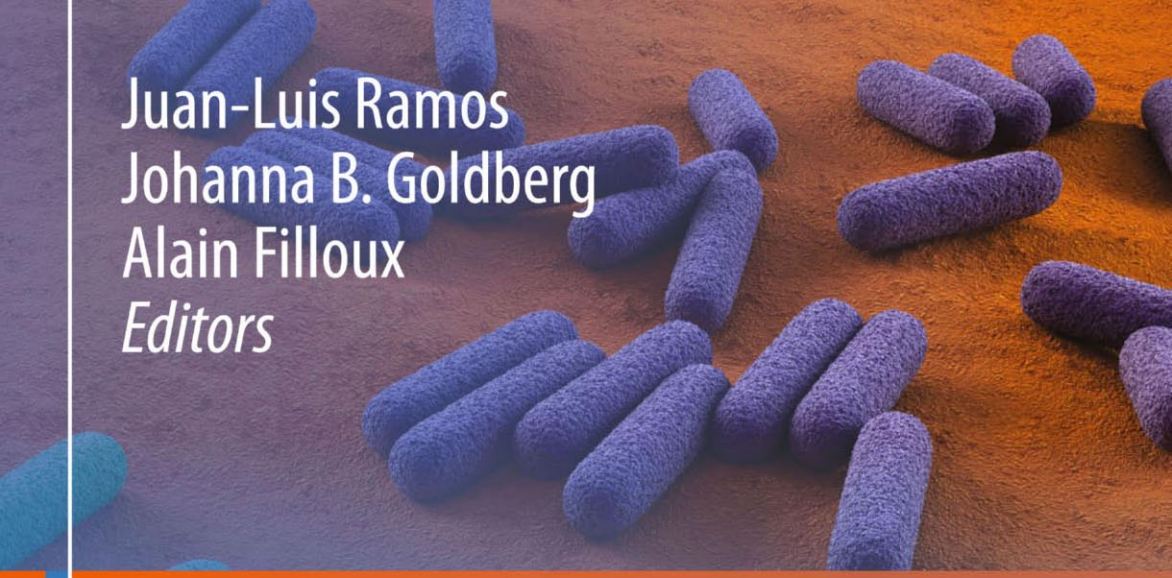

Juan-Luis Ramos
Johanna B. Goldberg
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Editors

Pseudomonas

Volume 7
New Aspects of Pseudomonas Biology

Springer

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Volume 7: New Aspects of *Pseudomonas* Biology

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Preface

Paris is cosmopolitan city where roaring life, wonderful museums and excellent science can be found. It was during the XI IUMS conference held in this city that the Pseudomonas book series was first envisaged. On the first row of the auditorium sat a group of outstanding scientists in the field, who after devoting much of the valuable time, contributed in an exceptional manner to the first three volumes of the series, which saw the light simultaneously. The volumes were grouped under the generic titles of "Vol. 1. *Pseudomonas*: Genomics, Life Style and Molecular Architecture", Vol. 2. *Pseudomonas*: Virulence and gene regulation; Vol. 3. *Pseudomonas*: Biosynthesis of Macromolecules and Molecular Metabolism.

Soon after the completion of the first three volumes, a rapid search for articles containing the word *Pseudomonas* in the title in the last 10 years produced over 6000 articles! Consequently, not all possible topics relevant to this genus were covered in the three first volumes. Since then three other volumes were published: *Pseudomonas* volume 4 edited by Roger Levesque and Juan L.Ramos that came to being with the intention of collecting some of the most relevant emerging new issues that had not been dealt with in the three previous volumes. This volume was arranged after the *Pseudomonas* meeting organized by Roger Levesque in Quebec (Canada). It dealt with various topics grouped under a common heading: "*Pseudomonas*: Molecular Biology of Emerging Issues".

Yet the "*Pseudomonas* story" was far from complete and a new volume edited by Juan L. Ramos and Alain Filloux was deemed necessary. The fifth volume was conceived with the underlying intention of collecting new information on the genomics of saprophytic soil *Pseudomonas*, as well as on the functions related to genomic islands and was published in 2006. It was followed by volume 6 is a sequel to the story and add some other items at the request of a number of scientists and colleagues working in the field, we have collected a new set of chapters that are called on to provide further views on the biology of *Pseudomonas.*

To further update the series we have produced this new volume for the series. Chapters in *Pseudomonas* volume 7 have been grouped under the following topics: Pathogenesis, Biochemistry and Physiology, Soil Microbiology, and we have incorporated a chapter of the history of *Pseudomonas* by Bruce Holloway.

It would not be fair not to acknowledge that this seventh volume would never have seen the light if it were not for a group of outstanding scientists in the field who have produced enlightening chapters to try to complete the story that began with the six previous volumes of the series. It has been an honor for us to work with them and we truly thank them.

The review process has also been of great importance to ensure the high standards of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. We would like to express our most sincere gratitude to:

Soeren Molin	Tino Krell	Lars Jesbalk
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Giovani Bertoni	Cristophe Bordi	

We would also like to thank Carmen Lorente-Vázquez once again for her assistance and enthusiasm in the compilation of the chapters that constitute this seventh volume.

