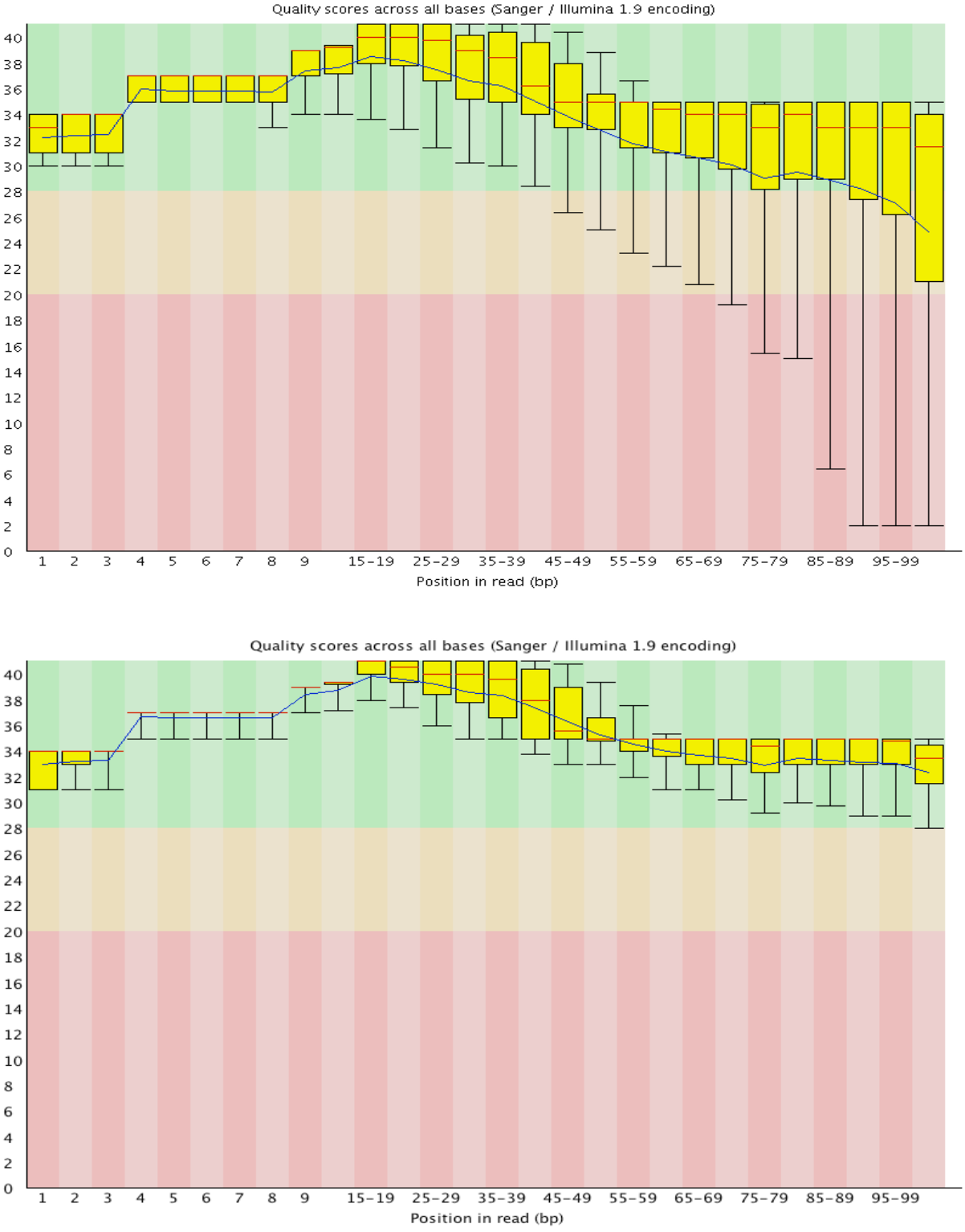


Fig. C-3.1.4 a: Quality scores of forward paired-end reads Illumina datasets before (top graph) and after trimming (bottom graph).

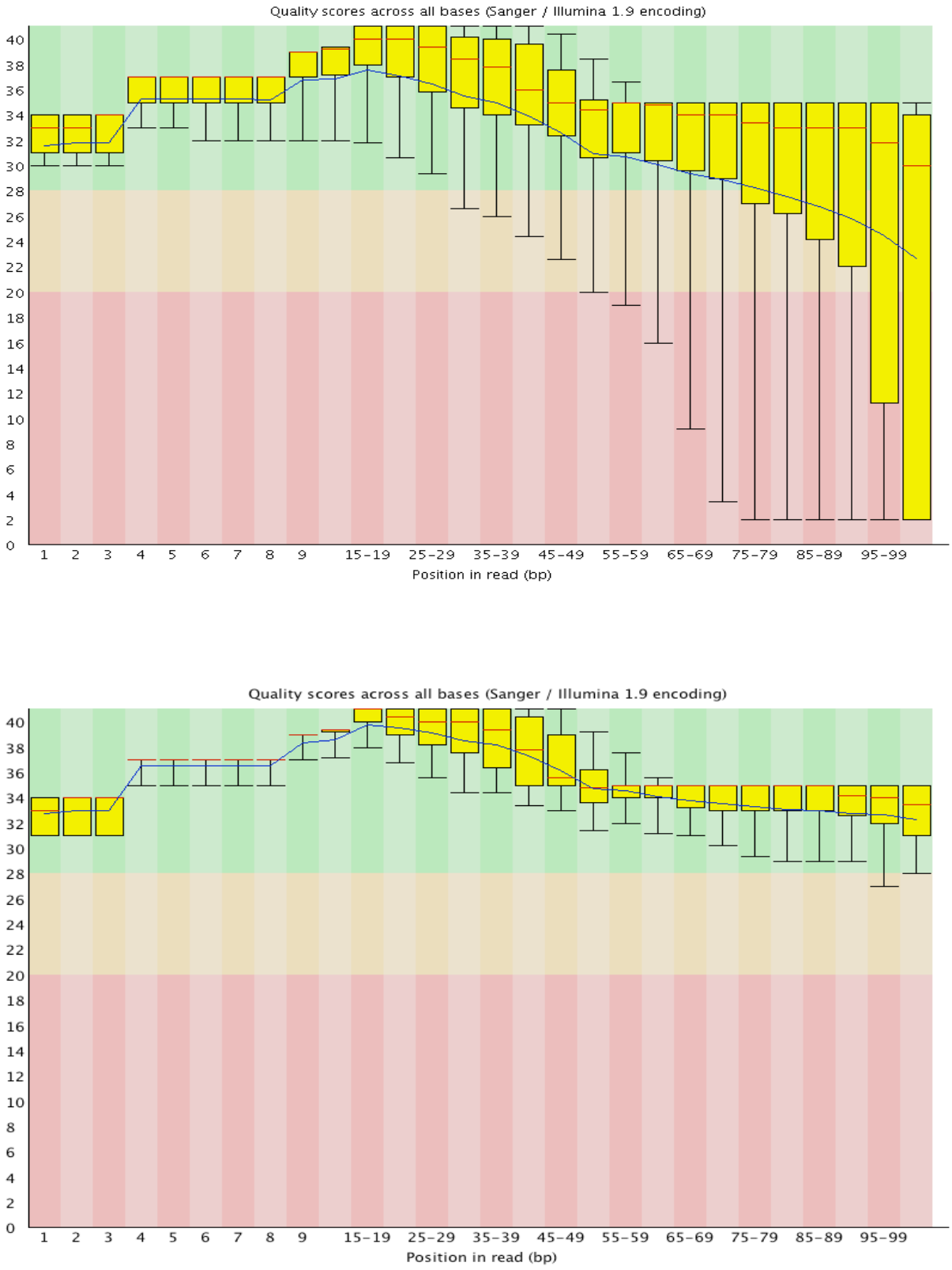


Fig. C-3.1.4 b: Quality scores of reverse paired-end reads Illumina datasets before (top graph) and after trimming (bottom graph).

C-3.2. Genome assembly

An assembly is a hierarchical procedure which allows the contruction of the original DNA sequence by align and joining groups of reads into contigs and contigs into scaffolds. The scaffolds, sometimes called supercontigs or metacontigs, define the contig order and orientation and the sizes of the gaps between contigs (Miller *et al*., 2010). The quality of an assembly is expressed as N50, that is the contig lenght such that the 50% of assembled bases are in equal or longer contigs. High values of N50 indicate a good assembly as the lenght of assembled contigs increases. N50, the total assembly length, the maximum contig length and the mean contig length represent the parameters for the evaluation of an assembly.

C-3.2.1 Assembly using reference genomes

Before starting assembly, a Blast2seq analysis performed between the two reference genomes (*P. aeruginosa* PAO1 *P. mendocina* ymp) showed the 50% of similarity. A reads dataset of KF707 genome was generated and used as subject for BLASTn analysis. 51.92% of KF707 reads were mapped on the reference genomes. In this respect, the assembly method using reference genomes needed to be complemented with other analyses such as *de-novo* assembly methods and PCR procedures to close the gaps between contigs.

C-3.2.2 de-novo assembly strategies

The 454 read dataset was assembled by using the Newbler software (§ C-1.1.2.2). 211.216 reads, corresponding to 77.029.069 bp (99.35% of genome coverage), were assembled. Assembly with default parameters returned 900 contigs, of which the longest was of 51.361 bp. A subset of 729 contigs longer

than 500 bp, with a total amount of 6.053.515 bp, was used for further analyses (i.e., gene prediction). The N50 of the assembly was 14.148 bp (§ C-3.2).

The Illumina dataset was assembled with the ABySS software (§ C-1.1.2.2). The best k (hashing) value was empirically evaluated by performing assemblies with increasing hash values, ranging from 20 to 96. The best assembly was obtained with k = 53, since it yielded 255 contigs and N50 = 81.842 bp; the longest contig was 367.837 bp. Illumina contigs dataset was aligned to the 454 contig dataset, in order to check whether datasets obtained from different sequencing technologies could complement each other. Reciprocal BLASTn of the two datasets reported that 93% of contigs from 454 dataset had an overlap in the Illumina contigs dataset.

C-3.3. Contigs scaffolding

An optical map (§ C-1.1.2.3) of the *P. pseudoalcaligenes* KF707 genome was constructed at the Canadian Food Inspection Agency (Lethbridge, Canada) with the BamHI restriction enzyme (Fig. C-3.3.1), yielding 650 ordered restriction fragments (the average fragment size = 9.1 kb; maximum contig size = 64.8 kb).

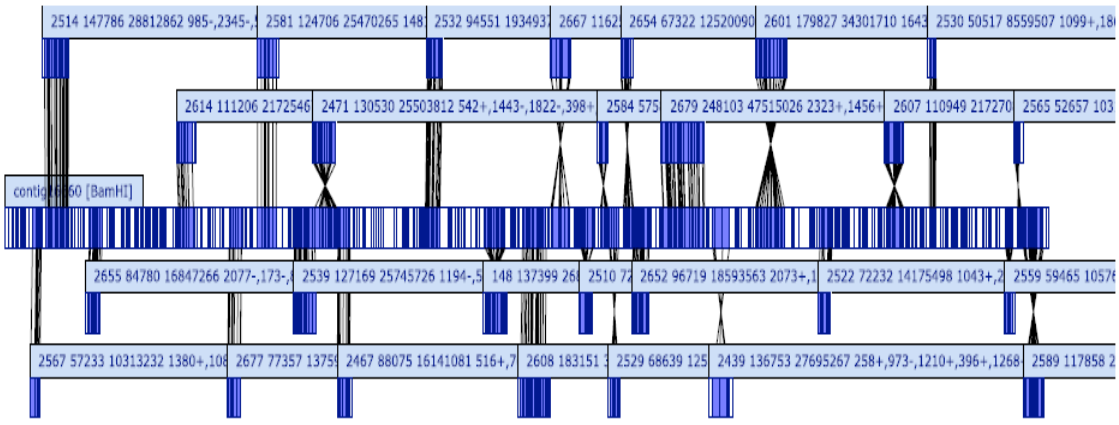


Fig. C-3.3.1: Optical map, obtained by perfroming BamHI restriction analysis.

The *P. pseudoalcaligenes* KF707 genome size was estimated to be approximately 5.95 Mb, with a GC content of 64.24%. The assembly was partially finished by scaffolding the contigs on the optical map, using the MapSolver software (OpGen). All the contigs longer than 40 kb (a suggested threshold value for a reliable mapping) were placed on the map, thus confirming the consistency of the assembly. This scaffold, supported by the contig connectivity obtained with the ABySS assembler software, was used to chain 33 contigs shorter than 50 kb, thus increasing the N50 of the assembly from 81.842 to 97.881 bp.

Contigs aligned on the optical map were used as guides to assemble other fragments. Concatenations of these fragments to the contigs already positioned on the Optical Map, increasing the map coverage up to 79.63%.

C-3.4 Genes prediction and annotation

Genes prediction and annotation were performed as described above (§ C-1.1.2.4).

Preliminary analyses for CDSs searching were performed using the GeneMark software, a bioinformatics tool which works scanning sequences and trying to find codifying genes in all of the possible ORFs on both DNA strands.

The actual gene prediction and annotation were performed via RAST system (Aziz *et al*., 2008). The bioinformatics analysis returned as output 6.512 CDSs (coding sequences), 81 tRNAs (representing all 20 amino acids), and 27 rRNAs. The annotated genes were grouped by RAST softwares in subsystems (Fig C-3.4.1).

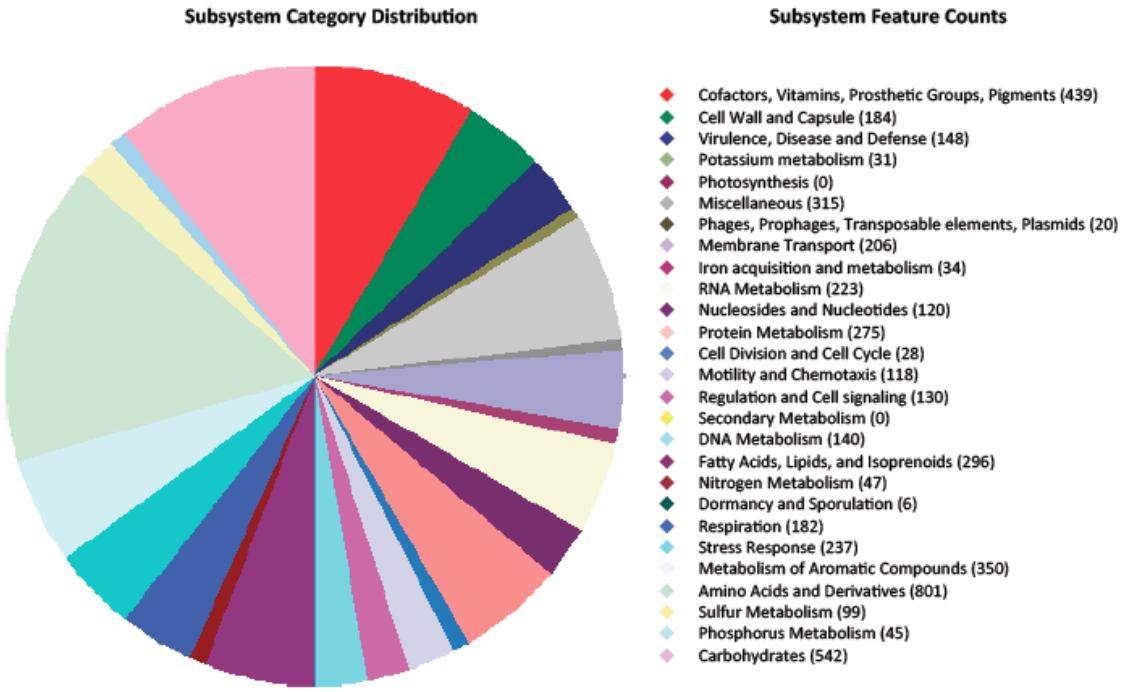


Fig. C-3.4.1.: Pie-chart representig *Pseudomonas pseudoalcaligenes* KF707 genes grouped in subsystems according to the RAST system prediction and annotation.

Genes involved in multiple functions were identified. Several of these genes are responsible for aromatic compounds biodegradation (Fig. C-3.4.2A), others are responsible for oxydative stress response (Fig. C-3.4.2B).

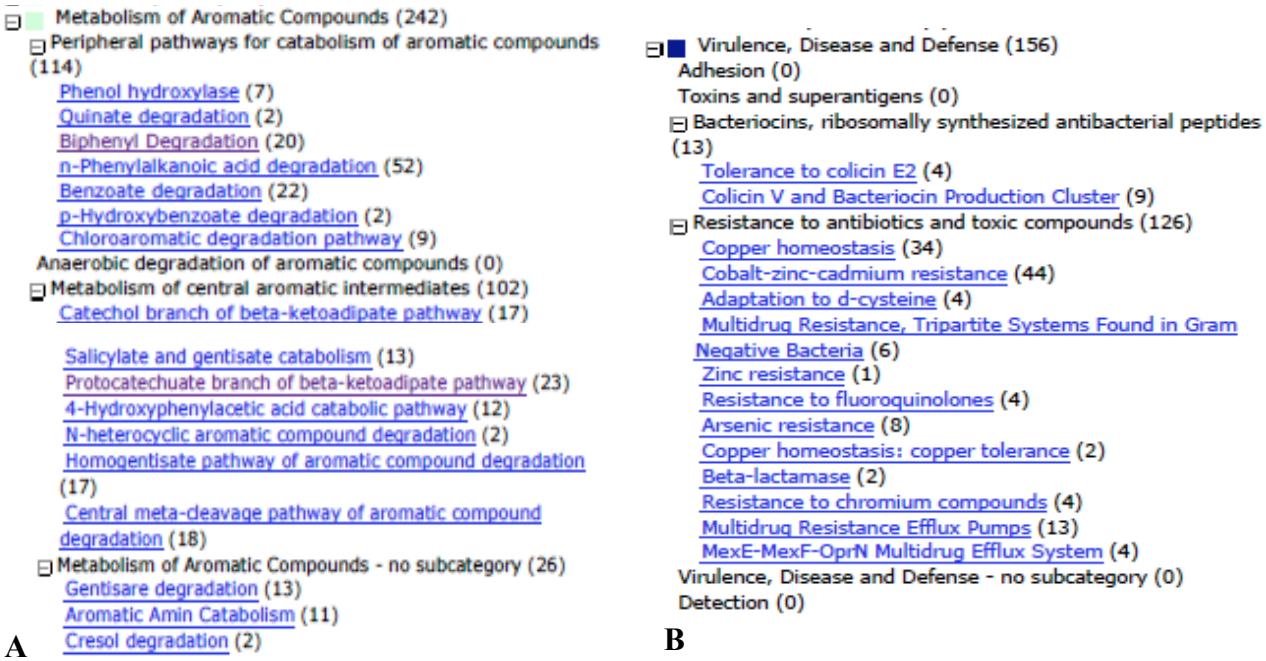


Fig. C-3.4.2 : Subsystems of genes codifying enzymes involved in the catabolism of aromatic compounds (A) and oxydative stress response (B).

Pseudomonas pseudolacaligenes KF707 has always been known for its ability to degrade xenobiotic compounds such as biphenyl and polychlorinated biphenyls (PCBs) (Furukawa *et al.,* 1986). In addition to the *bph* operon (Fig. C-3.4.3) cloned and characterized by Furukawa *et al*. (1986), genes involved in the degradation of aromatic compounds – including phenol, benzoate, p-hydroxybenzoate, cresol – were identified (Fig. C-3.4.2A).