Contents

Abbreviations

3OC12-HSL	N-3-oxododecanoyl-Lhomoserine lactone
ACC	Aminocyclopropane-1-carboxylic acid
ACIAR	Australian Centre for International Agricultural Research
AFP	Autofluorescent protein
AHL	Acylhomoserine lactone
AHL,	N-acyl-homoserine lactone
AHLs,	acyl homoserine lactones
AMEs	Aminoflycosides-modifying enzymes Antirepressor of MexR
BCA	Biological control agent
BCAs	Biological control agents
C4-HSL	N-butyryl-L-homoserine lactone
CAMPs	Antimicrobial peptides
CARD	Caspase recruitment domain
CFP	Cyan fluorescent protein
CLP	Cyclic lipopolypeptides
CLSM	Confocal laser scanning microscopy
CM	Cytoplasmic membrane
DAP	Diaminopimelic acid
DAPG	2,4-deoacetylphloroglucinol
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FIT	*Fluorescens* insect toxin
FORL	F. oxysporum f. sp. *radicis lycopersici*
FT	Pore-forming toxins
GABA	γ-aminobutyric acid
IAA	Indole-3-acetic acid
ICI	Imperial Chemical Industries
ISR	Induced systemic resistance
IVET	In vivo expression technology
LBD	N-terminal ligand binding domain
LPS	Lipopolysaccharide
LRR	Leucine rich repeat

MBLs	Metallo-β-lactamases
MDPR	Multidrug resistant *P. aeruginosa*
NAIPs	NLR apoptosis-inhibitory proteins
NLR	Nod-like receptor
NRPS	Non-ribosomal peptide synthase
OMV	Outer membrane vesicles
PAMPs	Pathogen associated molecular patterns
PBPS	Penicillin binding proteins
PCA	Phenazine-1-carboxamide
PCA	Phenazine-1-carboxylic acid
PGPR	Plant growth promoting rhizobacteria
PMBCs	Peripheral blood mononuclear cells
PQS	*Pseudomonas* quinolone signal
PRR	Pattern recognition receptor
PVF	*Pseudomonas* virulence factor
QS	Quorum sensing
RMTs	16S rRNA methylases
RND	Resistance, nodulation and cell division
ROS	Reactive oxygen species
SA	Salicylic acid
SAM	S-adenosyl-Lmethionine acyl-ACP) acyl carrier protein
SAR	Systemic acquired resistance
STM	Signature tagged mutagenesis
T3SS	Type III secretion system
TAD	Take-all decline
TCS	Two-component system
TLRs	Tol like receptors
TTSS	Type three secretion systems
VWO	Verticillium wilt of olive
YFP	Yellow fluorescent protein

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Part I
Pathogenesis

Chapter 1
Pseudomonas aeruginosa Host Immune Evasion

Taylor S. Cohen, Dane Parker and Alice Prince

Abstract The opportunistic pathogen *Pseudomonas aeruginosa* is a common cause of pneumonia in immunocompromised and mechanically ventilated patients. By virtue of its large genome and genetic flexibility, *P. aeruginosa* is able to adapt to its environment regulating the expression of a large repertoire of virulence factors. It is capable of forming biofilms that favor persistence and evasion of phagocytic clearance. Within a biofilm the bacteria communicate via soluble quorum sensors regulating production of elastases and proteases. *P. aeruginosa* is able to modify expression of pathogen associated molecular patterns such that recognition by host immune receptors is minimized. The bacteria are also able to subvert the innate immune system through direct interaction with host cells and host immune cytokines. These strategies of immune evasion and the genetic pathways that regulate them have contributed to the unusual success of *P. aeruginosa* as on opportunistic pathogen.

Keywords *P. aeruginosa* · innate immunity · biofilm

Pseudomonas aeruginosa is an opportunistic human pathogen especially common in health care associated facilities. Aspiration or contamination of the airways with *P. aeruginosa* is an infrequent cause of pneumonia in a normal host, but is a common pathogen in immunocompromised and mechanically ventilated patients (Richards et al. 1999; Rodríguez-Rojas et al. 2012; Lynch 2001; Craven and Hjalmarson 2010). Much of what we have come to understand about the ability of *P. aeruginosa* to evade immune clearance has come from the study of cystic fibrosis, a genetic disorder often associated with respiratory colonization with *P. aeruginosa*. This wealth of clinical and basic science information has been important in the understanding of host-pathogen interactions in the airway.

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P. aeruginosa utilize numerous strategies to avoid eradication by the immune system. Its large genome and genetic flexibility, allows *P. aeruginosa* to rapidly adapts to the milieu of the airway, and formation of biofilms promotes persistence and evasion of phagocytic clearance (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006). Once within the airways, organisms alter expression of pathogen associated molecular patterns (PAMPs) such that they limit detection by the host, and directly interact with host signaling through injection of effector proteins or release of vesicles. This chapter will review our current understanding of these methods of persistence and the interaction between *P. aeruginosa* and innate immune cells within the host.

P. aeruginosa Rapid Genetic Diversification

The *P. aeruginosa* genome consists of a conserved core set of genes and a number of accessory genes that allow for rapid adaptation within the host (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Wiehlmann et al. 2007). *P. aeruginosa* exhibits a high mutation rate during infection allowing the bacteria to respond to selective pressure in the host environment through diversification of the population's collective gene pool (Richards et al. 1999; Rodríguez-Rojas et al. 2012; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006). The mismatch repair system, essential to limiting mutation, can be turned off through down-regulation of the genes *mutS*, *mutL*, and *uvrD*. Loss of this system promotes increased mutation rates and genetic diversification, ultimately altering expression of other global regulators such as Vfr and OprF that influence expression of numerous virulence factors (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Fito-Boncompte et al. 2011; Fuchs et al. 2010; Oliver et al. 2002; Oliver et al. 2000). In addition to diversification of virulence expression, increased mutation allows members of the bacterial community to utilize difference sources of nutrition, survive in aerobic and anerobic conditions, and withstand antimicrobial peptides produced by the host (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006; Williamson et al. 2012; Zhao et al. 2012). Therefore while an individual bacterium might not be able to survive within the host, the population persists as subpopulations are resistant to various host pressures.

P. aeruginosa contains numerous two component systems that enable the organisms to respond to the environment or specifically to conditions encountered within a eukaryotic host. One of these regulons involves the two-component signal transduction system PmrAB that can be activated by host DNA. This two-component system regulates a variety of functions including: adding arabinose to lipid A of LPS altering its structural providing resistance to polymyxin (Moskowitz et al. 2004; Mulcahy et al. 2008). PmrAB also controls a group of genes with similarity to those involved in spermadine synthesis and provides a surface layer containing putrescine and spermidine that protects the organism from antimicrobial peptides

as well as polymyxin and gentamicin (Johnson et al. 2012). Consistent with its role in protection from host antimicrobial compounds, PmrAB is also induced in the presence of several cationic peptides including LL-37 as well (McPhee et al. 2003). Thus the organism has the genetic flexibility to adapt to conditions that would eliminate other microbes.

P. aeruginosa Communities: Biofilms

Within the host, *P. aeruginosa* forms biofilms, which protect the bacteria from innate immune clearance (Rodríguez-Rojas et al. 2012; Hoboth et al. 2009; Davies et al. 1997; Singh et al. 2000). Biofilms develop in a three step process involving bacterial attachment to the epithelial surface, microcolony formation, and formation of the mature biofilm (Fig. 1.1). Individual bacterial cells released from the mature

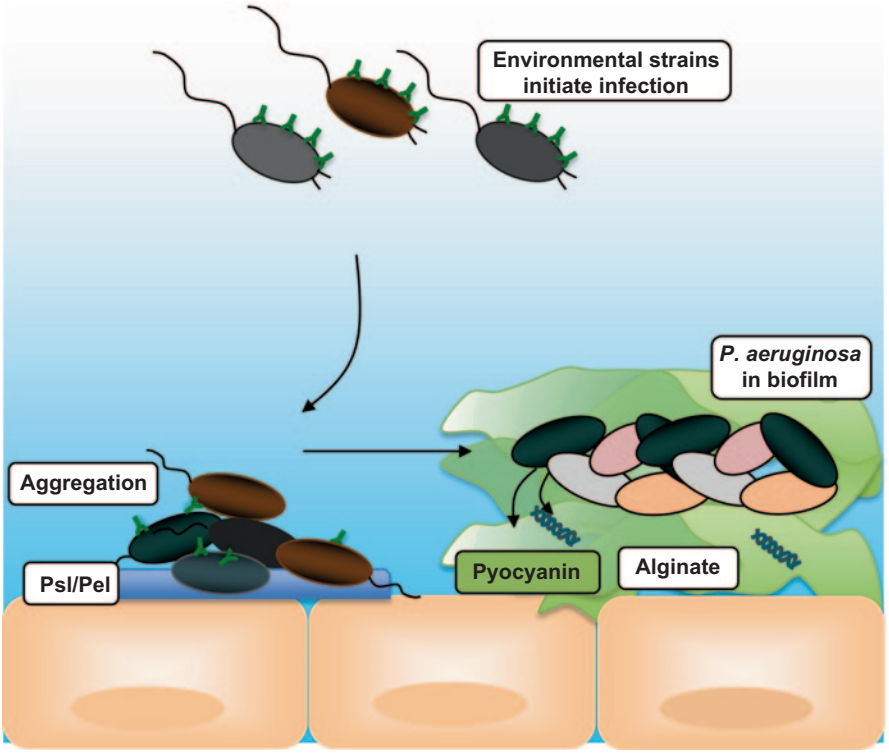

Fig. 1.1 Biofilm formation. Environmental isolates of *P. aeruginosa* aggregate within the host upon infection suppress expression of immunostimulatory virulence factors and initiate alginate production. Within the bacterial population gene expression in individual bacterium are diversified, allowing the population to adapt to the host's environment

biofilm known as planktonic cells facilitate dispersal of the infection (Stoodley et al. 2002). The biofilm matrix is composed of exopolysaccharides, nucleic acids and proteins. Initial formation is dependent on the *pel* and *psl* regulated exopolysaccharide production which firmly attaches bacteria to the epithelium (Colvin et al. 2011a, b; Ma et al. 2009; Jackson et al. 2004; Matsukawa and Greenberg 2004). Individual bacteria deposit Psl exopolysaccharide that attracts other bacteria and acts as the foundation of the biofilm (Zhao et al. 2013). Psl upregulates c-di-GMP levels in neighboring bacteria resulting in increased Psl production (Irie et al. 2012). Bacterial density and quorum sensing can suppress c-di-GMP and biofilm formation by repressing expression of genes within the *pel* locus (Ueda and Wood 2009). Upon initiation of the biofilm *P. aeruginosa* increases production of alginate, required for structural development of the bacterial community (Hentzer et al. 2001; Davies et al. 1998; Nivens et al. 2001). Production of this exopolysaccharide is regulated by *mucA*, and during the transition to a biofilm growth mode spontaneous selection of *mucA* mutants that promote alginate production allow the organisms to elude phagocytosis (Silo-Suh et al. 2002; Martin et al. 1993; Bjarnsholt et al. 2009). Host factors such as estrogen and mucus in the CF airway induce MucA mutations promoting colonization (Cattoir et al. 2012; Chotirmall et al. 2012). Alginate itself is immunogenic and elicits antibody production, which amplifies the host inflammatory immune contributing to pathology without resulting in clearance of the organisms (Pedersen 1992; Schiøtz et al. 1978).