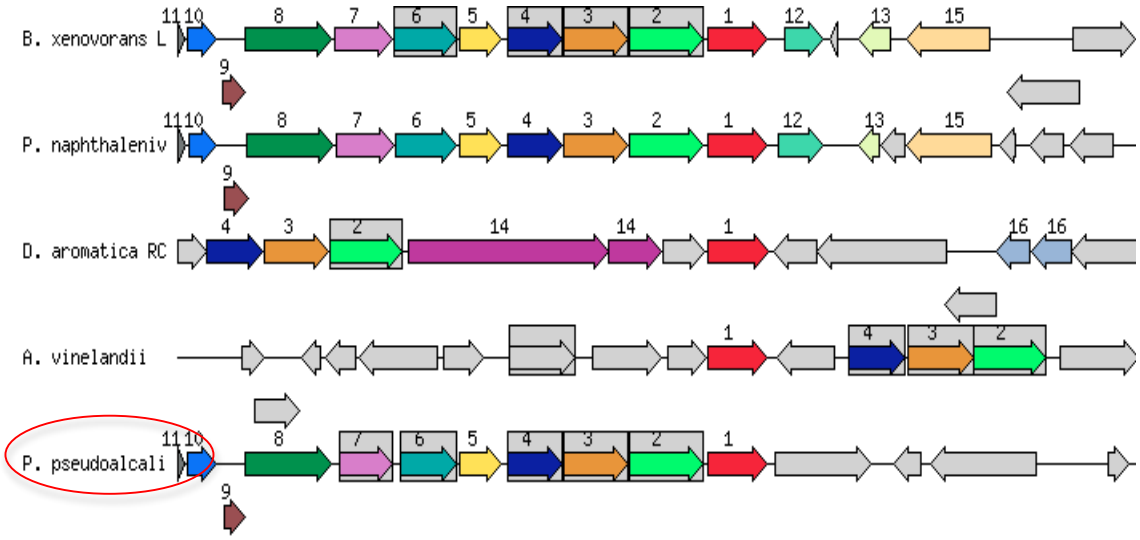


Fig C-3.4.3 : Organization of *bph* operon in *Pseudomonas pseudoalcaligenes* KF707 (at the bottom of the figure). Genes are indicated with numbers. Number **1** is *bph*D gene; number **7** represents *bph*B; **8**, **9**, **10**, **11** represents *bph*A4, *bph*A3, *bph*A1, *bph*A2 genes. The organization of KF707 *bph* genes cluster is homologue to that of the well known degrader strain *Burkholderia xenovorans* LB400 (at the top of the figure).

Interestingly, multiple cluster of putative genes involved in chemotaxis, were identified. In particular three *che*A genes, organized in different clusters, were found (Fig. C-3.4.4).

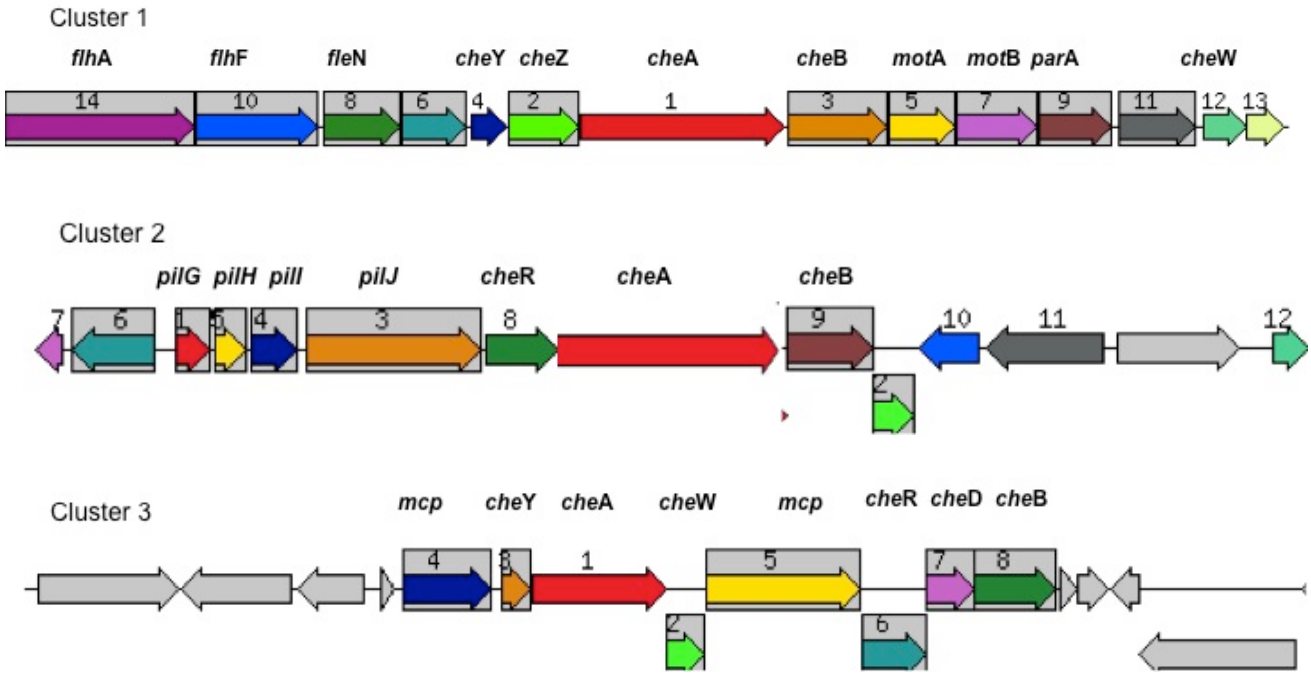


Fig. C-3.4.4: Multiple chemotactic genes clusters, containing three different *che*A genes in *Pseudomonas pseudoalcaligenes* KF707.

C-4 Discussion

In this chapter, the genome assembly and the genes prediction and annotation of *Pseudomonas pseudoalcaligenes* KF707 genome were described.

Assembly was performed starting from two different datasets of reads (the 454 and Illumina reads datasets). Two types of sequencing technologies were used and they provided different genome coverage. The Newbler and AbySS assemblers were employed. The Optical Map was also constructed and it was used as scaffold for contings orientation and concatenation. The Optical Map represented a useful approach because allowed us to get information on the position of the contigs, the size of the left gaps and even the genome size, which was estimated to be, approximately 5.95 Mb. The assembled contigs covered the 79.63% of the Optical Map. In this respect, a possible way to get the genome map closure might be the use of a chromosome walking approach (by PCR) or, alternatively, the use of new third generation sequencing technologies.

Genes prediction and annotation were also performed. RAST (Rapid Annotation based on Subsystem Technology) was the main bioinformatics tool for genes identification. Genes involved in interesting metabolic pathways were identified (§ C-3.4). Notably, multiple chemotactic pathways and two additional *che*A gene clusters, codifying for putative histidine kinase, were predicted. Several genes involved in the degradation of aromatic and xenobiotics compounds were also found. Moreover, genes involved in the oxydative stress response were identified.

In summary, it is evident that all these genetic features make KF707 an important strain for bioremediation procedures. Indeed, the sequencing, assembly and annotation of KF707 genome, provided a huge amount of genetic insights on its

chemotactic and degradative abilities. Consequently, KF707 can be considered a strong candidate for future studies regarding PCBs-degradative pathways so to provide the molecular basis for the construction of bacterial strains with improved performances in bioremediation of PCBs polluted sites.

CHAPTER D

Bioinformatics analysis of genes involved in motility and chemotaxis in *Pseudomonas pseudoalcaligenes* KF707 and contruction of chemotactic mutants

D-1 Introduction

The genome project of *Pseudomonas pseudoalcaligenes* KF707 provided a large set of data concerning the various physiological properties of this strain. Sequencing and annotation of KF707 genome were necessary since a wide range of phenotypic traits were poorly understood. One of the biggest issue was the understanding of the organization of the chemotactic pathway and the way it plays a crucial role in biofilm formation and development. Since the most of Gram negative bacteria possess more than one chemotactic pathway, not always involved in motility and chemotaxis but responsible of other physiological functions (§ A-5), we thought to look for homologous pathways in KF707 genome. In contrast to *E. coli*, which owns only one chemotactic pathway (Sourjik, 2004), other organisms have additional chemosensory operons and other *chemotaxis-like* pathways. For example, *Pseudomonas aeruginosa* PAO1 owns four operons named *Che, Che2, Pil-Chp* and *Wsp* (Kato *et al*., 2008) and some components are involved in the control of cyclic-di-GMP production and biofilm formation (Hickman *et al*., 2005; Guvener and Harwood, 2007). The bacterium *Myxococcus xanthus* has even eight *chemotaxis-like* pathways and some of their components play an important role in controlling genes expression and production of extracellular polysaccharides (Zusman *et al*., 2007)). The Gram positive *Bacillus subtilis* has only one

chemotactic pathway, although more complex as compared to the *E. coli* one (Garrity and Ordal, 1997; Szurmant *et al*., 2003; 2004). In the Gram negative *Rhodobacter sphaeroides* three pathways were found and their components are codified by genes organized in three independent operons, *CheOP1, CheOP2* and *CheOP3*. In this latter species, it was observed that the first two operons are both necessary for chemotaxis (Porter *et al*., 2002), while the third one is not expressed in laboratory conditions, therefore its function is still unknown (Poggio *et al*., 2007). In the pathogen *Vibrio cholerae*, three putative genes with strong homologies to the *E. coli che*A gene have been identified (Gosink *et al*., 2002). Moreover, many bacterial species own a high number of chemoreceptors, various CheY~P phosphatases and alternative adaptation systems (Porter *et al*., 2011).

This chapter describes how the putative chemosensory clusters and probable genes involved in motility, chemotaxis signal trasduction and adaptation systems in *Pseudomonas pseudoalcaligenes* KF707, have been identified. In particular, it has been of some interest to look for *che*A gene homologues as a histidine kinase CheA, previously identified by a mini-Tn5 transposon insertion, was shown to play an important role in motility, chemotaxis and biofilm formation (Tremaroli *et al*., 2011).

D-2 Materials and Methods

D-2.1. Identification of genes involved in motility and chemotaxis

RAST (Rapid Annotations based on Subsystem Technology) is a procaryotic genome annotation server, designed to find and annotate the genes of complete or almost complete bacterial genomes (§ C-1.1.2.4). KF707 assembled genome

sequence was uploaded in a FASTA and GenBank formats. Specific parameters were set up and the annotation process was launched. The resulting annotated genes were used for comparative studies. Homology searches were performed using BLASTn and BLASTp. The following parameters were used for nucleotide alignments: low complexity filter, word size of 28, match score of 1, mismatch score of -2 and gap penalty of 0.0. The parameters chosen for BLASTp were a word size of 3, gap penalty 11.1 and BLOSUM62 matrix. The amino acid alignment program Clustal W (http://www.ebi.ac.uk/clustalw/) was used for the amino acid comparative studies and putative conserved domains were detected by means of the Conserved Domain Database (CDD) available at the NCBI website.

D-2.2. Bacterial strains and growth conditions

Pseudomonas pseudoalcaligenes KF707, *E. coli* Top10 F' - harbouring pUC19 cloning vector - and *E. coli* JM109 - harbouring pG19II plasmid - were used in this study. Bacterial strains were grown at the optimal temperature on LB medium containing the appropriate antibiotics. Media compositions, antibiotic concentrations, relevant genotype features of all the strains and plasmid characteristics are described in the "General Materials and Methods Common to Chapters D, E, F" (§ B-1).

D-2.3. Amplifications of chemotactic target genes flanking regions and subsequent molecular fusion by using "Gene SOEing" method

DNA fragments for the construction of recombinant sequences with deleted chemotactic target genes, were obtained by performing the Gene SOEing (splicing

overlap extension) method, a PCR based approach which allows site-specific mutagenesis. Amplifications of DNA fragments flanking the target chemotactic genes were performed from KF707 wild type strain genomic DNA, which was extracted according to the protocol described in "General Materials and Methods Common to Chapters D, E, F" (§ B-2). Here the approach is described as a general procedure in order to allow the reader to undertsand. This procedure was used to obtain the recombinant DNA fragments and to construct of all the mutants in the chemotactic genes *che*A2, *che*A3, double mutants (Δ*che*A2*che*A1::*km*, Δ*che*A3*che*A1::*km*), *che*Y and *che*Z. It consists of three essential steps: primers design, PCR reactions to amplify regions flanking the target gene and, finally, a fusion PCR reaction to join the fragments. Fig. D-2.3.1 illustrates the principle steps of this method.

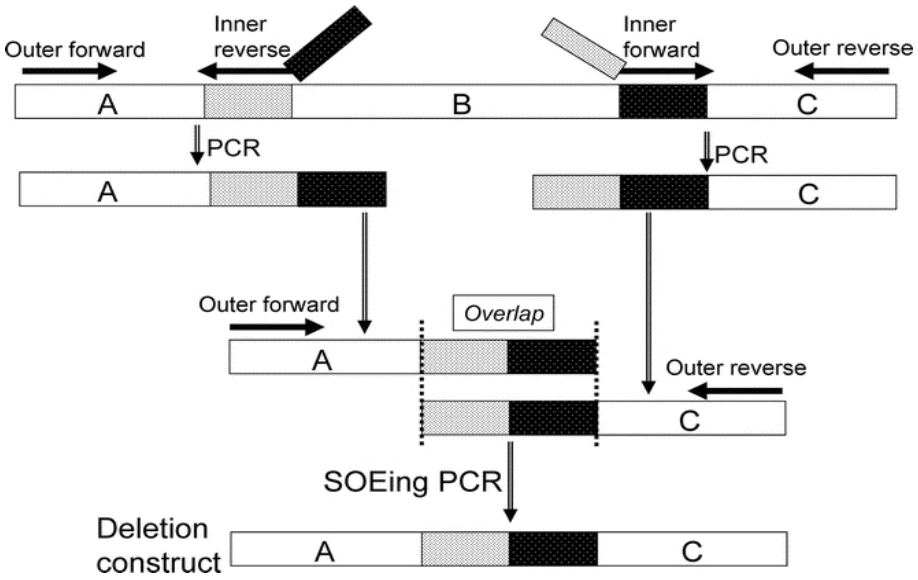


Fig. D-2.3.1. : Illustration of Gene SOEing method. This picture was taken from Izumi *et al*. (2007).

Primers design. According to the Gene SOEing method, two pairs of primers were used to amplify the flanking regions of each target gene. The reverse primer for the upstream region flanking the target gene, owns an oligonucleotide linker (at the 5'-OH extremity) which overlaps with that of the foward primer for the downstream flanking region. Moreover, the outer primers, specifically the forward for the upstream region and the reverse for the downstream one, own a sequence for restriction enzymes (in the present Thesis work, HindIII and BamH1, respectively).

Extension PCR. Two separated PCR reactions were performed. Reaction mixtures (50 µl) contained 5 µl of 10X PCR buffer containing Mg^{2+}, 0.5% (v/v) DMSO, 0.2 mM of each dNTP, 0.3 pmol of each primer, 1U High Fidelity DNA polymerase and 50 ng of DNA template. Amplifications were performed in a Bio-Rad-C-1000 T-gradient termocycler. Optimal conditions of denaturation, annealing and extension were used for each pair of primers. In general the following parameters were applied: initial denaturation at 96°C for 5 minutes followed by 30 cycles consisting of denaturation at 95°C for 1 minute, annealing at the the optimal temperature for each primers pair for 45 sec, elongation at 72°C for 1min/Kb and a final extension step at 72°C for 10 minutes.

PCR products were separated by electrophoresis on 1% (w/v) agarose gel and after staining in Gel-Red solution they were visualized under UV-light: when clear and clean bands were observed, the PCR reactions were cleaned-up using QIAGEN PCR purification kit, otherwise, if no specic products were observed, the correct bands were cut from gel and cleaned-up using the QIAGEN gel extraction kit.

Overlap PCR. Purified PCR products from the two separated reactions were

quantified and used as templates in the "overlap step": for each target gene, only the two outer primers were used in the reaction, since the overlapping linkers own the 3'-OH extremity to allow extension by Taq polymerase; the fragments formed an eteroduplex intermediate as mediated by the overlapping linkers. Subsequent extension of the eteroduplex led to the formation of the recombinant molecules.

Purification of joined amplicons. PCR products were separated by electrophoresis on 1% (w/v) agarose and after staining in Gel-Red solution they were visualized under UV-light; bands were cut from gel and cleaned-up using the QIAGEN gel extraction kit, according to the manifacturer's guide. The purified joined amplicons were stored at -20°C until use for subsequent experiments.

D-2.4. Construction of recombinat plasmids containing fragments with deleted chemotactic genes and conjugation into *Pseudomonas pseudolacaligenes* KF707 wild type strain.

Cloning in pUC19. Joined fragments obtained from SOEing method, were double digested with HindIII and BamHI restriction enzymes and cloned in pUC19 vector. Plasmids were introduced into *E. coli* Top 10 F' host by transformation of chemically competent cells, prepared according to the $CaCl_2$ method (Sambrook *et al*., 1989). Trasformants clones were selected *via* white/blue screening on LB ampicillin agar containing X-Gal at the final concentration of 50 μg/ml. In order to assess the presence of the insert, plasmid mini-preps were performed from white clones cultures and double digested with HindIII/BamHI restriction enzymes; after electrophoresis on 0,8% (w/v) agarose gel and staining in Gel-Red solution, digestions were visualized under UV-light. For further validation, white clones

were selected for colony PCR reactions: inserts were amplified using universal primers M13 foward and reverse and purified PCR products were sequenced to confirm the insertion of the DNA fragments.

Cloning in pG19II. Subsequently, each fragment was cloned into the conjugative plasmid pG19II double digested with HindIII/BamHI. pG19II is a pK19mobsacB derived conjugative plasmid, which carries its own origin of replication, the *oriV*, and an origin of transfer, named *oriT*. Moreover, this plasmid harbours two selection markers: Gm^R gene which confers resistance to the antibiotic gentamicin and *sac*B gene, codifying for the secreted enzyme levansucrase which causes sensitivity to sucrose (Maseda *et al*., 2004).

Recombinant plasmids were introduced into *E. coli* Top 10 F' host by transformation of chemically competent cells and transformant clones were selected for gentamicin resistance and *via* white/blue screening. Mini-preps were performed from cultures of positive clones and all the recombinant plasmids were sent for sequencing in order to verify the presence of the inserts.

Conjugation. pG19II recombinant plasmids carrying the constructs were transferred by conjugation to *Pseudomonas pseudoalcaligenes* KF707 wild type strain. Conjugation protocol is described in details in (§ B-5). Briefly, *E. coli* strains, each harbouring pG19II with one of the different contructs for each target gene, were used as donor strains, while *E. coli* HB101 pRK2013 was used as helper strain. Conjugation mixes were spotted on well dried LB agar plates; after 24 hours of incubation the biomass from each conjugation was collected and suspended in LB containing 10% (v/v) glycerol; the suspensions were serially 10-fold diluited and plated onto AB glucose medium containing gentamicin as selection marker.

Transconjugants were *tooth-picked* in fresh selective medium and the selection was repeated at least twice, in order to obtain pure cultures and remove both donor and helper strain backgrounds. KF707 transconjugant strains harboured a plasmid carrying the recombinat DNA fragment with the deleted copy of one of each target gene. In order to obtain deleted mutant strains, a double cross-over between the recombinat plasmid and the homologous genomic DNA sequence was stimulated. To force the double cross-over, strains were grown in medium containing a high concentration of sucrose. Since pG19II harbours the *sac*B gene which codifies for the levansucrase, an enzyme that doesn't allow growth on sucrose, this carbon source was added at high concentration to stimulate the expulsion of the plasmid.

Selection of mutant strains. Transconjugants were grown over-night in 10 ml of modified LB broth without NaCl. The next day, 1% inocula were grown in 4 ml of the same medium containing sucrose at the concentration of 10% (w/v), until they reached an $OD_{600\ nm}$ of ~0.4. 100 μl of each culture were spread onto modified LB agar (without NaCl). Plates were incubated until growth was visible. Grown clones were *tooth-picked* onto both LB agar 10% sucrose and LB agar 20 μg/ml gentamicin. After over-night incubation the growth of the selected clones in the two kinds of media was compared: double cross-over clones were those able to grow only on 10% sucrose plates and not on gentamicin plates. They were selected as probable double cross-over mutants and subsequently confirmed by performing colony PCR reaction using the outer primers for the flanking regions of each deleted gene.

D-3. Results

D-3.1. Motility and chemotaxis genes clusters in *Pseudomonas pseudoalcaligenes* KF707 genome

The bioinformatics analysis conducted on KF707 genome using the RAST tool for annotation (§ C-3.4), showed the presence of three putative clusters codifying for putative genes involved in chemotaxis (Fig. D-3.1.1); moreover, twenty-seven probable methyl accepting chemotaxis proteins (MCPs) (Table D-3.1.1), four genes codifing for proteins involved in flagellum biosynthesis (Table D-3.1.2) and eleven genes for its assembly (Table D-3.1.3) were identified.