The switch to the biofilm phenotype is highly correlated with persistent infection, however, it does not necessarily impair the ability of the bacteria to induce signaling in the host (Sanchez et al. 2013). Isolates from chronically colonized CF patients with altered immune phenotypes, loss of motility and increased mucosity, were not as lethal in a murine model as early isolates but were equally capable of inducing excessive lung inflammation and establishing a chronic infection (Bragonzi et al. 2009). A major component of the biofilm matrix is extracellular DNA, which can assist in initial biofilm development by attaching to F-actin released from neutrophils at the onset of infection (Parks et al. 2009; Whitchurch et al. 2002; Nemoto et al. 2003). Host pattern recognition receptors respond to the increase in DNA by upregulating inflammatory signaling, recruiting inflammatory monocytes to the lung, but not necessarily resulting in bacterial clearance (Fuxman Bass et al. 2010). Therefore, incorporated DNA from lysed bacteria into the biofilm structure maintains inflammatory signaling while preventing innate immune clearance.

Biofilm formation also facilitates the coordinate expression of numerous genes throughout the microbial population through secretion of highly soluble quorum sensors which have been identified as biomarkers of advanced infection (Struss et al. 2013). In *Pseudomonas* biofilms, homoserine lactones and quinolones act in concert with specific transcriptional activators to regulate gene expression (Guina et al. 2003; Wade et al. 2005). The Las system produces the homoserine lactone N-3-oxo-dodecanoyl that activates the transcriptional regulator LasR, influencing expression of elastases and proteases (Passador et al. 1993; Pearson et al. 1994). A second lactone, N-butanoyl, activates an alternate transcriptional regulator RhlR, which represses expression of the proteins that compose a major virulence factor,

the type III secretion system (TTSS). These signaling pathways regulate each other as LasR upregulates expression of RhlR and RhlR can control expression of LasR dependent factors in *lasR* mutant bacteria (Dekimpe and Déziel 2009). The *Pseudomonas* quinolone signal (PQS) activates its receptor PqsR which increases production of PQS, phenazine, and pyocyanin (Fito-Boncompte et al. 2011). PQS signaling is regulated by homoserine lactones, though through distinct mechanisms as LasR positively regulates and RhlR negatively regulates PQS production. Importantly, PQS correlates with bacterial virulence in animal models, potentially due to a relationship between PQS production and expression of inflammatory lipopolysaccharide (Guina et al. 2003).

Pyocyanin, regulated by transcriptional regulators LasR and OxyR, is produced by all *P. aeruginosa* strains, but at increased levels in biofilms (Vinckx et al. 2010; Schaber et al. 2004). It can directly interact with host cells inducing inflammatory signaling and reducing the function of antioxidants GSH and *N*-acetylcysteine by blocking the dual oxidase-based antimicrobial system (Rada et al. 2008; Look et al. 2005; Rada and Leto 2012). In the epithelium, the pyocyanin inhibits V-ATPase activity preventing endosomal acidification and perhaps inhibiting degradation of endocytosed bacteria (Ran et al. 2003). Pyocyanin may also negatively affect the function of the chloride channel CFTR, perhaps enhancing disease progression in cystic fibrosis (Schwarzer et al. 2008). Levels of pyocyanin have been correlated with decreased lung function in clinical studies, and in vivo experiments have documented decreased virulence of *P. aeruginosa* pyocyanin mutant strains (Fito-Boncompte et al. 2011; Lau et al. 2004; Hunter et al. 2012; Recinos et al. 2012). Therefore while cell-cell communication is especially important for formation and regulation of bacterial communities within the biofilm, it also results in the production of factors that interact directly with the host, inducing inflammatory signaling and interrupting host processes.

P. aeruginosa Altered PAMP Expression

P. aeruginosa presents an array of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins, and flagella that initiate host signaling through an numerous of receptor complexes. Toll like receptors (TLRs) such as TLR2 (bacterial lipoproteins) and TLR5 (flagellin) are expressed primarily on the cell surface and signal through the adapter protein MyD88 to activate innate immune signaling. TLR4 (LPS) is expressed both at the cell surface and within the endosome where it signals through the adapter protein Trif. Additional TLRs such as TLR3 (double stranded RNA), TLR7 (single stranded RNA), and TLR9 (CpG DNA) are intracellular receptors and activate similar signaling pathways. Host cells also express NOD-like receptors (NLRs) that activate caspase activation, cytokine release, and a form of inflammatory cell death called pyroptosis following recognition of bacterial peptidoglycans, flagellin, nucleic acids, and components of the type III secretion system (Akira et al. 2006). *P. aeruginosa* is a successful opportunist not

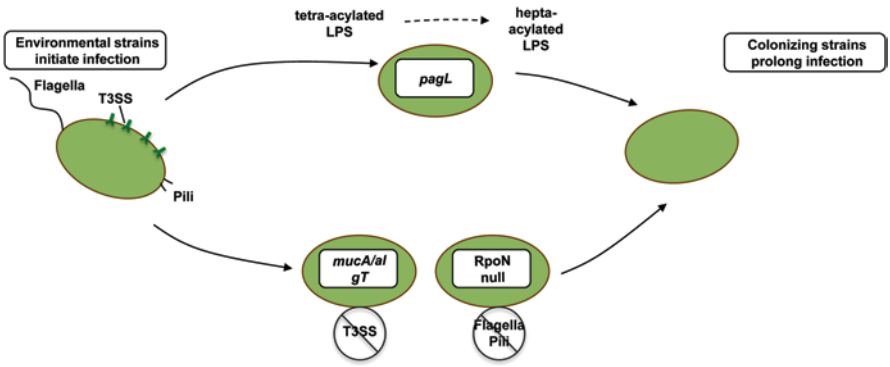

Fig. 1.2 Modification of virulence expression. Rapid genetic mutations upon infection result in alterations in *P. aeruginosa* virulence factor expression

only due to its many virulence factors but also due to its ability to adapt to the host environment minimizing activation of these host receptors and downstream signaling pathways (Fig. 1.2) (Bianconi et al. 2011).

Lipopolysaccharide (LPS), a major cell wall component all Gram-negative organisms is shed from the surface of bacteria into the airway and is recognized by TLR4 on host cells (Poltorak et al. 1998). The LPS-TLR4 interaction, mediated by adaptor proteins MD2 and CD14, results in activation of MyD88 (cell surface) and Trif (endosome) dependent signaling pathways (Kagan et al. 2008; Rowe et al. 2006; Visintin et al. 2001). The ability of LPS to activate host signaling pathways is influenced by the acylation state of side chains attached to the core signaling component of LPS, Lipid A, allowing for variation between the immunostimulatory capacity of LPS from various Gram-negative bacteria (Zughaier et al. 1999; Ganglloff et al. 1999). In fact mimics of triacylated LPS have been suggested as promising anti-inflammatories due to their inability to induce signaling while binding to TLR4 (Dunn-Siegrist et al. 2012). Lipid A alterations are controlled via transcriptional regulation (PhoP and PhoQ) of *pagP*, *pagL*, and *lpxO* genes that are responsible for acylation, deacylation, and hydroxylation of lipid A, respectively (Kawasaki et al. 2004; Geurtsen et al. 2006). In vivo selection of modified LPS, presumably in response to innate immune pressure, has been extensively studied. The affect of LPS structure on both acute and chronic infection has been clarified by analysis of isolates from newly infected infants with cystic fibrosis, and sequential isolates from colonized patients. Comparison of the LPS produced by *P. aeruginosa* isolates from infants with cystic fibrosis or non-CF patients with sepsis or bronchiectasis demonstrated that bacteria initially infecting the lungs of CF patients predominantly had a penta-acylated LPS lipid A with additional palmitate and aminoarabinose incorporated into the side chains (Ernst et al. 1999). These additions confer protection against cationic antimicrobial peptides (CAMPs) giving these organisms a significant survival advantage (Guo et al. 1998). The lipid A from CF isolates was more immunostimulatory when applied to human endothelial cells, resulting in more of

an interleukin-8 response than that of non-CF isolates. The differences in signaling were also attributed to palmitate additions to the side chains. Growth of the laboratory strain PAK in low Mg^{2+} media resulted in similar modifications to the side chain, indicating that these modifications could be in response to specific environmental stimuli within the CF lung (Ernst et al. 1999).

P. aeruginosa lipid A structure not only has an affect on the acute infection, but once in the CF lung further adaptations in side chain composition are observed. Analysis of sequential isolates from CF, confirmed to be clonal variants by pulsed-field gel electrophoresis and single nucleotide polymorphisms, demonstrated an enhanced ability of the later isolates to persist within the mouse lung (Lorè et al. 2012; Hajjar et al. 2002). While the murine data associated with these studies is controversial considering reports that murine TLR4 is unable to recognize alterations in lipid A, activation of NF-κB signaling in culture human epithelial cells was reduced in groups stimulated with later isolates. Analysis of the lipid A structure showed a blend of tetra- and penta- acylated lipid A in the early and mid isolates. Some samples from intermediate isolates also contained hexa-acylated lipid A, while the later isolates contained primarily hepta-acylated lipid A. These modifications persisted through serial passaging. Altered lipid A in the late isolate correlated with a mutation in the *pagL* gene that is responsible for deacylation, which was also observed in an independently published study of early and chronic infection in cystic fibrosis (Ernst et al. 2007; Cigana et al. 2009). These studies demonstrate that dynamic modifications to LPS in addition to other virulence adaptations enhance the ability of colonizing bacteria to persist within the airways.

In addition to changes in LPS structure, *P. aeruginosa* modifies expression of other virulence factors over the course of infection. The *mucA* mutant is associated with biofim formation and increased alginate production. Alginate and specifically the gene *algT* negatively regulate flagella production through inhibition of *fleQ* expression (Tart et al. 2005; Garrett et al. 1999; Cobb et al. 2004). The result is non-motile *P. aeruginosa* that lack flagella, but are also better able to evade immune clearance. Strains of *P. aeruginosa* lacking flagella have reduced virulence as they cannot activate TLR5 or NLRC4 signaling pathways, have an impaired ability to bind and colonize epithelial cell surfaces, and are resistant to macrophage engulfment (Hazlett et al. 1991; Saiman and Prince 1993; Saiman et al. 1990; Miao et al. 2008; Morris et al. 2009). Alternate regulation of flagella is also downregulated in colonizing infection (Mahenthiralingam et al. 1994). Flagellin and pilin synthesis are regulated by the alternative sigma factor σ54/RpoN and the majority of clinical isolates from long colonized patients are *rpoN* negative. Complementation of *rpoN* on a plasmid partially restores swimming ability. Therefore it seems that the presence of flagella, a highly inflammatory virulence factor, is not required for long term survival within the host, and a selective advantage exists for bacteria that lose flagellin expression.

Another group of virulence factors associated with strains of *P. aeruginosa* isolated from acute infections are those delivered by the type III secretion system (TTSS), which directly injects effector proteins into host cells and interrupts host signaling processes. The TTSS needle is composed of numerous proteins including

PscC, capable of activating the NLRC4 inflammasome independent of effector protein secretion (Miao et al. 2010). For pore formation in the host cell membrane, *P. aeruginosa* pilli must bind asialoGM1 on the host cell membrane (Saiman and Prince 1993; Sundin et al. 2002; Kang et al. 1997). Once the pore has formed, the bacteria inject a cocktail of proteins made up of a combination of ExoU, ExoY, ExoS, and ExoT. Individual bacterial isolates do not necessarily express each effector but they have major effects on the host.