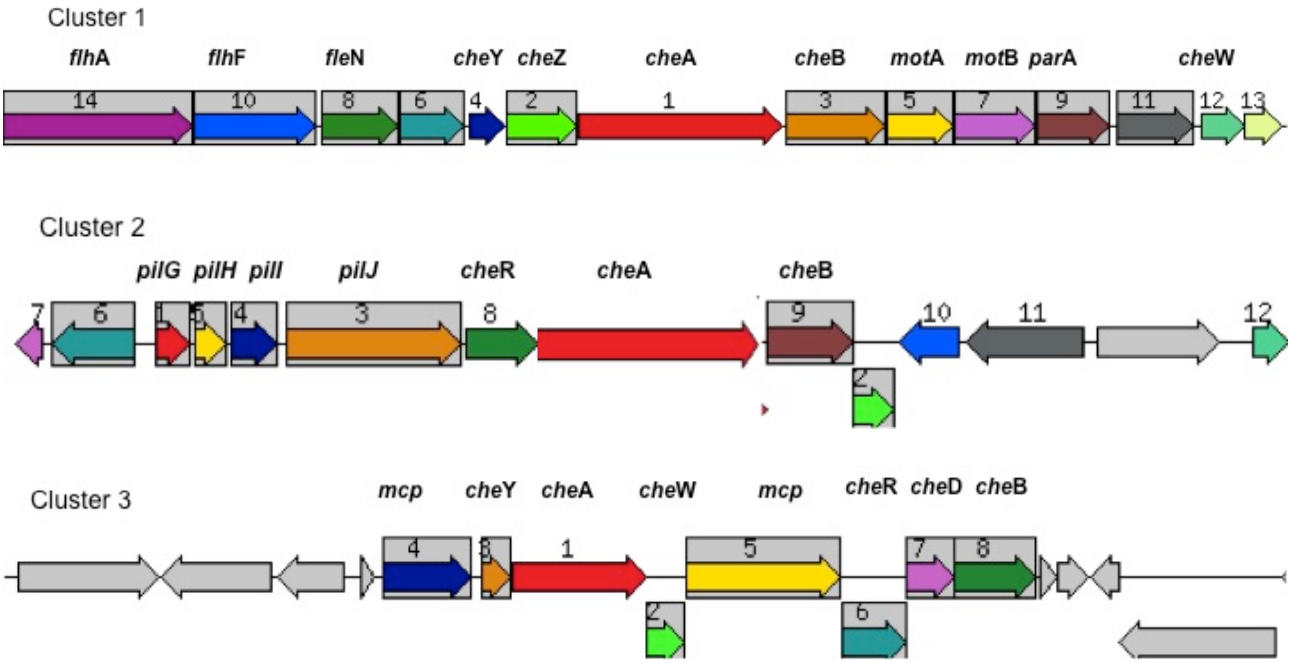


Fig. D-3.1.1: Chemotactic gene clusters in *Pseudomonas pseudoalcaligenes* KF707 containing the cheA genes.

In Fig. D-3.1.1, three of the multiple KF707 chemotaxis clusters are shown. The first one was identified and characterized after the isolation of a mutant impaired in motility due to the insertion of a Tn5 transposon in the *che*A1 gene codifying for a

histidine kinase (Tremaroli *et al*., 2011). As reported in "Chapter C" (§ C-3.4), genome sequencing and annotation showed the presence of two additional putative *che*A genes, named *che*A2 and *che*A3. BLAST similarity analyses of nucleotide sequences of the *che*A genes were performed. KF707 *che*A1 nucleotide sequence displayed high homology (from 81% to 95%) with *Pseudomonas sp*. chemotactic genes. The greatest Max Identity values were shown with *Pseudomoas putida* GB-1 (95%), *Pseudomonas putida* HB3267 (90%) and *Pseudomonas aeruginosa* PA7 (89%). The *che*A2 nucleotide sequence showed the maximum coverage (84%) with the nucleotide sequence of the *che*A signal transduction histidine kinase of *Pseumomonas mendocina* NK-01. The *che*A3 sequence showed a highest value of identity (83%) with the *Pseudomonas aeruginosa* UCBPP-PA14 gene codifying for a putative two-component sensor. However the three *che*A genes in KF707 did not show similarity between each other. In order to understand why the three genes were annotated as chemotactic *che*A genes, BLASTp analyses were performed to look for the presence of aminoacidic similarity and structural conserved domains. The CheA1 aminoacidic sequence displayed the maximum identity (81%) with the histidine kinase gene of *Pseumomonas mendocina* NK-01. The result was the same for the KF707 CheA2 protein. With regard to the CheA3 aminoacidic sequence, it showed the highest identity (72%) with the putative two-component sensor of *Pseudomonas aeruginosa* PA7. Moreover, the CheA1 and CheA3 proteins showed the same conserved domains (Fig. D-3.1.2): (i) the *HTP* (Histidine Phosphotransfer domain), involved in signalling through a two-part-component systems in which an autophosphorylating histidine protein kinase serves as a phosphoryl donor to a response regulator protein; (ii) the s*ignal transduction histidine kinase*

homodomeric domain which is a helical bundle domain at the interface of the signal transducing histidine kinase family; (iii) *histidine kinase-like ATPases*, which is part of a family including several ATP-binding proteins such as histidine kinase, DNA gyrase B, topoisomerases, heat shock protein HSP90, phytochrome-like ATPases and DNA mismatch repair proteins; (iv) *CheA regulatory domain* which belongs to the family of CheW-like proteins and has been proposed to mediate interaction with the kinase regulator CheW.

CheA2 protein showed also the *HTP,* the s*ignal transduction histidine kinase,* the *histidine kinase-like ATPases* and the *CheA regulatory* domains*,* but also additional *HPT* domains and one more *signal receiver domain* were found (Fig. D-3.1.3).

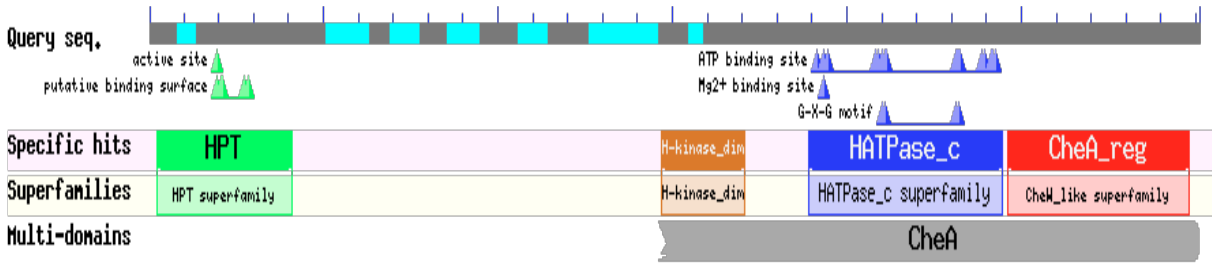


Fig. D-3.1.2 : Conserved domains in *Pseudomonas pseudoalcaligenes* KF707 CheA1 and CheA3 proteins

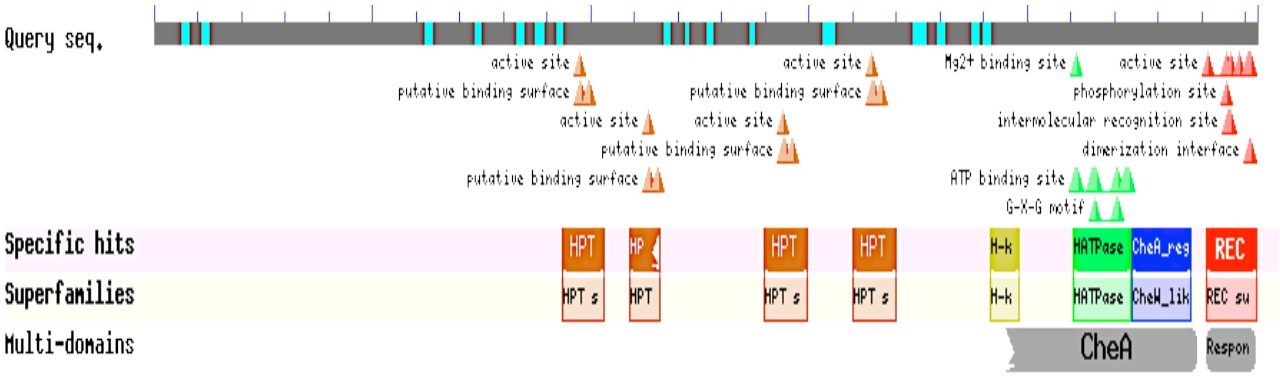


Fig. D-3.1.3 : Conserved domains in *Pseudomonas pseudoalcaligenes* KF707 CheA2 protein.

In addition to the *che*A genes, the presence of multiple copies of other genes involved in motility and chemotaxis was also investigated.

CheW proteins, called also adapters, are important components for the assembly of chemoreceptors clusters at the membrane level since their function in anchoring CheA dimers to the MCPs and to transmit the effect of the "piston-like" movement to the histidine kinase CheA, when ligands bind the periplasmic domain of MCPs. KF707 showed the presence of two *che*W genes (*che*W1 and *che*W3, codified at the *che*A1 and *che*A3 clusters repectively). With regard to the putative MCPs they are listed in Table D-3.1.1.

Table D-3.1.1: List of putative genes codifying for MCPs in KF707

Feature ID	Contig	Start	Stop	Lenght (bp)	Function
fig|1149133.5.peg.203	10002	77424	75850	1575	putative methyl-accepting chemotaxis protein
fig|1149133.5.peg.387	10003	32481	30850	1632	serine chemoreceptor protein
fig|1149133.5.peg.475	10003	116715	115573	1143	serine chemoreceptor protein
fig|1149133.5.peg.518	10004	39402	41018	1617	Methyl-accepting chemotaxis protein
fig|1149133.5.peg.805	10006	22921	21221	1701	serine chemoreceptor protein
fig|1149133.5.peg.1124	10006	343655	345241	1587	serine chemoreceptor protein
fig|1149133.5.peg.1170	10006	401088	399454	1635	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.1216	10006	456090	457481	1392	Serine chemoreceptor protein
fig|1149133.5.peg.1317	10006	566605	568569	1965	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.1514	10008	30422	32362	1941	Methylaccepting chemotaxis protein
fig|1149133.5.peg.1531	10008	48944	50569	1626	serine chemoreceptor

					protein
fig|1149133.5.peg.2778	1549	56243	54636	1608	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.2788	1549	66352	64346	2007	Serine chemoreceptor protein)
fig|1149133.5.peg.2837	1554	10271	8241	2031	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.3047	1555	209901	208219	1683	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.3436	1589	13939	12008	1932	Methylaccepting chemotaxis protein
fig|1149133.5.peg.3448	1589	31823	33787	1965	serine chemoreceptor protein
fig|1149133.5.peg.3531	1589	117658	115724	1935	methylaccepting chemotaxis protein
fig|1149133.5.peg.3846	1602	80234	81820	1587	methylaccepting chemotaxis protein
fig|1149133.5.peg.3957	1616	25063	23099	1965	Methylaccepting chemotaxis protein
fig|1149133.5.peg.4062	1621	43	1035	993	Methylaccepting chemotaxisprotein I
fig|1149133.5.peg.4483	1659	6958	5327	1632	Methylaccepting chemotaxis protein I
fig|1149133.5.peg.4612	1672	12967	11978	990	methylaccepting chemotaxis protein
fig|1149133.5.peg.5231	1698	219583	219014	570	Methylaccepting chemotaxis protein
fig|1149133.5.peg.5837	1729	75529	74387	1143	probable methylaccepting chemotaxis protein
fig|1149133.5.peg.5873	1732	35521	37155	1635	serine chemoreceptor protein
fig|1149133.5.peg.6130	1744	11755	10124	1632	Methylaccepting chemotaxis protein I

Chemotaxis pathways include also enzymes involved in response regulation (CheY), in signal termination (CheZ) and adptation mechanisms (CheR and CheB, a methyltransferase and methylesterase respectively). Two *che*Y genes (at the *clusters 1* and *3*, Fig. D-3.1.1) and only one *che*Z gene (at the *cluster 1*, Fig. D-

3.1.1) were found in KF707 genome. Furthermore, KF707 owns three *che*B genes (Fig. D-3.1.1). Three *che*R genes were found, two of which are part of the *cluster 2* and *cluster 3* (Fig. D-3.1.1) while the third was found in a different cluster and associated to one *che*V gene and other genes involved in flagellum assembly (Fig. D-3.1.4). Another *che*V, one *che*C and one *che*D genes were also found in other additional clusters.

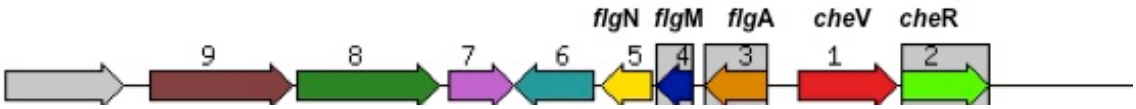


Fig. D-3.1.4: Additional *che*R gene in *Pseudomonas pseudoalcaligenes* KF707 genome.

Genes codifying for proteins involved in the flagellar byosinthesis and assembly, are listed in Tables D-3.1.2 and D-3.1.3 , respectively.

Table D-3.1.2: List of putative genes codifying enzymes involved in flagellar biosynthesis

Feature ID	Type	Contig	Start	Stop	Length (bp)	Function
	CDS	10008				flh
fig|1149133.5.peg.1595	CDS	10008	118971	120107	1137	Flagellar biosynthesis protein FlhB
fig|1149133.5.peg.1597	CDS	10008	122080	124203	2124	Flagellar biosynthesis protein FlhA
fig|1149133.5.peg.1598	CDS	10008	124219	125568	1350	Flagellar biosynthesis protein FlhF
fig|1149133.5.peg.1612	CDS	10008	137197	136862	336	FlhB domain protein

Table D-3.1.3: List of putative genes involved in flagellar assembly

Feature ID	Type	Contig	Start	Stop	Length (bp)	Function
	CDS	10008				flg
fig|1149133.5.peg.1545	CDS	10008	66148	66555	408	Flagellar basal-body rod protein FlgB
fig|1149133.5.peg.1546	CDS	10008	66570	67016	447	Flagellar basal-body rod protein FlgC
fig|1149133.5.peg.1547	CDS	10008	67033	67716	684	Flagellar basal-body rod modification protein FlgD
fig|1149133.5.peg.1548	CDS	10008	67762	69099	1338	Flagellar hook protein FlgE
fig|1149133.5.peg.1549	CDS	10008	69492	70235	744	Flagellar basal-body rod protein FlgF
fig|1149133.5.peg.1550	CDS	10008	70281	71066	786	Flagellar basal-body rod protein FlgG
fig|1149133.5.peg.1551	CDS	10008	71085	71798	714	Flagellar L-ring protein FlgH
fig|1149133.5.peg.1552	CDS	10008	71810	72910	1101	Flagellar P-ring protein FlgI
fig|1149133.5.peg.1553	CDS	10008	72920	74083	1164	Flagellar protein FlgJ [peptidoglycan hydrolase] (EC 3.2.1.-)
fig|1149133.5.peg.1554	CDS	10008	74103	76145	2043	Flagellar hook-associated protein FlgK
fig|1149133.5.peg.1555	CDS	10008	76157	77407	1251	Flagellar hook-associated protein FlgL

D-3.2. Amplification of cheA genes flanking regions and fusion by Gene SOEing (splicing overlap extension)

In order to obtain fragments with deletions of *che*A2, *che*A3, *che*Y1 and *cheZ* genes, the Gene SOEing method was applied (§ D-2.3). The first step consisted in primers design. The upstream flanking regions of the target genes were amplified with the primers pairs FcheA2up/FcheA2dw-overlap and FcheA3up/FcheA3dw-overlap for *che*A2 and *che*A3, respectively. The downstream flanking regions of both genes were amplified using the primers pairs FcheA2up-overlap/FcheA2dw and FcheA3up-overlap/FcheA3dw for cheA2 and cheA3 respectively. With regard

to the outer primers, they were designed with restriction sites, HindIII for the upstream outer primers and BamHI for the downstream ones. To construct the *che*Y1 and *che*Z mutant strains, the same approach was adopted (in Fig. D-3.2.1 is shown only the procedure for the *che*A2 gene).

The oligonucleotide linkers of the reverse primer for the upstream region and the forward primer for the downstream regions were designed with an overlapping sequence of 15 nucleotides (shown in Fig. D-3.2.1) for all the primers pairs.

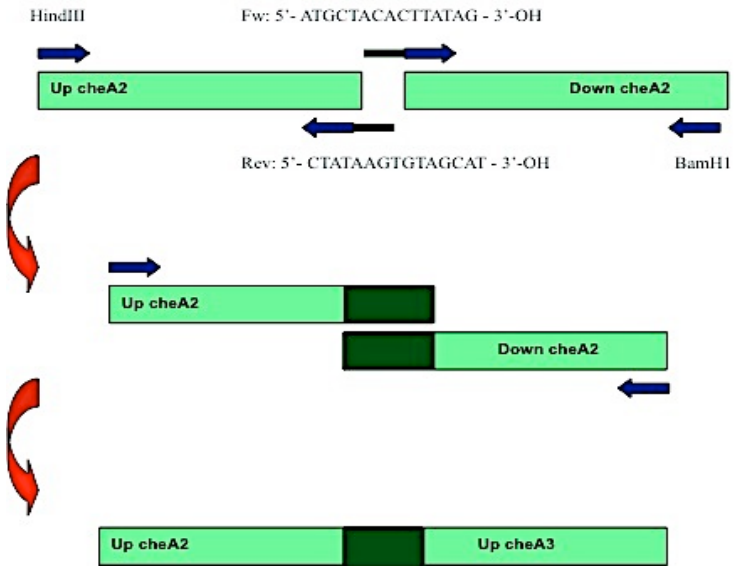


Fig. D-3.2.1.: Construction by SOEing of recombinant DNA fragments with *che*A2 gene deletion

For all the genes, the upstream and downstream flanking regions chosen for the amplification were about 500 bp in size. Two independent PCR reactions were set up for each gene in order to amplify the flanking regions. The PCR reactions using KF707 genomic DNA as template, resulted in the expected 500 bp products (Fig. D-3.2.2 A). Subsequently, purified PCR amplicons were quantified and used as templates in the "overlap step": for each target gene, only the two outer primers

were used in this reactions, allowing the formation of recombinant joined fragments (Fig. D-3.2.2 B).

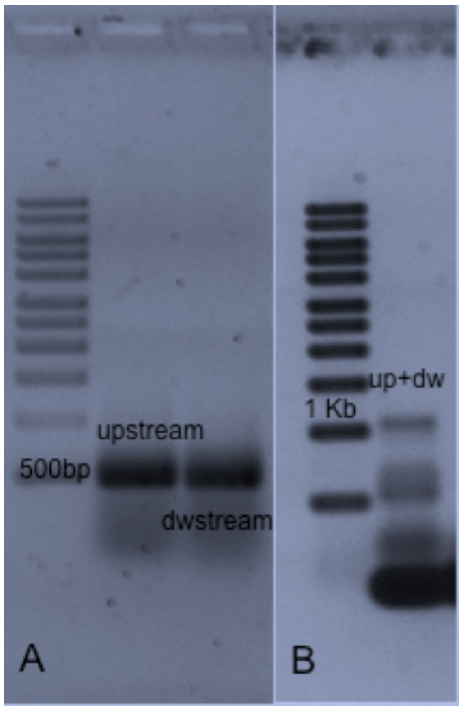


Fig. D-3.2.2: (A) Amplification of the upstream and downstream flanking regions of *che*A2 gene resulted in the production of 500 bp amplicons. (B) joined 1 Kb fragment obtained with the PCR fusion step. The results were similar for *che*A3, *che*Y1 and *cheZ* (not shown).

D-3.3. Construction of recombinant conjugative plasmids carrying fragments with deleted target chemotactic genes and conjugation into *Pseudomonas pseudolacaligenes* KF707 wild type strain

The 1 Kb bands were cut from gel and cleaned-up using the QIAGEN gel extraction kit; subsequently, they were double digested with HindIII and BamHI and after purification and quantification, they were used as inserts for a middle cloning step in pUC19 vector and finally they were cloned in the conjugative plasmid pG19II. Recombinant plamids were transformed in *E. coli* chemically competent cells and clones were selected by performing white/blu screening and for

Amp^R and Gm^R (for pUC19 and pG19II, respectively). The precence of the insert was tested by double digestions of plasmidic preps with HindIII/BamHI and visualization on agarose gel.

Moreover, at each step the sequences of the inserts were amplified using the universal M13 primers and sent for sequencing.

Specifically, the following recombinant plasmids were obtained: pG19IIΔ*che*A2, pG19IIΔ*che*A3 carrying the fragments with deleted *che*A2 and *che*A3 genes respectively. Moreover, the same procedure was used to contruct recombinant plasmids carrying fragments with deleted *che*Y1 (pG19IIΔ*che*Y1) and *che*Z (pG19IIΔ*che*Z) genes. Only transformant clones carrying the recombinant plasmids with correct sequences, were used as donor strains in the conjugation to KF707 wild type strain. pG19IIΔ*che*A2 and pG19IIΔ*che*A3 were also conjugated to the KF707 *che*A1::*Km* mutant (Tremaroli *et al*., 2011) for Δ*che*A2*che*A1::*Km* and Δ*che*A3*che*A1::*Km* double mutants construction.

Independent conjugations, one per each donor strain, were performed as described in "General Materials and Methods Common to Chapters D, E, F" (§ B-5). Transconjugants were selected in AB glucose medium containing gentamicin. The selection of mutant strains was based on the *sac*B system. pG19II harbours the *sac*B gene codifying for the secreted enzyme levansucrase, cause of sensivity to sucrose; normally KF707 strain is able to grow on sucrose. The reason for growing transconjugants on glucose was because they were not able to grow on sucrose since they harboured the recombinant pG19II plasmid. The procedure of mutants selection is illustrated in Fig. D-3.3.1.

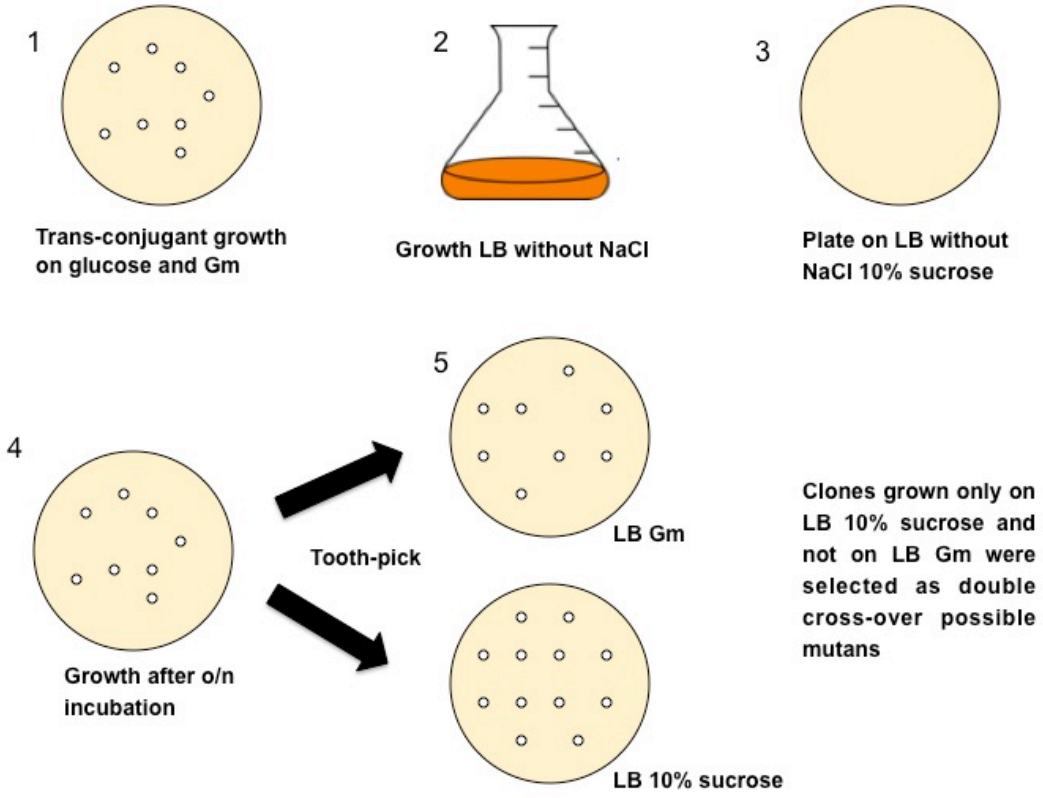


Fig. D-3.3.1: To stimulate the double cross-over in the transconjugants (1), they were grown in modified LB without NaCl (2). At the early exponential phase, they were plated onto LB containing 10% sucrose and without NaCl (3) and incubated over-night at 30°C (4). The next day, they were *tooth-picked* onto both LB 10% sucrose and LB 20 µg/ml gentamicin (5). Clones grown only on LB 10% sucrose and not on gentamicin were selected as probable double cross-over mutants.

The hypothetical mutants were then confirmed by performing colony PCR and sequencing of the obtained amplicons.

D-4. Discussion

In this study, putative genes involved in motility and chemotaxis were identified in *Pseudomonas pseudolacaligenes* KF707 genome. RAST tool (http://rast.nmpdr.org/) for rapid gene annotation was used. The output data showed that KF707 strain owns several putative chemotaxis pathways. Interestingly three

*che*A genes were found, each one organized in one independent cluster (Fig. D-3.1.1). Bioinformatics analyses on the aminoacidic sequences of the putative histidine kinases, codified by these new *che*A genes, were performed. The searching of conserved domains, revealed that both CheA2 and CheA3 possess the histidine kinase typical domains (Fig. D-3.1.2 and Fig. D-3.1.3), even though the nucleotide sequences did not show high similarity. Generally, CheA is divided into five structurally and functionally distinct domains ("General Introduction", § A-4.2): the histidine phosphotransfer domain (P1), the response regulator binding domain (P2), the dimerization domain (P3), the histidine protein kinase catalytic domain (P4), and the regulatory domain (P5). KF707 CheA1 and CheA3 proteins showed the same predicted conserved domains: the P1 domain belonging to the histidine phosphotransfer (HPT) family of proteins which transfer a phosphoryl groups between ATP and the phosphoaccepting aspartate side chains of response regulators; the response regulator binding domain, P2, which forms complex with CheY, allowing a conformational change in cheY active site that increases the accessibility of the phospho-acceptor aspartate, Asp57; the dimerization domain P3 and the kinase (HPK) catalytic core domain (P4) are also present in KF707 CheA1 and CheA3 proteins; the P5 was found too and it is homologous over its entire length to CheW: indeed it mediates binding to the chemoreceptor signaling domains. The KF707 CheA2 protein showed the same conserved domains as the other two CheA proteins, but also one additional signal receiver domain.

It has been reported that many environmental bacterial species have complex chemotaxis pathway: for example *Rhodobacter sphaeroides* has shown to have metabolic sensing, cytoplasmic chemoreceptors, alternative CheY~P phosphatases

and multiple chemosensory operons ("General Introduction", § A-5) . In addition to multiple *che*A genes, *P. pseudolacaligenes* KF707, showed multiple *che*W, *che*Y, *che*B and *che*R genes. Multiple CheW proteins could be involved in chemoreceptors arrays organization. The additional *che*Y gene, probably works as a phosphate sink in order to guarantee the signal termination; usually, multiple copies of *che*Y genes are present in bacteria lacking the *che*Z gene and since KF707 owns a *che*Z gene, the addictional CheY protein may act enhancing CheZ activity. KF707 also showed multiple copies of *che*B and *che*R that may be involved in adaptation mechanisms. Other bacteria, for example *B. subtilis,* show the CheC–CheD and the CheV circuits involved in adaptation ("General Introduction", § A-5)**.** The CheC–CheD circuit involves two proteins that are not found in *E. coli* but are found in about 40% of chemotactic bacteria and the putative genes for these proteins were also found in KF707. CheC is an alternative CheY~P phosphatase, whereas CheD is a chemoreceptor deamidase that converts glutamine residues to glutamic acid residues (Kristich and Ordal, 2002; Szurmant *et al*., 2004) In addition, CheD binds to the chemoreceptors to stimulate CheA autophosphorylation. The chemoreceptors and CheC– CheY~P compete with one another for binding of CheD, such that when CheY~P levels are low, most of the CheD binds to the chemoreceptors and stimulates CheA autophosphorylation. On the other side, when CheY~P levels are high, CheD dissociates from the chemoreceptors and forms the ternary complex with CheC and CheY~P (Rao *et al*., 2008). *che*V gene is also present in KF707 and it codifies for a protein with a CheW domain and a receiver domain. CheV function was studied in *Bacillus subtilis* and it works coupling chemoreceptor signalling to CheA kinase activity, and when phosphorylated by CheA, CheV inhibits CheA

kinase activity. In this way, phosphorylation of CheV by CheA establishes a negative feedback and adaptation by reducing CheA activity (Rao *et al*., 2008). All these results show that KF707 owns quite complex chemosensory pathways, which are probably interconnected. Indeed, as described in *Rhodobacter sphaeroides* (Sourjik and Armitage, 2010), gene products of the three different chemotaxis operons are involved in the regulation of gene products codified by genes on the other operons ("General Introduction", § A-5). It has been reported that, in addition to chemotactic pathways involved in motility under stimulating conditions, a number of bacterial species have pathways that are homologues to chemotaxis signalling pathways but which control other complex behaviours, such as developmental gene expression in *M. xanthus* (Zusman *et al*., 2007) and biofilm formation in *P. aeruginosa* (Hickman et al., 2005; Guvener and Harwood, 2007): hence it could be possible that in KF707, alternative chemotaxis pathways do not play a significant role in motility, but they may have a role in different cellular functions.

Since the genome annotation revealed all these informations regarding KF707 putative chemosensory pathways (§ C-3.4) and since previous works showed that the KF707 *che*A1 mutant strain is impaired in motility, chemotaxis and biofilm formation (Tremaroli *et al*., 2011), we thought to investigate the possible roles of the *che*A2 and *che*A3 genes. The procedure for the construction of deleted mutants in *che*A2 and *che*A3 genes and also of double mutants *che*A1/*che*A2 and *che*A1/*che*A3 is also described. These mutant strains, together with *che*Y and *che*Z mutants, were used in subsequent experiments to test their motile behaviour in the absence or in the presence of stimuli as well as biofilm formation.

CHAPTER E

Role of chemotactic genes in *Pseudomonas pseudoalcaligenes* KF707 motile behaviour and biofilm formation

E-1 Introduction

Bacteria may encounter variable conditions in sorrounding environments. In order to compete and survive in particular kinds of ecological niches they must be able to sense the environment, integrate external and internal stimuli and, accordingly, react with a behavioural response. The capacity to distinguish among the different stimuli and the ability to move towards to or away from chemical compounds is referred to as *chemotaxis* (§ Chapter A). Some so-called biodegrader bacteria, have the property to sense concentration of carbon sources (even toxic) and to move towards them. In this respect, *Pseudomonas pseudoalcaligenes* KF707 is known for its ability to degrade compounds such as biphenyl, polychlorynated biphenyls (Furukawa *et al*., 1986), benzoic and naftenic acids. In the case of highly hydrophobic chemicals adsorbed in the non-aqueous-phase liquid (NAPL), bacteria have been shown to gain access to the target contaminants by adhering directly to the NAPL-water interface, possibly through biofilm formation. Indeed, in natural environments bacteria are commonly found in association with biotic surface and KF707 is able to grow as a biofilm, even in the presence of toxic metal oxyanions such as tellurite and selenite. Biofilm-mediated bioremediation is a proficient alternative to the environmental clean-up with planktonic microorganims because

of the increased capability of adaptation and survival of biofilm cells and the distinct physiological properties displayed by bacteria in a biofilm.

This chapter describes in details the chemotaxis behavior of both *Pseudomonas pseudoalcaligenes* KF707 wyld type and chemotactic mutant strains. Biofilm formation, in relation with the possible role of CheA2 and CheA3 proteins similarly to previos data obtained with CheA1 mutants (Tremaroli *et al*., 2011), was also investigated.

E-2 Materials and Methods

E-2.1 Bacterial strains and growth conditions.

Pseudomonas pseudoalcaligenes KF707 wild type and mutant strains in the chemotactic genes *che*A1, *che*A2, *che*A3, *che*Y1, *che*Z and the double mutants in *che*A1/*che*A2 and *che*A1/*che*A3 genes, were tested. Bacterial strains were grown at the optimal temperature on LB medium. For motility assays they were grown in both rich and minimal media with defined agar concentration. Genotypic features of the strains and media compositions are described in the "General Materials and Methods common to Chapters D, E, F" (§ B-1).

E-2.2 Motility assays

The swimming, swarming and twitching behaviour of *Pseudomonas pseudolacaligenes* KF707 wild type and mutant strains were analysed as described in the following protocols.

E-2.2.1 Swimming

Tryptone swim plates (10 g/l tryptone, 5 g/l NaCl, 0.3% w/v Difco Bacto agar), were inoculated with a toothpick from over-night LB agar plates. Plates were wrapped in saran-wrap to prevent dehydration and incubated at 30°C for 24 h and then at room temperature for a week.

The same kind of plates were used to inoculate exponentially growing bacteria. 1 ml of exponentially growing culture in minimal medium (OD_{600nm} ~ 0.5) was centrifuged and resuspended at the same OD in 0,9% saline; 10 µl of this suspension were spotted in the middle of each plate. Plates were incubated for 24 h at 30°C and then for a week at room temperature. Motility was qualitatively assessed by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

E-2.2.2 Swimming in presence of metals

In order to asses the effect of metals on swimming motility, different metals were added to the plates at the following concentrations: $Al_2(SO_4)_3$ 0.4 mM, $NaAsO_2$ 0.25 mM, $CdCl_2$ 0.05 mM, $CuSO_4$ 0.05 mM, $K_2Cr_2O_7$ 0.06 mM, $NiCl_2$ 0.05 mM, $Pb(NO_3)_2$ 0.4 mM, K_2TeO_3 0.01 mM and 0.1 mM, $ZnCl_2$ 0.05 mM. Succinate was used as carbon source at the concentration of 5 mM. The swimming ring was measured every 24 hours for 72 h of incubation. The same inoculum described above (§ E-2.2.1) was used for this experiment.

E-2.2.3 Swimming chemotaxis assay

Swim plates for the qualitative analysis of chemotaxis were prepared in MSM containing 0.2% Difco Bacto Agar. Cells exponentially growing in 5mM succinate (OD_{600nm} ~ 0.5) were washed and re-suspended in saline solution (NaCl 9 g/l) at the same optycal density and a 10 μl drop of cellular suspension was spotted at the centre of the plate. Crystals of succinate, BA, 2-, 3- and 4-CBAs or biphenyl were added as chemoattractants on the right side of the plate while a saline solution (3x20 ul drops) was added on the left side of the plate as a negative control.

E-2.2.4 Plugs chemotaxis assay

Plugs contained 2% of low-melting-temperature agarose in chemotaxis buffer (40 mM potassium phosphate pH 7.0, 0.05 % glycerol, 10 mM EDTA) and the chemoactractant to be tested. 10 μl of the melted agarose mixture was placed on a microscope slide and a coverslip, supported by two plastic strips, was then placed on top to form a chamber. Cells were harvested in exponential phase (OD_{600} ~ 0.7), resuspended in chemotaxis buffer to the same OD_{600}, and flooded into the chamber to surround the agarose plug. Tryptone, succinate and glucose were assayed as chemoattractants and were provided at 1% (w/v) in plug assays. Control plugs contained 0.9 % saline instead of the attractant.

E-2.2.5 Quantitative chemotaxis assays

The quantitative analysis of chemotactic response was performed in modified capillary assays: cells exponentially growing in succinate were washed and resuspended in chemotaxis buffer (10mM Tris- HCl, pH 7.4). 100 μl aliquots

of a cellular suspension were placed in 200 μl pipette tips and a disposable 2-cm 25-gauge needle was attached to a 1-ml tuberculin syringe and was subsequently used as the chemotaxis capillary. Capillaries held 200 μl of the compound to be tested (1 and 10mM succinate; 0.01 and 0.1mM biphenyl; 1mM BA and CBAs) dissolved in a chemotaxis buffer or, in the case of biphenyl, in a chemotaxis buffer with 1.4% hexane. Control assays contained buffer only or buffer and 1.4% hexane for assays in which chemotaxis to biphenyl was tested. Cells were incubated with the capillary at room temperature for 90 min and then the content of the syringe was serially diluted in saline solution. Aliquots of appropriate dilutions were spot plated onto Luria–Bertani (LB) agar plates. Counts were performed after 24 h of incubation at 30°C.

To increase the riproducibility of this assay, a hot 25-gauge needle was used to make holes in two sealed lids for a 96-multiwells microtitre plate. 25-gauge needles attached to a 1-ml tuberculin syringe were used as capillary helding 200 μl of the compounds to be tested and were inserted through the holes of the two sealed lids. This platform was used to cover the bottom of the microtitre plate containing 100 μl of culture of each strain in parallel and alternate rows. After 90 minutes of incubation the lid was lifted and capillaries were rinsend twice in mutiwells microtitre plates cointaining 200 μl of sterile water; the contents of the capillaries were expelled into 1.5 ml eppendorf tubes containing 800 μl of saline solution and serial 10-fold diluitions were plated onto LB agar containing 50 ug/ml of ampicillin. Plates were incubated at 30°C and counts were performed after 24 hours.

E-2.2.6 Contrast phase microscopy

All strains were grown in MSM succinate 5 mM. At OD_{600nm} ~ 0.5 they were washed and resuspended at the same OD in chemotaxis buffer. A 10 µl drop was placed on a microscopy glass and covered with a coverslip glass. Video were recorded with Motic 3.0 software at 40X magnification.

E-2.2.7 Swarming

Swarm plates consisted of :

1) 0.5% (w/v) Difco Bacto agar with 8 g/l Difco nutrient broth (5 g /l bacto beef extract , 3 g/l bacto peptone extract) supplemented with 5 g/l dextrose.

2) modified M9 medium [20 mM NH_4Cl; 12 mM Na_2HPO_4; 22 mMKH2PO4; 8.6 mM NaCl; 1 mM $MgSO_4$; 1 mM $CaCl_2$ 2 H_2O; 11 mM dextrose; 0.5% casamino acids (Difco) solidified with Bacto-agar (Difco). Swarm plates were allowed to dry at room temperature under laminar flow for different period of time (Tremblay *et al.,* 2008).

These two kind of media were inoculated with a toothpick from both over-night LB agar plates and swim plates and incubated at 30°C for at least 24 h and then at room temperature for a week. The same plate were used to inoculate exponentially growing bacteria suspensions prepared as follow: 1 ml of exponentially growing culture in minimal medium (OD_{600nm} ~ 0.5) was centrifuged and resuspended at the same OD in 0,9% saline; 10 µl of this suspension were spotted on each plate. Plates were incubated for 48 h at 30°C and then for a week at room temperature.

3) Since swarming motility could be influenced by sugar composition of the extracellular matrix, swarm plates with different kind of sugar as carbon source

were used in this study; they consisted of: 0.01% K_2HPO_4, 0.01% NaCl, 0.02% $MgSO_4 7H_2O$, 0.04% KH_2PO_4, 0.4% Yeast Extract, 0.7% Difco Bacto Agar; glycerol or glucose or sucrose were added at the concetrations of 0.1 and 0.5% (w/v). The plates were allowed to dry at room temperature for 17-48 h. Bacterial strains were grown for 24 h in the same medium broth and with the different carbon sources and a spot of 2 μl was inoculated at the centre of each plate. They were wrapped in saran wrap to prevent dehydration and incubated at room temperature for 3-4 weeks.

E-2.2.8 Twitching

Twitch plates (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, 1% (w/v) Difco Bacto Agar) were stab inoculated with a sharp toothpick to the bottom of a petri dish form an over-night grown LB agar plate. After incubation for 48 h at 30°C, if bacteria are able to performe twitching motility, a hazy zone of growth at the interface between the agar and the polystyrene surface can be observed. The ability of bacteria to adhere on the polystyrene surface was then examined by removing the agar, washing unattached cells with water stream and staining with 1% (w/v) crystal violet solution prepared in 10% ethanol.

E-2.3. Evaluation of biofilm growth

KF707 wild type and mutant strains biofilms were grown on the Calgary Biofilm Device (CBD), commercially available as MBEC™ assay and produced by Innovotech™, Edmonton, Canada), as described by Ceri *et al*. (1999) and by the manifacturer. The MBEC™ high –throughput assay consists of two parts: the top

half of the device is a polystyrene lid with attached 96 identical pegs, which perfectly fit into a 96 well microtitre plate. CBD is a powerful method for biofilm growth and it can be used for multiple purposes. Here the use of the CBD for time course studies of biofilm formation is described. The procedure consists of 3 steps: (i) preparation of a standard inoculum of each bacterial strain; (ii) growth in the Calgary Biofilm device and (iii) determination of CFU/ml (counts of planktonic cells) and CFU/peg (counts of cells attached to each peg). The entire protocol is shown in Fig. E-2.3.1.

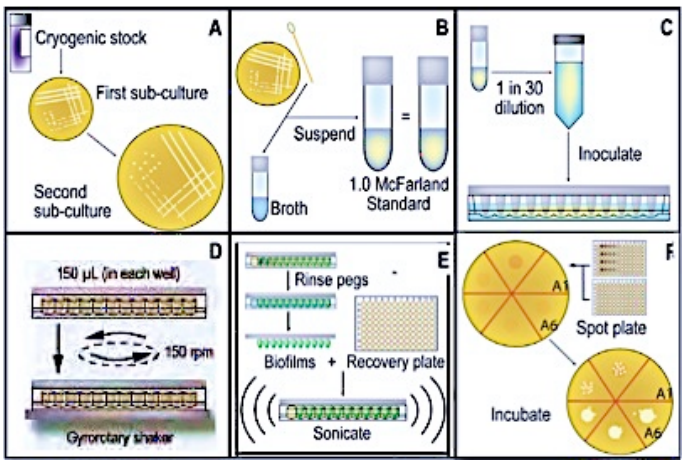


Fig. E-2.3.1: Biofilm cultivation on the CBD. The picture has been adapted from Harrison *et al. (2006)*.