While important for bacterial dissemination during acute infections, expression of TTSS components are reduced in colonizing strains of *P. aeruginosa* (Jain et al. 2004). The bacterium is able to sense the host and regulate expression and activation of the TTSS through the RsmAYZ regulatory cascade (O'Callaghan et al. 2012). Furthermore, *mucA* is part of the regulatory pathway upstream of TTSS expression, and selection of *mucA* mutants also selects for bacteria lacking TTSS expression (Wu et al. 2004). Therefore, as with other virulence factors such as flagella, expression of TTSS needle and effector proteins is repressed facilitating the selection of mutants that evade detection by the innate immune system and eradication by the host.

Evasion from Host Cells

The large array of virulence factors expressed by *P. aeruginosa* are also utilized to interact directly with host immune cells to avoid detection and clearance by the immune system (Table 1.1). *P. aeruginosa* can directly interact with host structural proteins and signaling pathways to promote colonization and dissemination from the airways. *P. aeruginosa* secretes several virulence factors that suppress the immune response. The pigment pyocyanin is able to suppress the production of the cytokines RANTES and MCP-1 as well as leukotriene B_4 and 5-hydroxyeicosateraenoic acid, both proinflammatory mediators (Müller and Sorrell 1991; Denning et al. 2003). The alkaline protease and elastase can degrade RANTES and MCP-1 as well as the neutrophil-activating protein-78 (ENA-78) (Leidal et al. 2003). The quorum sensing molecules *Pseudomonas* quinolone signal (PQS) and *N*-3-oxododecanoyl reduce IL-12 (Skindersoe et al. 2009). *N*-3-oxododecanoyl has significant immunomodulatory activity able to also inhibit TNF and high concentrations have inhibited antibody production in spleen cells (Telford et al. 1998; Hooi et al. 2004). Rhamnolipids have been shown to prevent induction of human beta defensin 2 (hBD-2), an important antimicrobial peptide, in response to flagellin of *P. aeruginosa* as well as in response to host cytokine IL-1β (Dössel et al. 2012). Outer membrane vesicles (OMV) of *P. aeruginosa* containing immunostimulatory bacterial components are released during infection and can directly interact with the host (Wessel et al. 2012; Bomberger et al. 2009). One protein found in OMVs, Cif, was shown to redirect cystic fibrosis transmembrane conductance regulator (CFTR) from recycling endosomes to the lysosome where it is degraded (Bomberger et al. 2011). Exotoxin A can inhibit interferon gamma synthesis, elastase can degrade interferon gamma

Table 1.1 *P. aeruginosa* virulence factors and their host targets

P. aeruginosa	Host Target	Reference
Rhamnolipid (alginate)	hBD-2	Dössel et al. 2012; Pedersen et al. 1990; Song et al. 2003; Mai et al. 1993; McClure and Schiller 1996; Krieg et al. 1988
	Phagocytosis	
	Neutrophil chemotaxis	
Elastase	Rantes	Leidal et al. 2003; Hong and Ghebrehiwet 1992; Theander et al. 1988; Mariencheck et al. 2003
	Complement	
	Phagocytosis	
Pyocyanin	VATPase	Müller and Sorrell 1991; Denning et al. 2003; Usher et al. 2002; Prince et al. 2008; Bruno et al. 1998; Rada and Rada 2013; O'Malley et al. 2003; Gloyne et al. 2011; Wilson et al. 1987
	Cytotoxic	
	CFTR	
	T-Cell proliferation	
	Ciliary beat	
	Cytokine production	
Tuf/Lpd	Complement	Kunert et al. 2007; Hallstrom et al. 2012
Outer membrane vesicles	CFTR	Wessel et al. 2012; Bomberger et al. 2009; Bomberger et al. 2011
Protease	Complement	Schmidtchen et al. 2003; Hong and Ghebrehiwet 1992
Alkaline phosphatase	Complement	Leidal et al. 2003; Schmidtchen et al. 2003; Kharazmi et al. 1986; Kharazmi et al. 1984
	Cytokine production	
	Myeloperoxidase	
ExoU/ExoT/ExoS	Actin polymerization	Parmely et al. 1990; Chung et al. 2009; Rieber et al. 2013; Bruno et al. 1998; Geiser et al. 2001; Soong et al. 2008; McMorran et al. 2003; Diaz and Hauser 2010; Cuzick et al. 2006; Galle et al. 2008; Jia et al. 2003
	Cytotoxic	
	T-Cell proliferation	
	Rho GTPases	
	Inflammasome	
Exotoxin A	Cytotoxic	Schmidtchen et al. 2003; Parmely et al. 1990; Michalkiewicz et al. 1999; Puri et al. 1994
	T-Cell proliferation	
	Cytokine production	
N-3-oxododecanoyl/PQS	T-Cell proliferation	Skindersoe et al. 2009; Telford et al. 1998; Hooi et al. 2004; Ritchie et al. 2005
	Cytokine production	

and the antimicrobial peptide LL-37 (Schmidtchen et al. 2003; Parmely et al. 1990; Michalkiewicz et al. 1999). Exotoxin A is a tremendously potent cytotoxin, and has been adapted for use in cancer therapy by fusing the toxin to cancer cell specific antibiodies (Kreitman et al. 2012; Mazor et al. 2012).

Another mechanism whereby *P. aeruginosa* can maintain a presence in the host is to prevent complement binding and activation. Particularly in the absence of *P. aeruginosa* specific IgG, complement deposition becomes important in the

opsonization and clearance of the bacteria. On the bacterial surface the elongation factor Tuf is able to bind the human complement regulator Factor H as well as plasminogen, preventing complement activity (Kunert et al. 2007). The enzyme, dihydrolipoamide dehydrogenase (Lpd) is also able to bind Factor H and plasminogen as well as Factor H-like protein-1 and complement Factor H-related protein 1 and (Hallstrom et al. 2012). Lpd, like Tuf is surface exposed and has been shown to promote survival in serum. Alkaline protease and to a lesser extent, elastase, are both able to degrade hemolytic complement (Schmidtchen et al. 2003; Hong and Ghebrehiwet 1992). Alkaline protease is able to inhibit opsonization by cleaving C2, which blocks deposition of C3b via both the classical and lectin pathways as well as the formation of C5a (Laarman et al. 2012). Each type of host cell (neutrophils, macrophages, dendritic cells, natural killer cells, lymphocytes, and epithelial cells) has a specific role in the innate immune response to *P. aeruginosa*. How each is affected differently by secreted and injected virulence factors is reviewed below.

Neutrophils

Neutrophils are thought to be essential to the clearance of *P. aeruginosa* and numerous virulence factors directly target neutrophil function. The mechanisms utilized by *P. aeruginosa* target three general functions of the neutrophil, their abilities to sense, move and kill pathogens. Direct physical interaction of *P. aeruginosa* with the neutrophil allows *P. aeruginosa* to absorb host sialic acids and bind to siglec-9 on the surface of neutrophils (siglecs are sialic acid recognizing immunoglobulin superfamily type I transmembrane lectins) (Khatua et al. 2012). The engagement of *P. aeruginosa* with siglec-9 inhibits neutrophil engagement, decreasing elastase and ROS production and reducing neutrophil NET (neutrophil extracellular trap) production.

The secreted virulence factors, alkaline phosphatase (AP) and elastase act upon neutrophils. Although the mechanism is largely unknown they are both able to inhibit chemotaxis (Theander et al. 1988). The surface protein alginate is also able to inhibit chemotaxis and prevent clearance of *P. aeruginosa* (Pedersen et al. 1990; Song et al. 2003; Mai et al. 1993). AP and elastase also inhibit myeloperoxidase-based killing, influencing oxidative burst and superoxide production (Kharazmi et al. 1986; Kharazmi et al. 1984). Both proteins at physiological concentrations inhibit phagocytosis, possibly by cleaving surface receptors required for uptake (Kharazmi et al. 1986). The secreted type III toxins ExoS, ExoT and ExoU through their ADP ribosyltransferase activity leads to neutrophil apoptosis (Diaz et al. 2008; Sun et al. 2012). Apoptosis has also shown to be induced by the phenazine, pyocyanin. Mediated by oxidative stress, pyocyanin causes early lysozyme dysfunction, change in pH leading to loss of acidification, membrane permeabilization and caspase-3 cleavage (Usher et al. 2002; Prince et al. 2008).

Macrophages and Dendritic Cells

Macrophages and dendritic cells are also targets of *P. aeruginosa* factors that reduce their ability to clear bacteria both directly and indirectly by affecting opsonization. The initial step in clearance by macrophages is the phagocytosis of invading pathogens. Rhamnolipids produced by *P. aeruginosa* inhibit this process as demonstrated in vivo by decreased uptake of zymosan particles by macrophages in the presence of exogenous rhamnolipid (McClure and Schiller 1996; Krieg et al. 1988). *P. aeruginosa* elastase degrade lung surfactant proteins, allowing the organism to avoid opsonization and phagocytosis (Mariencheck et al. 2003). The type III toxins ExoS and ExoT inhibit bacterial uptake by macrophages by interfering with actin polymerization. These toxins also kill macrophages by inducing apoptosis, as does exotoxin A, the quorum sensing molecule *N*-3-oxododecanoyl and the secreted type III toxin ExoU (Garrity-Ryan et al. 2000; Shaver and Hauser 2004; Ritchie et al. 2005; Diaz et al. 2008). Exotoxin A inhibits interferon gamma induced activation of the co-stimulatory molecules CD80 and CD86 while ExoU, once injected into macrophages by the type III secretion machine, inhibits inflammasome activation and causes cell death in the majority of immune cell types encountered by *P. aeruginosa* (Michalkiewicz et al. 1999; Sutterwala et al. 2007; Diaz et al. 2010).

Dendritic cells act as a link to the adaptive immune response. In vivo models have shown that chronic infection with *P. aeruginosa* leads to decreased dendritic cell expression of the co-stimulation molecules CD40, CD80, and CD86 (Mukae et al. 2010). Ex vivo analysis of dendritic cells from infected animals demonstrated enhanced apoptosis of dendritic cells and reduced capacity to stimulate and recruit T cells (Boontham et al. 2008). The quorum signaling molecules *Pseudomonas* quinolone signal (PQS) and *N*-3-oxododecanoyl have been shown to reduce DC induced T cell proliferation in vivo (Skindersoe et al. 2009). T cells are required for clearance of *P. aeruginosa*, therefore inhibition of T cell activation and recruitment could provide this opportunist the chance to colonize (Cohen and Prince 2013).