From the corresponding cryogenic glycerol stock, a first subculture of each bacterial strain was streaked out on LB ampicillin 50 µg/ml agar plates and they were incubated at 30°C until growth was visible. From the first sub-culture, a second one, for each strain, was streaked out on LB agar plates without antibiotic

selection. Bacteria were grown over-night at the optimal temperature and these cultures were used to prepare the standard inoculm for the CBD. After growth, the biomass of all bacterial strains, was collected using a sterile cotton swab and resuspended into fresh LB medium to reach the optical density value matching with the 1.0 McFarland standard; 1:30 diluitions from each suspension was prepared in the same kind of medium. 150 µl of these standard inocula, each containing approximatively $1.0x10^7$ CFU/ml, were added to each well and the peg lid was then fitted on the top of the microtitre plate to assemble the CBD, which was subsequently placed on a gyrorotatory shaker at 100 rpm at 30°C and 95% of relative humidity.

E-2.3.1. Biofilm and planktonic growth curves

For biofilm growth curve analysis, CFU/peg were determined by viable cells counts. Sterile microtiter plates were set up with 200 µl of physiologycal saline solution in every well. Since the pegs are designed to be easily removed from the peg lid, at each time point 4 pegs for each strain, were broken with a sterile pair of pliers and rinsed twice in the saline solution in order to remove loosely adherent planktonic cells from the biofilms. Subsequently, they were transferred into fresh saline solution to which 0.1% (v/v) Tween-20 was added and the biofilms attached to the pegs were removed by sonication using an Aquasonic 250 HT ultrasonic cleaner (VWR International, Mississauga, ON, Canada) set at 60 Hz for 10 minutes. 20 µl of the resulting cellular suspensions were serially 10-fold diluited into 180 µl of fresh saline solution. Aliquotes of each diluition were plated onto well dry LB agar and the plates were incubated at 30°C for 24 hours.

As a control, viable cell counts of planktonic cells for each strain were performed: at each time point 20µl of bacterial suspension from the 4 wells, corresponding to those of the broken pegs, were serially 10-fold diluited in 180 µl of saline solution. Diluition were spotted onto LB agar, the plates were incubated at 30°C for 24 hours. For both biofilm and planktonic cells, counts were carried out in order to determine the CFU/peg and CFU/ml, respectively.

E-2.3.2 Confocal Laser Scanning microscopy (CLSM)

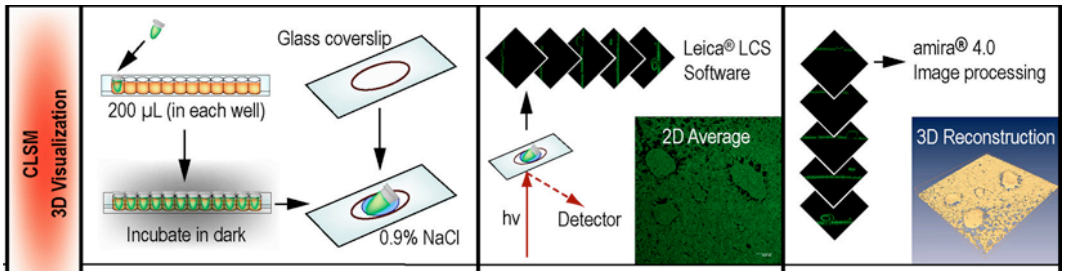


Fig. E-2.3.2.1: visualization of biofilm by CLSM

For microscopy techniques biofilms were fixed to the surface of the pegs of the MBEC™ Assay. The following protocol (Fig. E-2.3.2.1) was used to prepare samples for confocal laser scanning microscopy (CLSM). At each time point pegs were broken from the MBEC™-HTP device using a pair of flamed pliers, rinsed in 0.9% saline for 1 min to eliminate loosely-adherent planktonic bacteria. Pegs were fixed in 5% glutaraldhyde in phosphate buffered saline (pH 7.2) at room temperature for 1 hour and then rinsed again in 0.9% saline for 1 min. Pegs were subsequently stained in the dark for 5 minutes in 0.1% (w/v) acridine orange and examined at the confocal laser scanning microscope. Acridine orange is a membrane permeant nucleic acid stain, emitting fluorescence at λ~505-535 nm

when excited at λ = 488 nm. This fluorescent stain functions as a general indicator of the bacterial biomass attached to the solis durface of the pegs. Stained biofilms were placed on the top of a microscope glass and immersed in drops of water and visualized using a Leica IDR2 confocal microscope with a Leica TCS SP2 system. The excitation beam splitter was selected in order to detect acridine orange wavelenght range from the excitation light. 10X, 20X and a water immersion 63X magnifications objectives were used. The xyz mode scanning was chosen to record image stacks from the xy-sections in the z direction. Scans in series, 2D projections of z-stacks and 3D recontructions were performed using Leica Confocal Software (Leica Microsystem).

E-3 Results

E-3.1 Motile behaviour in *Pseudomonas pseudoalcaligenes* KF707 wild type and chemotactic mutant strains

Recent findings have shown that KF707 mutants in the *che*A1 gene are impaired in swimming motility, chemotaxis and biofilm development (Tremaroli *et al*., 20011). Annotation of KF707 genome revealed the presence of additional *che*A genes, both codifying for a histidine kinase. In order to assess the role of these genes in KF707 cellular functions, deleted mutants in *che*A2 and *che*A3 genes and the double mutants Δ*che*A2/*che*A1::Km and Δ*che*A3/*che*A1::Km were constructed. Mutant strains in *che*Z and *che*Y1 genes were also constructed and included in this study.

Swimming. The swimming ability of *P. pseudoalcaligenes* KF707 wild type and mutant strains, was assessed on swimming plates containing a low percentage (0.3% w/v) of Difco Bacto agar. Single colonies or 10 μl of exponentially growing bacteria were inoculate on this semi-solid plates, wrapped and incubated at 30°C. Cells able to swim formed a ring starting from the inoculation point as a result of flagellum-driven motility. The swimming behaviours of KF707 wild type and mutant strains are shown in Fig E-3.1.1.

Notably, a turbid ring zone was observed in KF707 wild type and all mutant strains, except in *che*A1::Km, Δ*che*A2/*che*A1::Km, Δ*che*A3/*che*A1::Km and *che*Y mutants. This suggests that among the three *che*A genes, only *che*A1 is involved in swimming motility since the *che*A1::Km mutant does not show swimming behaviour. This conclusion is also supported by the absence of swimming motility

in the double mutants. As expected, also the *che*Y mutant did not show swimming behaviour.

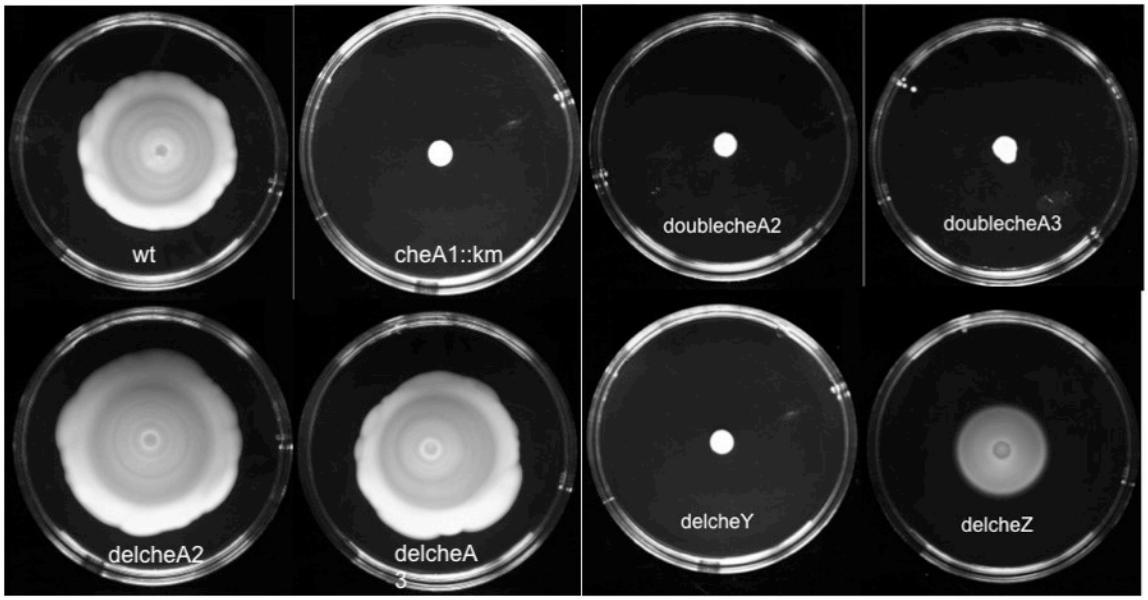


Fig. E-3.1.1: Swimming behaviour of Pseudomonas pseudoalaligenes KF707 wild type and mutant strains in tryptone swim plates.

Swimming in the presence of metals. *P. pseudoalcaligenes* KF707 strain is known for its ability to grow in the presence of toxic and heavy metals (Se<As<Ni<Cd<Al<Te) (Tremaroli *et al*., 2008). In order to asses the effect of metals on swimming motility, different metal cations and anions were added to the plates. By measuring the diameter of the turbid zone, it was observed that all the tested metals did not affect swimming motility; in addition wild type and mutants strains behaviours were similar to control plates with no metals added (Fig. E-3.1.2).

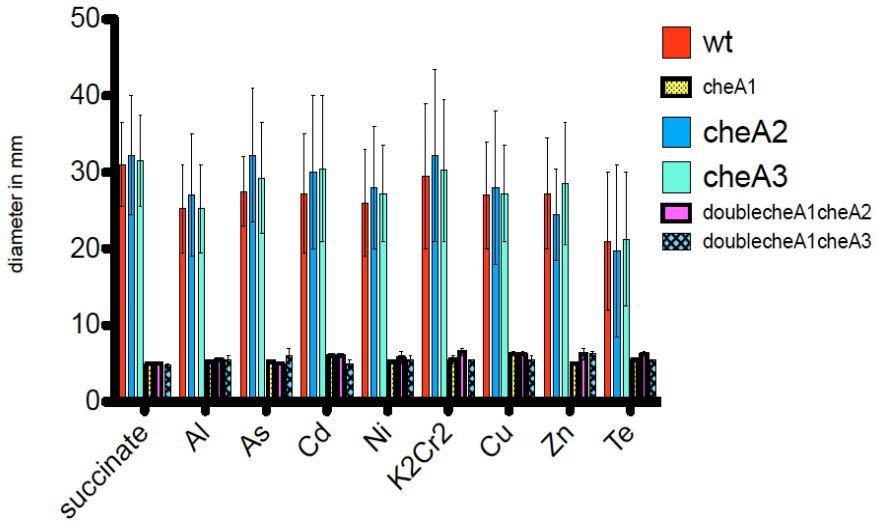


Fig. E-3.1.2.: Swimming motility in presence of toxic metals in KF707 wild type and mutant strains.

Qualitative and quantitative chemotaxis swimming assays

Chemotactic swimming behaviour was tested in the presence of chemo-attractants. Since KF707 strain is able to degrade xenobiotic compounds such as polychlorinated biphenyls (PCBs), both wild type and mutant strains were tested for their motility and chemotactic response to biphenyl, its chloroderivatives and chlorobenzoates. Generally, chemotaxis towards these compounds correlates with their use as carbon and energy sources. Minimal salt medium containing 0,2% (w/v) pure Difco Bacto Agar, was prepared. Crystals of the compounds to be tested (succinate, byphenil, benzoic acid and 2-,3-,4- chlorobenzoic acid) were added on the right side of the plate; saline solution was used as negative control and drops were added on the left, top and bottom side of the plate. 10 µl of exponentially growing cultures of the strains, all at the same value of optical density, were spotted onto the plates, which then were wrapped and left on the bench at room temperature. The chemotactic behaviours towards the different chemicals were observed daily for a period of seven days.

Fig E-3.1.3 shows that KF707 wild type and Δ*che*A2, Δ*che*A3 and *cheZ* mutants are motile and chemotactic towards succinate, byphenil and benzoic acid. With regard to *che*A1::Km, double mutants Δ*che*A2/*che*A1::Km and Δ*che*A3/*che*A1::Km and *che*Y mutant strains, they were not motile being incapable to move towards the chemo-attractans.

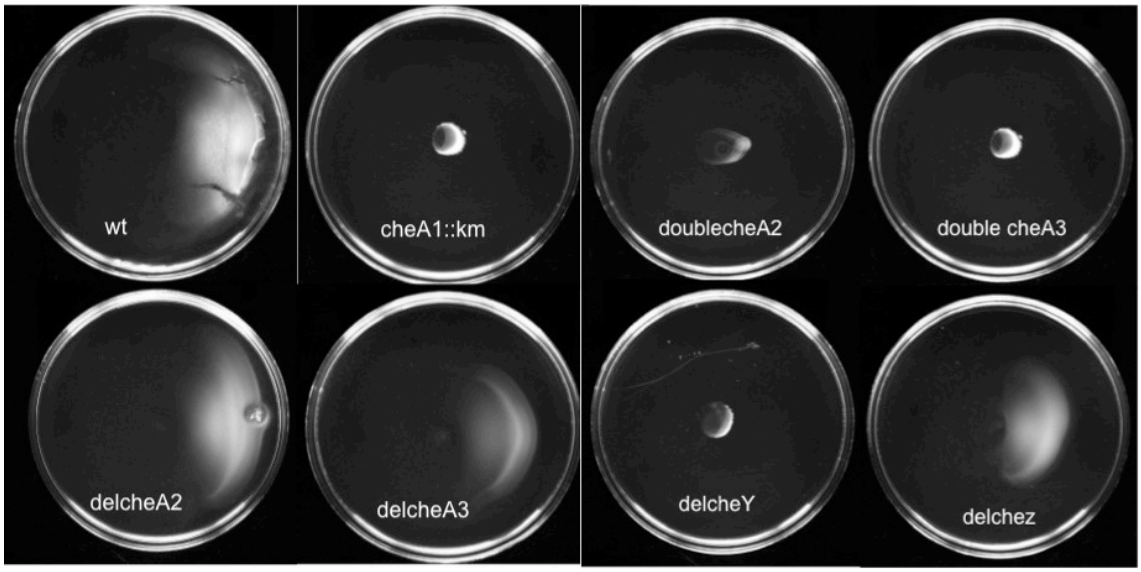


Fig. E-3.1.3.: Swimming motility in the presence chemo-attractants in KF707 wild type and mutant strains.

The quantitative analysis of chemotactic response toward these compounds was also performed in modified capillary assays (§ E-2.2.5.), but with no clear results, since the method is known for its limitation for reproducibility.

Contrast phase microscopy Chemotaxis towards tryptone, succinate and glucose was also investigated, using plugs containing 2% (w/v) of low-melting-temperature agarose in chemotaxis buffer. 10 µl of the melted agarose mixture were placed on a microscope slide and a coverslip, supported by two plastic strips, was then placed

on top to form a chamber. Exponentially growing cells were flooded in the chamber and bacterial behaviour was observed at the contrast phase microscope. It was observed that motile strains (KF707 wild type and Δ*che*A2, Δ*che*A3 and *che*Z mutants) swam towards the agarose plugs, while non motile strains (*che*A1::Km, double mutants Δ*che*A2/*che*A1::Km and Δ*che*A3/*che*A1::Km and *che*Y mutant) were simply transported by the fluid flow (data not shown).

Further, KF707 wild type and mutant strains behaviours in the absence of stimuli were observed through the use of a contrast phase microscope. Interstingly, all motile strains swam more or less similarly, except the Δ*che*A2 mutant which appeared to swim faster. It is noteworthy the difference between *che*A1::Km and *che*Y mutant strains: they both are not motile, but while the first did not swim because it was not able to move, the latter did not swim in any direction because it was continuosly tumbling at a very high speed. The double mutants Δ*che*A2/*che*A1::Km and Δ*che*A3/*che*A1::Km showed the same behaviour of *che*Y and *che*A1::Km, respectively (data not shown).

Swarming In the presence of an agar concentration of 0.5-0.7 % (w/v) swarming motility can be observed. This kind of motility is different from swimming, because of the irregular branching that appears at the periphery of the colony. It has been reported that swarming motility and biofilm development in *P. aeruginosa* are not only nutritionally dependent, but they are also both affected by quorum sensing (QS) (Shrout *et al*. 2006). Previous results (Tremaroli *et al*., 2011) have shown that KF707 wild tipe and *cheA1::Km* mutant strains did not show swarming motility in the tested conditions (the number **1** medium was used, § E-2.2.7). In the present

study, swarming experiments were repeated and no swarming motility was shown in all the strains tested. Consequently, swarming motility was verified in various media and under different conditions such as variable agar concentration, temperature and humidity.

Modified M9 medium (§ B-1) was used; swarm plates were allowed to dry at room temperature under UV-light and sterile laminar flow cabinet for different period of time. Single colonies were picked onto the plates, which were wrapped and incubated over-night at 30°C and then at room temperature. All strains did not show swarming motility, even though the Δ*che*A2 mutant showed an attempt of movement in 0.5 % (w/v) agar.

As previosly reported (Tambalo *et al*., 2010) different factors are involved in swarming motility such as the carbon source, the growth phase of the cells, the production of biosurfactants. Swarm plates with different kind of sugar as carbon source (glycerol or glucose or sucrose were added at the concetrations of 0.1 and 0.5% (w/v)) were used in this study. Bacterial strains were grown for 24 h in the same medium broth along with different carbon sources and a spot of 2 μl was inoculated at the centre of each plate. They were wrapped in saran-wrap to prevent dehydration and incubated at room temperature for 3-4 weeks.

The results indicated that KF707 wild type was not able to swarm as well as Δ*che*A2 and the double mutant strains under all tested conditions. *che*A1::Km showed the formation of pink/orange branches patterns, even though it did not expand on the plate. The Δ*che*A3 mutant showed swarming motility with the formation of branches patterns and production of the pink/orange pigment in all the media containing the variable carbon source; interestingly, the shape of the

branches patterns was different depending on the carbon source (Fig. E-3.1.4)

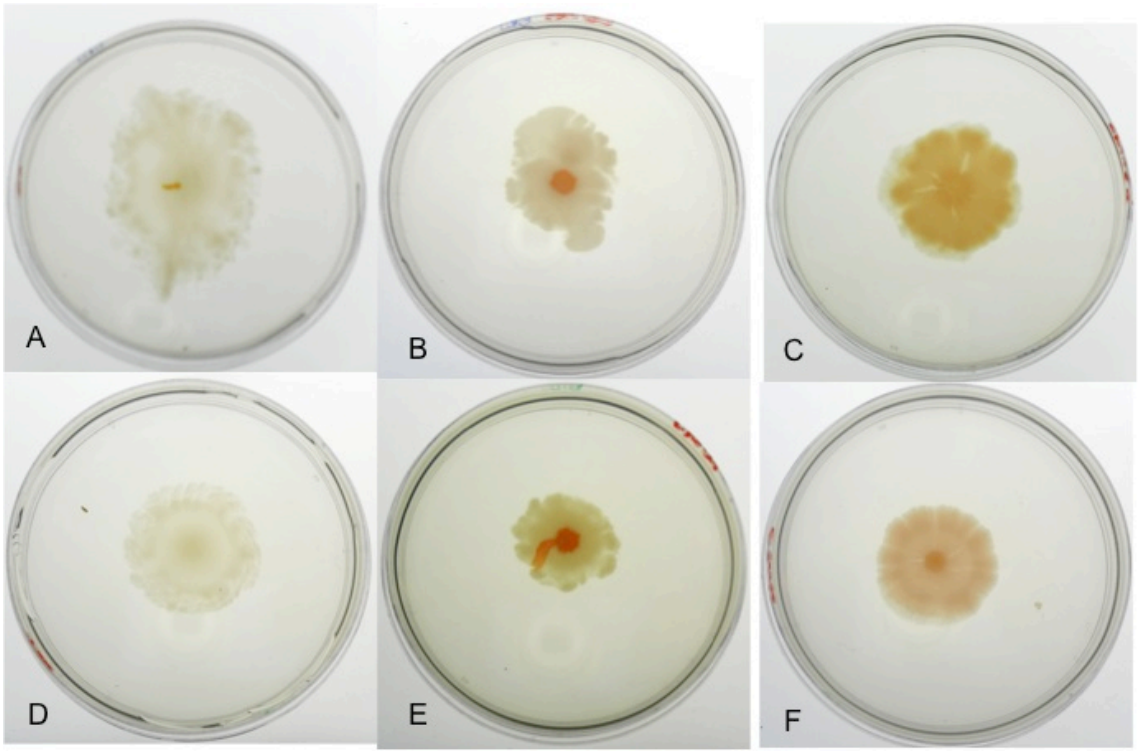


Fig. E-3.1.4: Swarming motility in KF707 wild type (A and D) and mutant strains *che*A1::*km* (B and E) and Δ*che*A3 (C and F) in the presence of different carbon source (A-B-C, glucose; D-E-F, sucrose).

Twitching Twitching motility is a form of translocation on solid surfaces which is dependent on pili-assisted motility (Henrichsen 1972; 1983) and it occurs at the interstitial surface between the agar and the polystyrene, when cells are inoculated through a thin 1 % agar layer to the bottom of a Petri dish. However, phenotype was not displayed by *P. pseudoalcaligenes* KF707 wild type and mutant strains under the conditions tested.

E-3.2. Role of *che*A genes in *Pseudomonas pseudoalcaligenes* KF707 biofilm formation and development.

The formation of biofilm in *Pseudomonas pseudoalcaligenes* KF707 wild type and mutant strains was investigated using the Calgary Biofilm device (CBD). The CBD is a batch system that allows the formation of 96 statistically equivalent biofilms (Ceri *et al.,*1999). This approach reproduces the bacterial lifestyle, in which free swimming (planktonic) bacteria attach to abiotic surface and produce an organized multicellular structure (biofilm) in response to environmental signals. Cells forming a biofilm on the CBD are probably released and colonize the surrounding medium, thus closing the planktonic-biofilm bacterial life cycle. Acridine orange (AO) staining and confocal laser scanning microscopy (CLSM) were used to visualize the evolution of biofilms organization.

Since it was reported that KF707 mutant in the *che*A1 gene is able to attach to the abiotic surface of the CBD but not able to form a mature biofilm (Tremaroli *et al*., 2011), we thought to define the role of the other two addictional *cheA* genes (*che*A2 and *che*A3) in *P. pseudoalcaligenes* KF707 biofilm formation. The time course of biofilm development in the *wild type, cheA1::Km,* Δ*che*A2 and Δ*che*A3 mutant strains were investigated, both at the confocal microscope (Fig. 7) and by the viable counts of cells attacched at the pegs of the CBD (Fig. 8 A).

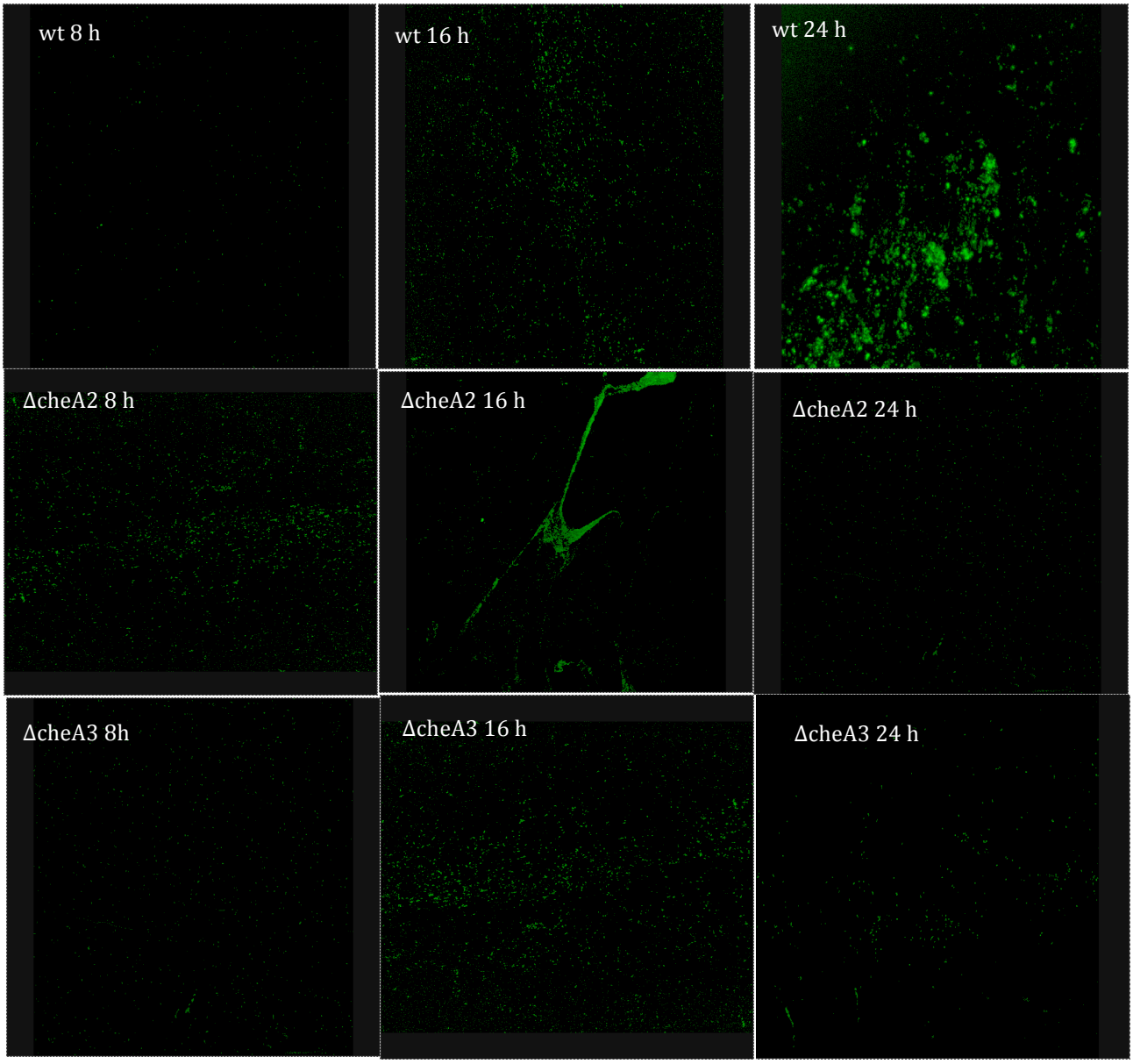
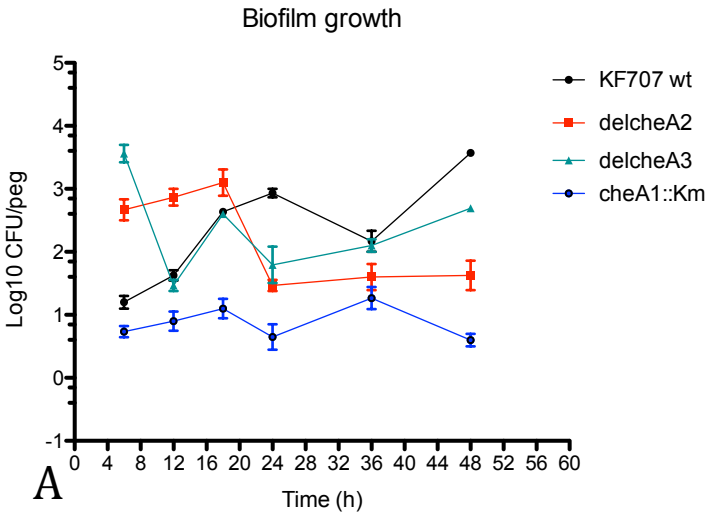


Fig. E-3.2.1.: Time course of biofilm formation of *P. pseudoalcaligenes* KF707 and mutant strains in LB medium. Images display the 2D averages of z-stacks.

Figure E-3.2.1. displays images of the time course of *P. pseudoalcaligenes* KF707, Δ*che*A2 and Δ*che*A3 mutant strains biofilms in LB medium at 8, 16 and 24 h of growth. At 8 h of biofilm development, the number of KF707 wild type cells attached to the surface was lower than the Δ*che*A2 mutant strain. At 8 h of growth, the latter showed a number of attached cells similar to that showed by the wild type

strain at 16 h. At 16 h, wild type and Δ*che*A3 strains showed a similar organization; notably, the Δ*che*A2 reached the maximum of cells density at 16 h, which showed a strong decrement between 16 and 24 h of growth. Only the wild type strain, showed the formation of a mature biofilm after 24 h of growth. Indeed the Confocal-Laser-Scanning-Microscopy (CLSM) image (Fig. E-3.2.1) shows a complex developed biofilm. The EPS matrix , composed of short oligonucleotides to which AO stain is bound, proteins and polysaccharides, can be observed as a diffuse fluorescence sheltering the biofilm cells and linking adjacent cell.



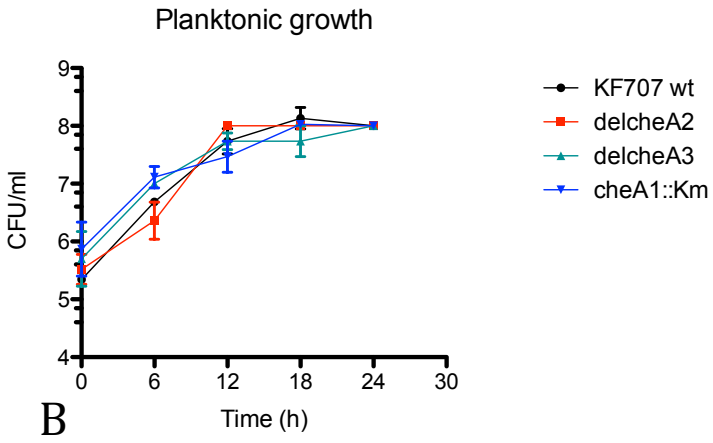


Fig. E-3.2.2.: Growth curves of biofilms in LB medium (8 A). Viable cell counts were carried out to determine CFU per peg during biofilm development. The curves show the CFU per peg as a function of time for KF707 wild type, Δ*che*A2 and Δ*che*A3 mutand strains The values presented and standard deviations are the means of at least three independent experiments, each performed in quadruplicate. Growth curves of planktonic cells in LB medium are reported as a control (8 B).

Figure E-3.2.2.A shows biofilm growth curves of the Δ*che*A2 and Δ*che*A3 mutant strains as compared to the wild type strain. In this experiment KF707 wild type strain showed a low attachment at 8 h and reached the maximum biofilm cell density at 24 h of growth. Δ*che*A2 mutant strain showed a high attachment in the early stage, but the number of the cell attached at the pegs strongly decreased between 16 and 24 h of growth. Δ*che*A3 mutant biofilm growth curve showed a dramatic decrease in biofilm cell density after the initial attachment, but also a consistent recovery between 24 and 48 hours. *che*A1::Km, as reported by Tremaroli *et al*, showed an initial attachment but no further development of the biofilm. CLSM visualizations showed that the *cheA*1*::*Km mutant was indeed capable of early attachment

Planktonic growth of the wild type and mutants strains was also monitored, indicating that planktonic growth was similar in both wild type and mutant strains (Fig. E-3.2.2).

From all these observations, we can conclude that KF707 wild type strain is able to grow as a biofilm, with the formation of a mature structure after 24 h by following a gradual increasing of the number of the cells attached to the abiotic surface of the CBD and a maintenance of the structure for a prolonged period (50 h). Conversely, the Δ*che*A2 mutant strain, is able to attach to the pegs reaching the maximum of density within 16 h of growth; subsequently, it shows a decrement of biofilm

attached cell. Δ*che*A3, was able to attach strongly during the first few hours of biofilm formation, maintaining the structure stable for a prolonged time period.

E-4 Discussion

KF707 is an environmental organism able to use xenobiotic and aromatic compounds, such as PCBs, as sole carbon source and it is also chemically attracted to them. Chemotaxis is considered an important and necessary trait for degrading bacteria, since it enhances the ability of motile bacteria to locate and degrade low concentrations of organic compounds; for this reason, it may also direct the movement of motile bacteria to toxic, but metabolizable, compounds present in contaminated environments. There is evidence that chemotaxis can not only enhance biodegradation but also promotes the formation of microbial consortia (Pedit *et al*., 2003; Wu *et al*., 2003), presumably by bringing cells into close contact with degradable substrates.

In this chapter, KF707 wild type and mutant strains were tested for their capacity to sense and degrade compounds such as byphenil, PCBs, benzoic acid and chlorobenzoic acids. Wild type and mutant motile strains were able to move towards these compounds, while non motile mutants, even though they were not able to move, they were able to grow. In addition KF707 motile and motile strains showed the ability to grow using xenobiotics as carbon source also in presence of toxic metal oxyanions and cations; moreover, regarding motile strains, they were not affected in swimming behaviour by the presence of metals. All these feature make KF707 an important strain for bioremedation application as t has been shown that the efficiency of microbial remediation procedures depens not only on the

metabolic abilities and chemotactic behaviour but also the capacity to tolerate the intermediates formed during the degradation process.

The role of chemotactic genes, with particular enphasis on *che*A2 and *che*A3, was invastigated. Motile and chemotactic behaviour of *Pseudomonas pseudoalcalignes* KF707 wild type and mutant strains *che*A1::Km, Δ*che*A2, Δ*che*A3, Δ*che*A2*che*A1::Km, Δ*che*A3*che*A1::Km, *che*Y and *che*Z were analyzed. Swimming motility assay results, showed that the mutans *che*A1::Km, Δ*che*A2*che*A1::Km, Δ*che*A3*che*A1::Km, *che*Y are impaired in motility, remarking the fact that only the *che*A1 gene is responsible for swimming of KF707. Further support to this conclusion derives from the Δ*che*A2, Δ*che*A3 and double mutants phenotypes.

The ability of KF707 and chemotactic mutant strains in adhering to a solid surface and to form biofilm were also assessed. Interestingly the Δ*cheA*3 mutant indicated swarming motility only under specific nutrient conditions. It was also observed that the mutant was able to form a branches pattern and produce a pink/orange pigment. The present findings on swimming motility, chemotaxis, swarming and biofilm formation, taken together, tend to suggest that in *Pseudomonas pseudoalcaligenes* KF707 strain, multiple factors are involved in this network, as they might play different roles depending on the environmental conditions. Regarding to the hypothetical implication of KF707 *che*A genes in swarming and biofilm formation,they could be involved in regulating the choice between a planktonic and/or sessil modes of growth. When KF707 *che*A1 gene is mutated the bacterium is not able to swim and form a mature biofilm; moreover it was observed the formation of branches patterns in swarm plates. On the other hand, the Δ*che*A2

mutant is able to swim but doesn't show swarming; in addition it is able to form a developed biofilm, but the density of sessile cells was observed to decrease after 16 h of growth. With regard to the Δ*che*A3 mutant it showed both swimming and swarming motility; it was able to grow as a biofilm, with a high attachment in the first stage of growth and without a further development. All these data can be interpreted to show that:

1. only the *che*A1 gene is involved in swimming motility;
2. both *che*A1 and *che*A2 are important for adhesion and biofilm development, since in their absence an early attachment without development is observed; notably, the attachement seems to be stronger and lasting for a longer time in Δ*che*A2 mutant;
3. *che*A3 gene seems to inhibit swarming motility, as its deletion restore swarming behaviour.

Depending on the above reported conclusions, it seems that *che*A2 gene plays an important role on swarming motility; indeed, when it is deleted, cells do not show swarming; in addiction to this, the fact that the mutant in *che*A1 gene showed branches patterns that do not expand, could be related to the positive regulation of CheA2 (which stimulates swarming in certain conditions) and the negative regulation by CheA3 that does not allow branches to expand.

Finally, it can be assumed that KF707 wild type strain doesn't show swarming motility because of the negative regulation exerted by CheA3 which is stronger than the positive regulation due to CheA2.

CHAPTER F

Searching for a Quorum Sensing (QS) system in *Pseudomonas pseudoalcaligenes* KF707

F-1 Introduction

F-1.1 Bacterial Quorum Sensing: general features

In natural habitats, bacteria exist as members of communities and communication whitin these communities modulates the activities of individual cells in the entire population, thus imparting a social behaviour. Bacteria synchronously control gene expression in response to changes in cell density, switching between two distinct programs: one that is favored at low-cell-density (LCD) for individual, asocial behaviors, and another that is favored at high-cell-density (HCD) for social, group behaviours (Parsek and Greenberg, 2005; Waters and Bassler, 2005; Williams *et al*., 2007; Novick and Geisinger, 2008). This is viewed as an evolutionary adaptation to survive in changing environment and to benefit the population as a whole.

Cell-to-cell communication is possible thanks to the production, release and detection of extracellular chemicals and is referred to as 'quorum sensing' (QS). The concentration of these signal molecules depends both on biotic and abiotic factors such as the amount of signal producers, diffusion and presence of inactivating factors.

During bacterial growth, different molecules from the cellular metabolic turnover diffuse or are secreted in the culture media and any of these may be a potential QS signal. Hence it is important to define the features that distinguish a probable QS

signal molecule from other metabolites (Torres *et al*., 2007). To act as a QS signal a molecule requires:

- accumulation of a critical threshold concentration in the extracellular milieu during a specific growth stage or under certain physiological conditions or in response to particular environmental changes;
- recognition by a specific cell surface or cytoplasmic receptor;
- ability to induce a concerted cellular response that extends beyond the physiological adaptations required to metabolize the molecule (Winzer *et al*., 2002);
- positive, autoinductive feedback loop to amplify QS signal molecule production, therefore the term "autoinducer" is sometimes used to describe the QS signal molecule.

To date QS circuits have been found in many different bacterial species and shown to control the expression of a wide spectrum of functions, including bioluminescence, plasmid conjugative transfer, synthesis of antibiotics and extracellular hydrolytic enzymes, motility and production of virulence factors (Laue *et al*., 2000).

F-1.2 Bacterial QS systems

The principles of QS mediated gene expression is common among bacteria, even though the molecular mechanisms and signal molecules involved are different (Camara *et al*., 2002).

F-1.2.1 *Vibrio fischeri* luxI/luxR system: the QS paradigm in gram negative Bacteria

The first observed QS system was that of the marine *Vibrio fischeri* and is considered a model of the basic mechanism of QS. This bacterium lives in symbiotic association with some eukaryotic hosts: they supply *V. fischeri* with a safe and nutrients rich environment while the bacterium provides the host with light (Ruby et al., 1992; Ruby, 1996; Visick and McFall-Ngai, 2000) which is used for specific purposes. For example, in the squid *Euprymna scolopes–V. fischeri* association, the squid has evolved an antipredation strategy; the fish, *Monocentris japonicus* uses the light produced by *V. fischeri* to attract a mate (Nelson and Hastings, 1979). Although the purposes are different, the regulation of light production by *V. fischeri* in the specialized light organs is identical: as the *V. fischeri* culture grows, it produces via the LuxI synthase the autoinducer N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) which diffuses in the extracellular environment and trapped inside the light organ. At a critical threshold concentration, HSL signals form complexes more efficiently with LuxR. The latter functions both to bind the autoinducer (sensor) and to activate transcription (response regulator) of the *lux*ICDABE operon (Engebrech, 1983; Hanzelka and Greenberg, 1995; Schaefer *et al*., 1996; Stevens *et al*., 1999), which encodes the luciferase enzymes required for light production. Interaction of LuxR with the autoinducer unmasks the LuxR DNA binding domain, allowing LuxR to bind the *lux*ICDABE promoter (at the lux-box site) and activate transcription (Hanzelka and Greenberg, 1995). This action results in an exponential increase in both autoinducer production and light emission. The LuxR-AHL complex also acts as a negative

regulator on *lux*R expression, thus establishing a negative feedback loop, i. e. a compensatory mechanism that decreases *lux*ICDABE expression in response to the positive feedback circuit.

F-1.2.2 *lux*-like QS systems in Gram⁻ Bacteria

Members of the LuxI/LuxR-type proteins families are widely distributed among a large number of Gram negative bacteria (Manefield and Turner, 2002). Some examples, associated to their functions, are listed in the Table F-1.2.2.1.

Table F-1.2.2.1: *lux*-like QS systems

Organism	LuxI/LuxR homologues	Signal molecules	Phenotype
Aeromonas hydrophila	AhyI/AhyR	C4-HSL, C6-HSL	Biofilms, exoproteases, virulence
Aeromonas salmonicida	AsaI/AsaR	C4-HSL, C6-HSL	Exoproteases
Agrobacterium tumefaciens	TraI/TraR	3-Oxo-C8-HSL	Plasmid conjugation
Burkholderia cenocepacia	CepI/CepR	C6-HSL, C8-HSL	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence
Chromobacterium violaceum	CviI/CviR	C6-HSL	Exoenzymes, cyanide, pigment
Erwinia carotovora	ExpI/ExpR CarI/CarR	3-Oxo-C6-HSL	Carbapenem, exoenzymes, virulence
Pantoea (Erwinia) stewartii	EsaI/EsaR	3-Oxo-C6-HSL	Exopolysaccharide
Pseudomonas aeruginosa	LasI/LasR RhlI/RhlR	C4-HSL; C6-HSL, 3-oxo-C12-HSL	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, secondary metabolites, 4-quinolone signalling, virulence
Pseudomonas aureofaciens	PhzI/PhzR	C6-HSL	Phenazines, protease, colony morphology, aggregation, root colonization
Pseudomonas chlororaphis	PcoI/PcoR	C6-HSL	Phenazine-1-carboxamide
Pseudomonas putida	PpuI/PpuR	3-Oxo-C10-HSL, 3-oxo-C12-HSL	Biofilm development
Rhizobium leguminosarum bv. viciae	RhiI/RhiR CinI/CinR	C14 : 1-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL	Root nodulation/symbiosis, plasmid transfer, growth inhibition; stationary phase adaptation
Rhodobacter sphaeroides	CerI/CerR	7-cis-C14-HSL	Aggregation

Table F-1.2.2.1: *continued*			
Serratia liquefaciens MG1	SwrI/?	C4-HSL, C6-HSL	Swarming motility, exoprotease, biofilm development, biosurfactant
Yersinia enterocolitica	YenI/YenR	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	Swimming and swarming motility
Yersinia pseudotuberculosis	YpsI/YpsR YtbI/YtbR	C6-HSL, 3-oxo-C6-HSL, C8-HSL	Motility, aggregation

The figure F-1.2.2.1 shows *Pseudomonas aeruginosa* QS systems.

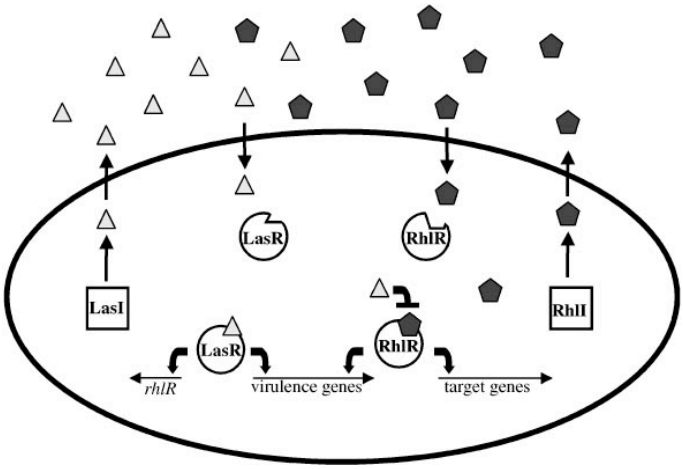


Fig. F-1.2.2.1: QS system in *Pseudomonas aeruginosa.* Taken and adapted from Miller and Blasser, (2001). *Pseudomonas aeruginosa* owns two LuxI/LuxR-like QS systems. The LasI protein produces N-(3-oxo-dodecanoyl)-homoserine lactone (triangles), and the RhlI protein synthesizes N-(butryl)-homoserine lactone (pentagons). The LasR protein binds the N-(3-oxo-dodecanoyl)-homoserine lactone when this signal molecule reaches a critical threshold level. The LasR-autoinducer complex induces the transcription of several genes and also rhlR is expressed. RhlR binds the N-(butryl)-homoserine lactone and activates the transcription of different subsets of genes. The LasI autoinducer interferes with binding of the RhlI-autoinducer to RhlR, guaranteing a hierarchic order of the QS systems. The Pseudomonas quinolone signal (PQS) is an additional regulatory link between the Las and Rhl quorum sensing circuits (not shown in the figure). The expression of PQS requires LasR, and PQS in turn induces transcription of *rhl*I.

These systems are used for intraspecies communication as the structures of LuxR proteins suggest that they possess specific binding pockets that allow each LuxR to bind and be activated only by a cognate signal (Vannini *et al*., 2002; Zhang *et al*., 2002).

F-1.2.3 Structure and function of the LuxR proteins family

The response regulators consist of two domains: the amino-terminal domain is involved in binding to the HSL autoinducer, and the carboxyl-terminal helix-turn-helix motif domain is required for DNA binding and transcriptional activation (Shadel *et al*., 1990; Slock *et al*., 1990; Choi and Greenberg, 1991; 1992). In each case, the LuxR type proteins bind a similar DNA promoter element termed "lux box." The lux box consists of a 20-base-pair palindromic DNA sequence situated at approximately -40 from the start site of transcription of a given target gene (Fuqua *et al*., 1994). Moreover, the amino-terminal domain inhibits DNA binding by the carboxyl-terminal domain and this inhibitory function is eliminated when LuxR binds the autoinducer. Although the LuxIs produce a highly related family of molecules, in general, the HSL autoinducers are not capable of cross-stimulation of a noncognate system.

However many response regulators can be activated by related compounds, which explains their utility as components of HSL biosensors based on LuxR protein and the promoter of a target gene fused to a reporter such as *lac*Z (Bainton *et al*., 1992; Winson *et al*., 1998). Furthermore, in several cases autoinducer analogues inhibit cognate autoinducer binding to LuxR and, therefore, inhibit target gene activation (Gray *et al*., 1994; Schaefer *et al*., 1996; Zhu *et al*., 1998).

Not all the members of the LuxR family function as transcriptional activators but like repressors. As more bacterial genomes are sequenced, the presence of additional LuxR homologues has become evident. Many of these do not have an associated synthase on their genome and are therefore referred to as *orphan* LuxR

homologues (Fuqua, 2006). Their predicted protein sequences have the amino-terminal-binding domain and carboxy-terminal domain, typical of the LuxR family of proteins. Unlike synthase-associated LuxR proteins, *orphan* LuxR homologues do not directly control the synthesis of autoinducers, but they can interact with non-cognate ones to expand the existing regulatory network of the bacterium even responding to signals produced by nearby cells belonging to different species.

F-1.3 Structural Diversity in QS Signal Molecules

Several chemically distinct classes of QS signal molecules have been identified: (i) N-acyl homoserine lactones (AHLs) produced by many Gram-negative proteobacteria and composed of a homoserine lactone ring carrying C4–C18 acyl chains; (ii) g-butyro-lactones produced by streptomycetes (Yim *et al*., 2006); (iii) 4,5-dihydroxy-2,3-pentandione (DPD) derivatives referred to as AI-2 synthesized by different Gram-negative and Gram-positive bacteria and thought to function as interspecies signal molecule (Winzer *et al*., 2002; Winzer & Williams 2003 ; Vendeville *et al*., 2005); (iv) linear, cyclic, or modified peptides (autoinducing peptides, AIPs) used by Gram-positives (Yim *et al*., 2006); (v) diffusible signal factors (DSF) produced by pathogens such as *Xanthomonas campestris* (Torres *et al*, 2007), *Xylella fastidiosa* (Chatterjee *et al*., 2008), and members of the *Burkholderia cepacia spp* (Boon *et al*., 2008; Deng *et al*., 2010), and (vi) the *Pseudomonas aeruginosa* quinolone signal (PQS) 2-heptyl-3-hydroxy-4-quinolone (Pesci *et al*., 1999).

F-1.4 Synthesis and detection of AHLs signal molecules

Presently, the most studied QS molecules belong to the AHL family produced by various Gram-negative bacterial genera. All AHLs reported to-date are characterized by a homoserine lactone ring unsubstituted in the b- and g- positions which is N-acylated with a fatty acyl group at the a-position 1. The fatty acyl chain is linked to the lactonized homoserine ring via an amide bond. The compound is synthesized from two substrates, S -adenosyl-L–methionine (SAM), from amino acid biosynthesis, and an acylated-acyl carrier protein (acyl-ACP), from lipid metabolism (More *et al*., 1996; Schaefer *et al.,* 1996). LuxI and its homologous proteins synthesize AHL by catalyzing the transfer of the acyl group from the acyl-ACP to the amine of SAM. This leads to the release of ACP, followed by homoserine lactone ring formation and dissociation of the AHL from the synthase (Pappas *et al*., 2004).

The acyl chain has various lengths, saturation levels and oxidation states. In addition, some AHLs also have unsaturation with Z stereochemistry in the 7 position in a chain of 14 carbons 4 (Pesci *et al*., 1999; Williams *et al*., 2007). Stereochemistry at the a-centre of the homoserine lactone (HSL) ring has been established to be L for the *V. fischeri* autoinducer and by analogy all other natural AHLs have the same configuration. In some cases D-isomers have been synthesised and shown to lack activity (Bainton *et al*., 1992; Ikeda *et al*., 2001).

To date, the large number of AHL QS systems have been identified *via* the use of bacterial biosensors that are able to detect the presence of AHLs. These biosensors do not produce AHLs and contain a functional LuxR-family protein

cloned together with a cognate target promoter (usually the promoter of the cognate luxI synthase), which positively regulates the transcription of a reporter gene. As many biosensors detect a limited range of AHLs, it is crucial, when testing a bacterium for AHL production, to use several biosensors, each responding to AHLs with different structural features (McClean *et al*., 1997; Shaw *et al*., 1997; Camara *et al.,* 1998; Ravn *et al.,* 2001). Separation by TLC coupled with detection by AHL-biosensors gives a rapid and direct visual index of the AHL(s) produced by the tester bacteria. AHLs cannot be clearly identified using TLC and their structures are unequivocally determined by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) (Schaefer *et al*., 2000). MS has been shown as the most valuable tool for the identification and characterisation of AHLs with detection levels in the picomole range.

F-1.5 Role of QS in swarming motility and biofilm development

Biofilms represent the natural bacterial lifestyle and are thought to be ubiquitous in nature (Costerton *et al*., 2001). It has been reported that biofilm development proceeds through a temporal series of stages (Palmer and White, 1997). In the initial phase, bacteria attach to a surface, aggregate and then proliferate to form microcolonies. These microcolonies are hydrated structures in which bacterial cells are enveloped in a matrix of selfproduced slime, referred to as exopolysaccharide (EPS). When the nutrients availability becomes limiting due to increased diffusion distances, growth will decrease and biofilm development will reach a steady-state. Mature biofilms typically consist of “mushrooms” of cells separated by channels which allow a convective flow to transport nutrients to

interior parts of the biofilm and remove waste products. The involvement of QS in the regulation of biofilm formation was originally reported in *P. aeruginosa* (Davies *et al*., 1998) architecture. A role for AHL-mediated quorum sensing in biofilm formation has also been demonstrated in *B. cepacia* (Huber *et al*., 2001; 2002) *Aeromonas hydrophila* (Lynch *et al.,* 2002) *Pseudomonas putida* (Steidle et al., 2001). Quorum sensing systems are usually involved in biosurfactant production and swarming motility, allowing bacteria to disseminate and colonize new solid surfaces. It was reported that swarming behavior requires the integration of chemical and physical signals, which leads to the physiological and morphological differentiation of the bacteria into swarmer cells. Moreover, it's clear that many of these regulatory pathways that lead to swarming behavior also affect the formation of biofilms. *Pseudomonas pseudoalcaligenes* KF707 is able to grow in the form of a biofilm (Tremaroli *et al*., 2011); we have also observed (this Thesis work) that the *che*A3 gene, codifying for a histidine kinase signal transduction, is possibly involved in a regulatory pathway which allows swarming motility (§ E). Owing to this, we thought necessary to search for the presence of a QS system in KF707 cells as QS could also be linked to chemosensory pathways driving KF707 cellular adhesion to solid surfaces.

F-2 Materials and Methods

F-2.1 Bacterial strains and growth conditions

Pseudomonas pseudoalcaligenes KF707 was grown on either reach (LB, LB supplemented with Mops 50 mM and LB without NaCl) and minimal media (SA and MSM with biphenyl). Biosensor strains (*E. coli* pSB401, *Agrobacterium*

tumefaciens NTL4 pZLR4, *Agrobacetrium tumefaciens* WCF47 (pCF218)(pCF372) and *Chromobacterium violaceum* CV026), were grown on LB medium with the appropriate antibiotics. All the biosensor strains used in this study do not produce AHLs and contain a functional LuxR-family protein cloned together with a cognate target promoter which positively regulates the transcription of a reporter gene
The composition of media and strains genetic features and resistance phenotypes are described in the "General Materials and Methods common to Chapters D, E, F".

F-2.2 T-streak bioassay

Biosensor strains *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 pZLR4 were used in T-streak bioassay. *Pseudomonas pseudoalcaligenes* KF707 was streaked and grown at the optimal temperature on LB agar close to the biosensors to form a 'T': if AHLs are produced a phenotypic change (violacein and 5,5'-dibromo-4,4'-dichloro-indigo, respectively) is observed as a gradient with most response observed at the meeting point of the two strains.

F-2.3 Extraction of N-acyl-homoserine lactones

The organic extraction of AHLs increases many-fold the probability of detection by the biosensors. *Pseudomonas pseudoalcaligenes* KF707 was streaked on LB plates; a single colony was inoculated in LB and grown o/n at 30°C at 150 rpm. 1% v/v subcultures in 750 ml of both fresh rich (LB, LB supplemented with Mops 50mM and LB without NaCl) and minimal (SA and MSM with biphenyl) media in a 3 L Erlenmeyer flasks, were grown until the exponential, late exponential and stationary phases. At each time point, 250 ml of each culture were

centrifuged at 4°C, 6000 rpm for 30 minutes. The supernatants were transferred in 1 L Erlenmeyer flasks, 1 volume of acidified ethyl acetate (0.1 ml glacial acetic acid in 1 L ethyl acetate) was added and the supernatans were extracted twice by mixing with a magnetic stirrer for 4 hours. Mixed solutions were allowed to separate by centrigugation at 4°C, at 6000 rpm for 30 minutes; the organic phases from each culture were collected in clean glass beckers and the combined extracts were dried down under N_2 flow. Extracts were resuspended in 100 µl of acidified ethyl acetate and used for analytic TLC or stored in glass vials tightly capped and wrapped in parafilm at -80°C until use.

F-2.4 AHL reporter plate bioassays

Biosensor strains *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 pZLR4 were used for this assay. 10 ml of molten semi-solid LB agar (1%) were seeded with 100 µl of an over-night LB culture of *C. violaceum* CV026 and Agrobacterium tumefaciens NTL4 pLZR4 and poured immediately over the surface of prewarmed LB agar plates prepared in Petri dishes. When the overlaid agar had solidified, wells were punched in the agar with a sterile borer (diameter 5 mm). The wells were filled with the preparation to be assayed (solvent extracts, synthetic AHLs). Positive (standard AHL molecules) and negative (sterile LB broth) controls were included in each assay plate. The Petri dishes were incubated in the upright position for 48 h at 30°C, then examined for the stimulation of violacein in the case of *C. violaceum*; when *A. tumefaciens* NTL4 was used as the reporter strain, the eventual production of signal molecules was measured by monitoring the hydrolysis of the chromogenic substrate X-Gal

(previously added to the plates).

F-2.5 TLC and detection of AHLs

Each AHL migrates with a characteristic mobility and results in a spot shape. 5 ul of synthetic AHLs (purchased from Sigma and made up as 1 mM solutions in acified ethyl acetate) and extracted samples, were applied to C18 reversed-phase TLC plates (200 mm layer, Merck) and dried in a stream of cold air (as described by Shaw *et al*., 1997). Samples were separated using methanol in water (60 %, v/v). Once the solvent front had migrated to within 2 cm of the top of the chromatograms, the plates were removed from the chromatography tank, dried in air and overlaid with a culture of the indicator bacterium prepared as follows. For 20**x**20 cm TLC plates, 100 µl of a 10 ml over-night cultures of all reporter strains were used to inoculate 10 ml of LB medium and the new cultures were grown to late exponential phase. 5 ml of each culture was added to 45 ml of the same melted medium containing 0,7% agar maintained at 45°C (50 µg/ml of X-Gal were added when *Agrobacterium tumefaciens* strains were used as biosensors since they have the *lux-like* promoter fused to *lac*Z reporter gene). The cultures were mixed thoroughly and immediately spread over the surface of the developed plates. After the agar solidified, the coated plates were incubated at 28°C in a closed plastic container until when blue and purple spots appeared (*Agrobacterium tumefaciens* strains and *Chromobacterium violaceum* strain respectevely); for AHLs detection using *E. coli* pSB401 biosensor strain based on the *lux* reporter system, the TLC plate overlaied with the culture, was incubated over-night at 37°C and then exposed for 10 minutes to a chemiluminescence sensitive film (Biomax Light Film, Kodak,

Perkin Elmer); finally, the signals were detected by developing and fixing the film with Kodak developer and fixing liquids.

F-2.6 KF707 growth as biofilm for AHLs extraction

Biofilms were grown on the inside of silicone tubing in a method similar to that previously described (Booth *et al*., 2011). LB medium from a reservoir was pumped through 1 m long silicone tubing preceded by a bubble trap. After initial setup, the tubing was inoculated with 1 ml of over-night subculture using a 30 gauge needle. This was allowed to sit for 2 h to allow the cells to attach to the tubing wall, then the flow was started at approximately 0.5 ml/min. Biofilms were grown for 48 h. This growth time was necessary in order to allow sufficient biomass to accumulate on the interior of the tubing. Medium and loosely attached cells were allowed to drain from the tubing to a sterile Ermenleyer flask capped with a silicone lid. The cell mass was extruded from each tube section, using a metal clamp, into microfuge tubes. AHLs were extracted as described above (§ F-2.3), from both cell mass and collected supernatant and used for TLC analyses (§ F-2.5).

F-2.7. Genome analysis for lux homologous searching

KF707 genome data were used to search for *luxI/luxR* homologues. Nucleotide and protein BLAST were performed using a dataset of *lux*I homologue sequences to assess the percentage of similarity. Conserved domains were also identified and KF707 genome data were used to investigate the presence of these domains typical of LuxI/LuxR proteins.

F-3. Results

In this study, the ability of *Pseudomonas pseudoalcaligenes* KF707 to produce signal molecules involved in quorum sensing (QS) was assessed. Since KF707 strain belongs to Gram negative bacteria, having in general acyl-homoserine-lactone (AHLs) as quorum sensing molecules, the presence of these compounds was investigated. Based on the regulation of QS system in Gram negative bacteria, biosensors sensitive to a wide range of AHLs were developed (reviewed in Venturi *et al*., 2006). These biosensors usually carry a plasmid where a reporter gene transcription is regulated by the bound of the complex signal molecule/response regulator LuxR-like to the *lux-box* promoter region. Generally, LuxR-like response regulators are able to bind only the signal molecule produced by its cognate LuxI-like synthase. The specifity of LuxR-like proteins in binding cognate AHLs seems to depend on the presence of a box region in LuxR which can fit perfectly with a specific ligand, ensuring an intraspeciees communication. However, some LuxR were shown to be sensitive to similar compounds, while others can bind a wide range of AHLs.

Here, reporter strains *E. coli* pSB401, *Chromobacterium violaceum* CV026, and *Agrobacetrium tumefaciens* WCF47 (pCF218)(pCF372) and NTL4 pZLR4 were used.

F-3.1. Agar-Bioassays for the detection of QS molecules

Chromobacterium violaceum CV026 (violacein pigment based biosensor, sensitive to C6-3-oxo-AHL, C8-AHL, C8-3-oxo-AHL and C4-AHL) and

Agrobacterium tumefaciens NTL4 pZLR4 (a β-galactosidase based biosensor, sensitive to all 3-oxo-AHLs, C6-AHL, C8-AHL, C10-AHL, C12-AHL, C14-AHL, C6-3-hydroxy-AHL, C8-3-hydroxy-AHL and C10-3-hydroxy-AHL) were used for a preliminary investigation by T-streak assay. *Pseudomonas pseudoalcaligenes* KF707 was streaked and grown at the optimal temperature on LB agar close to the biosensors to form a 'T'; as a positive control, drops of synthetic AHLs were spotted close to the biosensors. This test showed that KF707 was not able to trigger a response in both the biosensor strains.

Usually, bacteria owning a QS system produce AHLs at a very low concentration, that is far below the threshold of sensitivity of the biosensor. In order to abtain concentrated signal molecules, the extraction in organic solvent was performed from KF707 cultures (§ F-2.3). Moreover, as the synthesis of hypothetical QS molecules depends on bacterial growth phase and nutrient conditions, the extractions were performed from cultures grown in different media and stopped at different growth phases (exponential, late exponential and stationary growth phases). Indeed, it has been reported (Yates *et al*., 2002) that the AHL lactone ring is readily hydrolysed under alkaline conditions to form the corresponding N-acyl-homoserine compound, which is inactive as a QS signal molecule. In this respect, we added buffer to culture media. Extractions were performed from planktonic cultures with acidified ethyl acetate (0.1 ml glacial acetic acid in 1 L ethyl acetate); the organic phases were collected and dried down under N_2 flow. Extracts were resuspended in 100 µl of acidified ethyl acetate and used for analytic TLC or stored in glass vials tightly capped and wrapped in parafilm at -80°C until use. The extracts were at first used for the reporter plates

bioassay. As shown shown in Fig. F-3.1.1 KF707 is not able to producesignal molecules belonging to th AHLs class.

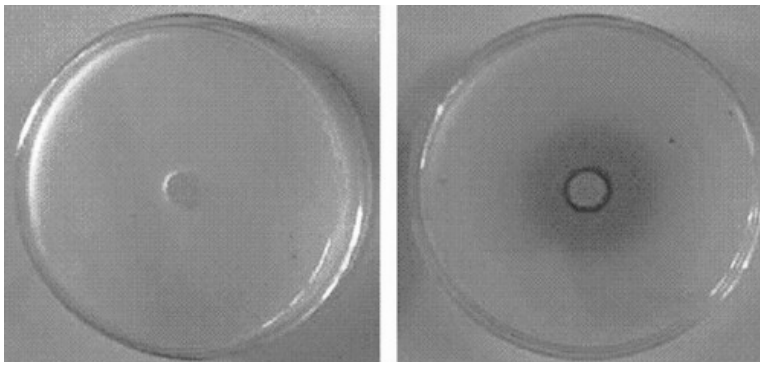


Fig. F-3.1.1: Biosensor strains *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 pZLR4 were used for this assay. The figure shows the results only for the reporter *Agrobacterium tumefaciens* NTL4 pZLR4. 10 ml of molten semi-solid LB agar (1%) were seeded with 100 µl of an overnight LB culture of C. *violaceurn* CV026 and *Agrobacterium tumefaciens* NTL4 pLZR4 (X-Gal was added to the agar since it is a *lac*Z based biosensor) and poured immediately over the surface of prewarmed LB agar plates prepared in Petri dishes. When the overlaid agar had solidified, wells were punched in the agar with a sterile borer (diameter 5 mm). The wells were filled with the preparation to be assayed (solvent extracts, synthetic AHLs). Positive (synthetic AHLs) and negative (sterile LB broth) controls were included in each assay plate. On the left is shown the plate with extracts from KF707 spent medium. On the right the positive control, which allowed the formation of a coloured ring around the well, is shown.

F-3.2. TLC analyses on planktonic and biofilm organic extracts

Thin layer chromatography was also performed on extracts obtained from both planktonically and biofilm grown bacteria; samples, positive and negative controls, were applied to C18 reversed-phase TLC plates and where allowed to separate using methanol/water (60 % , v/v). After separation, the TLC plates were removed from the chromatography tank, dried in air and overlaid with a culture of the indicator bacterial strains in soft agar. Biosensor strains (*E. coli* pSB401, *Chromobacterium violaceum* CV026, *Agrobacterium tumefaciens* NTL4 pZLR4 and *Agrobacetrium tumefaciens* WCF47 (pCF218)(pCF372) were all used in this

assay. X-Gal was added to the soft agar in which *Agrobacetrium tumefaciens* were suspended, since they own a reporter system based on the *lac*Z gene. All the TLC plates, each overlaied with one of the different reporter strains, were incubated at the optimal temperature, until when spot were visible. Only plates overlaided with *E. coli* pSB401 (based on lux operon reporter system and sensitive to C6-AHL, C8-3-oxo-AHL and C8-AHL), after over-night incubation were exposed for 10 minutes to a chemiluminescence sensitive film (Biomax Light Film, Kodak, Perkin Elmer) and the signals were detected by developing and fixing the film with Kodak developer and fixing liquids (Fig. F-3.2.1 and Fig. F-3.2.2). TLC analysis and detection with all these resporter strains showed the lack of AHLs production by KF707 strain.

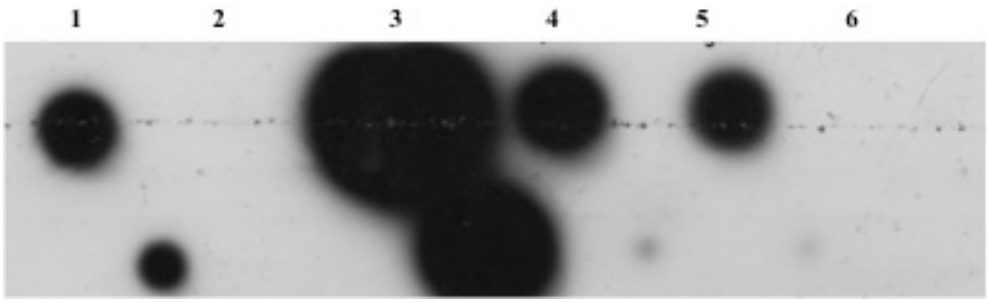


Fig. F-3.2.1: AHLs detection using *E. coli* pSB401. C6 (lanes 1 and 2), 3-oxo-C6 (lanes 3 and 4) and 3-hydroxy-C6 (lanes 5 and 6) were purchased from Sigma. This positive control experiment was performed to set the optimal condition for detection of AHLs. The same results were obtained when all the other reporter strains (§ F-2.1) were used.

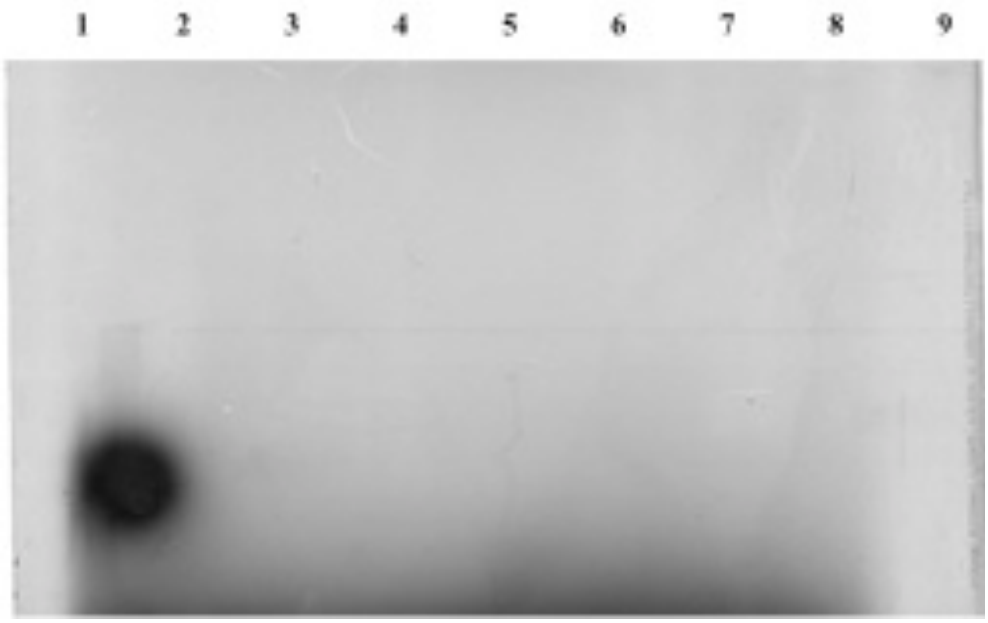


Fig. F-3.2.2: AHLs detection after TLC. Lane 1: standard 3-oxo-C6 AHL. Lane 2-9: KF707 extracts from culture grown in different nutritional conditions, pH value and stopped at different growth phases. The results here shown were obtained using *E. coli* pSB401 as reporter strains. The same results were obtained when all the other reporter strains (§ F-2.1) were used.

F-3.3. Bioinformatics analysis on *Pseudomonas pseudoalcaligenes* KF707 genome for luxI/luxR homologues systems searching

RAST tool was used for KF707 genome annotation. The presence in the genome of gene codifying for LuxI/LuxR proteins was invastigated. Twenty-one genes codifying for LuxR trancriptional regulator proteins were found. A BLASTp run was performed using aminoacidic sequence of known autoinducer binding proteins LuxR as queries against KF707 genome sequence. Results showed that all the 21 hypotetical LuxR response regulators in KF707 genome lack the autoinducer binding domain (Fig. F-3.3.1) which is typical of LuxR response regulator able to bind AHL molecules. Moreover they can not be even considered as LuxR orphan proteins (§ F-1.2.3).

KF707 putative transcription regulators LuxR showed the HTH (helix-turn-helix) superfamily domain which is the C-terminal DNA-binding domain of LuxR-like proteins. Proteins belonging to this group are response regulators; some act as transcriptional activators, others as transcriptional repressors. Many are active as homodimers. Many are two domain-proteins in which the DNA binding property of the C-terminal DNA binding domain is modulated by modifications of the N-terminal domain. The group also includes small proteins which lack an N-terminal signaling domain, such as Bacillus subtilis GerE. These LuxR family regulators may share a similar organization of their target binding sites. For example the LuxR dimer binds the lux box, a 20 bp inverted repeat, GerE dimers bind two 12 bp consensus sequences in inverted orientation having the central four bases overlap, and the NarL dimer binds two 7 bp inverted repeats separated by 2 bp.

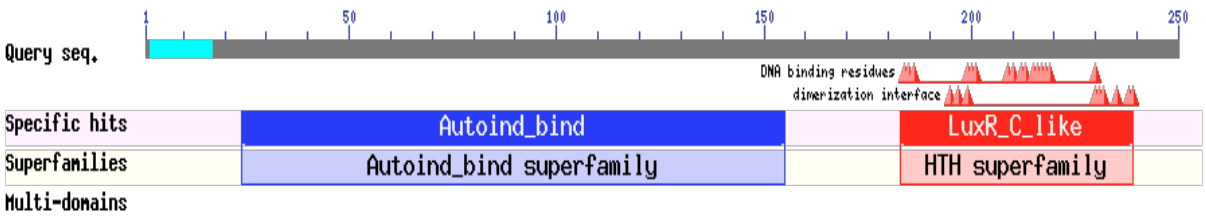


Fig. F-3.3.1: LuxR transcripational regulator domains. LuxR homologues found in KF707 do not possess the Autoinducer Binding Domain (blue colour in the figure), typical of LuxR with the ability to bind QS signal molecules.

The presence of LuxI-like proteins was also investigated. It has been reported that they don't show a high degree of similarity at the aminoacidic sequence. All the LuxI synthase were found only after the isolation of mutants, by means of screening using biosensors, not able to produce AHLs molecules.

Conserved domains analysis showed that the NAT (N-acyl-transferase) domain

(Fig. F-3.3.2) is typical of all the known autoinducers synthases. Proteins with NAT superfamily domain were found in KF707 genome too, but none of them is associated to the production of signal molecule involved in QS circuit.

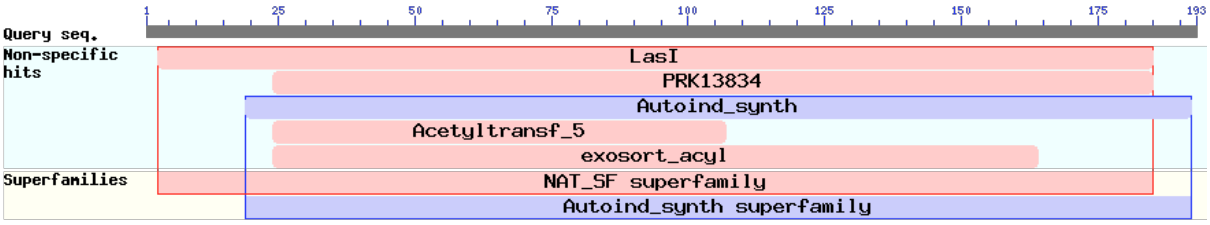


Fig. F-3.3.2: Conserved domains of aiutoinducer synthases in Gram negative bacteria.

F-4. Discussion

In this study the ability of *Pseudomonas pseudoalcaligenes* KF707 to produce signal molecules belonging to the AHL (acyl-homoserine-lactone) class was investigated. Cell-to-cell communication is a necessary trait for bacterial populations inhabiting the soil or particular hostile environments. In general QS systems facilitate the coordination of population behaviour to enhance access to nutrients or specific environmental niches, collective defence against other competitor organisms or community escape where survival of the population is threatened (Williams, 2007). Bacteria belonging to genera which occupy a wide variety of environmental niches – from marine and freshwater environments to soil, plants and animals, including many pathogens, symbionts, extremophiles and plant-growth-promoting bacteria – produce AHLs. Examples include species of *Acidithiobacillus, Acinetobacter, Aeromonas, Agrobacterium, Brucella, Burkholderia, Erwinia, Enterobacter, Chromobacterium, Hafnia, Mesorhizobium, Methylobacter, Paracoccus, Pseudomonas, Ralstonia, Rhodobacter, Rhizobium,*

Rhanella, Serratia, Sinorhizobium, Vibrio and Yersinia (Williams *et al*., 2007). Many of these bacteria produce multiple AHLs and possess more than one AHL synthase. So far, no AHL-producers have been found among strict anaerobes. A study using *Pseudomonas* strains revealed that, in contrast to soil isolates, all those isolated from plants produced AHLs. These signal molecules have been detected in natural habitats such as naturally occurring biofilms or bacterial aggregates.

Since the most of environmental Gram-negative bacteria own a *lux-like* quorum sensing system, it was appealing look for a homologue system in *P. pseudoalcaligenes* KF707. Moreover, KF707 strain must own a cell-cell communication system, since it is able to grow as biofilm and this imply the need of exchanging informations in the bacterial population.

Notably, this present study clearly shows the absence from KF707 cells of quorum sensing molecule belonging to the *lux-like* group.

Our finding suggests that KF707 may produce either structurally novel AHLs that were not detected by the biosensors used here or AHLs molecules were present at a concentration far below the threshold of the biosensor sensivity. Indeed, it has been reported in the past, that long chain AHLs may exhibit low permeability through the cell membrane, which could affect purification and detection; in this latter case, AHL extraction using the whole culture would be compelling. An alternative explanation for the results reported here, is that even though KF707 cells do not produce AHLs like compunds, they may use other molecules such as aminoacids, small peptide or metabolic intermediates as QS signals.

Future investigation focused on the isolation of possible QS signal molecules produced by KF707 might be based on metabolomics analysis of KF707 cells

grown under variable growth conditions, such as for example, plancktonic cultures versus biofilms. This approach will hopefully allow us to identify those metabolites and/or metabolic precursors forming the complex QS signal network of *Pseudomonas pseudoalcaligenes* KF707.

Conclusions

Prokaryotes, generally known as microorganisms or bacteria, represent the most ancient and simplest form of life on our planet. They evolved and adapted to various and even harsh surrounding enviroments with a great impact on all living organisms. Although a large number of microorganisms are related with infectious diseases, "friendly bacteria" also do exist: they are naturally involved in biogeochemical cycles and they establish symbiotic associations with both plants and animals. Furthermore, bacteria play a preminent role in numerous human activities such as for example agriculture, genetic engineering - for antibiotics and drugs production - and in environment cleaning up procedures.

The degradation of toxic compounds through the use of microorganisms is referred to as *bioremediation* and it has been recognized as an important means for the recovery of polluted sites. The efficency of this approach depends non only on the ability of microorganisms to degrade pollutants, but also in their capacity to tolerate intermediate metabolites produced during degradation of these compounds. Owing to this, bacteria must be capable to sense the surrounding environment and performe a chemotactic response towards nutrients necesarry for survival.

Moreover, in natural environments, bacteria are associated to a solid surface as biofilms, that consist of organized cellular communities where multiple bacterial species may coexist and form consortia. In bacterial biofilms, intra- and inter-species cell-to-cell comunications are important traits which allow bacteria to modulate and synchronize their activities, thus imparting a social behaviour. Communication in the microbial world is referred to as *quorum sensing* (QS), a

phenomenon dependent on production, release and perception of specific molecules known as *autoinducers*.

Pseudomonas pseudoalcaligenes KF707 is a soil Gram negative bacterium isolated in the ’80 in Japan near a biphenyl manifacturing plant and able to grow using biphenyl as the sole carbon source (Furukawa *et al*., 1986), to which is chemically attracted. Moreover, KF707 strain tolerates the oxydative stress due to toxic metal oxyanions such as tellurite and selenite (Di Tomaso *et al*., 2002; Zanaroli *et al*., 2002; Tremaroli et *al*., 2007). Since many polluted sites are contaminated not only by biphenyl (BP), but also by BP-chloroderivatives known as PCBs - and metal oxyaniones, KF707 owns all the necessary features to be used in bioremediation procedures for the recovery of co-contaminated sites.

Based on the above reported considerations underlining the peculiar phenotypic and physiological features of KF707, an important fraction of this Thesis work has been focused on determining the molecular nature of such a unique microorganism.

The genome project (see Chapter C) provided a large set of data concerning the various physiological properties of this strain. Interestingly, multiple putative operons encoding for different chemotactic pathways and therefore multiple *che*A genes (*che*A1, *che*A2, *che*A3) were found (see Chapters C and D). Their functions were compared with those previously attributed to a *che*A1 gene in a KF707 mutant strain constructed by a mini-Tn5 transposon insertion (Tremaroli *et al*., 2011), thus the role of *che*A multiple genes in motility, chemotactic response and biofilm

formation were assessed (see Chapter E). Finally, the ability of KF707 strain to produce signal molecules possibly involved in cell-to-cell communication, was also investigated (see Chapter F).

The findings described in this Thesis can be divided into three main sections:

I. Genome assembly and annotation;
II. Molecular and functional characterization of chemotactic genes, with particular interest on *che*A genes codifying for histidine kinase involved in chemosensory signalling pathways;
III. Investigation on a probable *lux-like quorum sensing* (QS) mechanism in KF707.

"The genome project of *Pseudomonas pseudoalcaligenes* KF707", started in 2010, has been performed in collaboration with Prof. R.J.Turner (University of Calgary, Calgary, Ca) and Prof. M.Attimonelli (University of Bari, Bari I). The genome analysis, not only has provided insights on the numerous and unique physiological traits of KF707 strain, but it has also been useful for the acquisition of bioinformatics skills with regard to the assembly and the annotation of a microbial genome. 454-pyrosequencing and Illumina sequencing technologies were employed and the datasets obtained were assembled using the Newbler and AbySS softwares. Generally, the use of different sequencing and assembly approaches is highly recommended as they provide complementary data, thus allowing to obtain a high value of genome coverage. Moreover, the KF707 Optical Map was also constructed and provided information on the position of the contigs, the size of the left gaps and even the genome size, which was estimated to be approximately 5.95 Mb with a GC

content of 64.24%. Assembled genome was annotated using the RAST tool and 6.512 CDSs were identified. Notably, biochemical pathways involved in the degradation of biphenyl, PCBs and other aromatic compounds such as phenol, benzoate and chlorobenzoic acids were identified. Additionally, genes involved in cobalt, zinc, cadmium, arsenic and tellurium tolerance were also found (Triscari *et al*., 2012). Genome annotation data showed that KF707 strain owns several putative chemotaxis pathway and in particular two additional *che*A genes, named *che*A2 and *che*A3. Since previous works showed that the KF707 *che*A1 mutant strain is impaired in motility, chemotaxis and biofilm formation (Tremaroli *et al*., 2011), we investigated the possible roles of the *che*A2 and *che*A3 genes. Deleted mutants in *che*A2 and *che*A3 genes and also double mutants *che*A1/*che*A2 and *che*A1/*che*A3were constructed. These mutant strains, together with *che*Y and *che*Z mutants, were used to test their motile behaviour in the absence or in the presence of stimuli as well as biofilm formation. We found that the mutants *che*A1::Km, Δ*che*A2*che*A1::Km, Δ*che*A3*che*A1::Km, *che*Y are impaired in motility and chemotaxis remarking the fact that only the *che*A1 gene is responsible for swimming of KF707. Further support to this conclusion derived from the Δ*che*A2, Δ*che*A3 and double mutants phenotypes. The ability of both KF707 and chemotactic mutant strains to form biofilm were also assessed. Interestingly, the Δ*cheA*3 mutant showed swarming motility only under specific nutrient conditions. The present findings on swimming motility, chemotaxis, swarming and biofilm formation, taken together, were interpreted to suggest that in *Pseudomonas pseudoalcaligenes* KF707 strain, multiple factors are involved in these networks, as they might play different roles depending on the environmental conditions.

The possible presence in KF707 growing cultures of signal molecules belonging to the AHLs class, typical of Gram negative bacteria QS systems, was investigated. By means of reporter strains sensitive to a wide range of AHLs it was found that KF707, tested under different conditions of growth, was not able to produce these types of QS signal molecules. This finding tend to suggest that other classes of molecules might be involved in KF707 cell-to-cell communication.

In conclusion, the present experimental work provides the bases for future investigation about the genetic and physiological features of *Pseudomonas pseudoalcaligenes* KF707. The genetic informations given by genome analysis represent a fundamental tool for the identification of enzymes involved in the degradation of toxic pollutants and it can be used to construct strains with improved catabolic skills. Moreover, the results obtained on the KF707 chemotactic attraction to aromatic compounds, its ability in swarming and in adhering to solid surfaces in the form of biofilms might support new strategies for the colonization of polluted sites. Notably, the data indicating the lack in KF707 cells of a *lux*-like QS system - which is conversely widely present in Gram negative bacteria – keeps open the question about the actual molecular nature of KF707 quorum sensing mechanism.

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