Natural Killer Cells

Natural killer (NK) cells are activated by interferons and macrophage derived cytokines to tackle both intracellular and extracellular infections (Small et al. 2008). They are critical in inflammation and their role in *P. aeruginosa* infection varies depending upon the infection model (Hazlett et al. 2007; Newton et al. 1992; Laghvani et al. 2005). Although these lymphoid cells are less studied some *P. aeruginosa* virulence factors have been shown to influence their function. Inhibition of NK cell function is thought to occur by cleavage of surface receptors necessary for binding of NK cell to their targets (Pedersen and Kharazmi 1987). Exotoxin A, already implicated in bacterial killing of macrophages can also inhibit NK cell cytotoxic activity involved in oncogenesis (Michalkiewicz et al. 1999). Exotoxin A exposure leads to apoptosis due to the generation of reactive oxygen species resulting from

PI3K, caspase-9 and MAPK (Chung et al. 2009). This process is thought to offer an explanation for disease relapse in immunocompromised individuals as studies have shown *P. aeruginosa* to enhance tumor metastasis.

T Cells

T lymphocytes act as a connection between the innate and adaptive immune systems. Interference with T cell cytokine production prevents bacterial clearance and blocks the hosts ability to mount an adaptive response (Cohen and Prince 2013). Several *P. aeruginosa* virulence factors affect the ability of T cells to proliferate in the response to IL-2. Shed flagella recruit myeloid-derived suppressor cells which restrict T-cell function (Rieber et al. 2013). Alkaline protease, elastase, exotoxin A, ExoS, pyocyanin and quorum sensing molecules all have been shown to inhibit T cell proliferation (Hooi et al. 2004; Ritchie et al. 2005; Bruno et al. 1998; Puri et al. 1994; Nutman et al. 1987; Ulmer et al. 1990; Gupta et al. 2011; Smith et al. 2002). Alkaline protease and elastase inhibit IL-2 by binding to its cognate sensor (Theander et al. 1988). Exotoxin A is toxic to T cells and has shown promise in the potential removal of activated lymphocytes in allograft rejection by killing $CD8^+$ cells when conjugated to IL-4 (Puri et al. 1994). The two quorum sensing molecules *N*-3-oxododecanoyl and PQS also inhibit proliferation (Hooi et al. 2004; Gupta et al. 2011). *N*-3-oxododecanoyl causes apoptosis in CD4+ cells and T cell lines by changing mitochondrial transmembrane potential (Ritchie et al. 2005; Jacobi et al. 2009).

Epithelial Cells

Epithelial cells in many cases are the first cell type to come into contact with pathogenic organisms. It is not surprising then that the virulence factors of *P. aerguinosa* influence their function in a variety of ways to persist within the host and avoid immune clearance.

One mechanism of avoiding host detection is co-opting cells to hide. *P. aeruginosa* is able to invade and survive, avoiding immune detection in epithelial cells. Inside epithelial cells it forms pod-like clusters akin to those observed with uropathogenic *E. coli* (Garcia-Medina et al. 2005; Anderson et al. 2003; Fleiszig et al. 1994). This intracellular niche also allows *P. aeruginosa* to avoid antibotics, even those capable of permeating cells. The invasion of *P. aeruginosa* into epithelial cells requires src kinases and lipid rafts. Mice lacking caveolin-1, an important component of lipid rafts, are resistant to infection (Zaas et al. 2009; Zaas et al. 2005; Esen et al. 2001). The decision to invade cells is also dependent on the availability of nutrients. When iron is low *P. aeruginosa* invades and when high, such as in the case of cystic fibrosis sputum it forms biofilms, which are also recalcitrant to clearance (Berlutti et al. 2005).

More commonly expressed effectors such as ExoS or ExoT interfere with activation of the Rho GTPases preventing host cells from internalizing bacteria, promoting reorganization of key tight junction proteins, and allowing invasion of the bacteria across epithelial barriers (Geiser et al. 2001). The GTPase-activating protein domain of ExoS in addition to targeting the Rho pathway also prevents ERM protein phosphorylation preventing their interaction with the actin cytoskeleton, and disrupting epithelial barrier function (Soong et al. 2008). ExoU, a phospholipase, is only found in a small fraction of isolated bacteria, but is the most potent of the effector proteins and is commonly associated with strains causing ventilator associated pneumonia (Schulert et al. 2003). Following injection into host cells it binds ubiquitin and is activated (Anderson et al. 2011). ExoU can activate signaling through MAPK signaling cascades and activates transcription factor AP-1 (McMorran et al. 2003; Diaz and Hauser 2010; Cuzick et al. 2006). Furthermore, ExoU and ExoS can inhibit activation of caspase-1, limiting production of inflammasome dependent cytokines and cell death pathways (Sutterwala et al. 2007; Galle et al. 2008). ExoS is also able to induce apoptosis in epithelial as well as other cells types via caspase-3 and 9 activation (Jia et al. 2003). Pyocyanin is also toxic to epithelial cells. It induces superoxide production in cells while inhibiting catalase (Rada and Rada 2013; O'Malley et al. 2003; Gloyne et al. 2011). Pyocyanin has also been shown to inhibit epithelial ciliary beat frequency, an important component of mucociliary clearance (Wilson et al. 1987).

Infection of polarized epithelial monolayers is thought to require bacterial access to components of the epithelial basal membrane. During infections in which epithelial integrity is not compromised, *P. aeruginosa* recruits components of the basal membrane to the apical surface of the cell (Kierbel et al. 2007). Rapidly following bacterial interaction with confluent epithelial monolayers, PI3K is recruited to the apical surface, resulting in local PIP3 production and localized increases in actin. Through a proposed vesicle transport mechanism components of the basal membrane are then transported to the apical surface without disrupting tight junction integrity. *P. aeruginosa* are then able to bind and invade epithelial monolayers at these sites. It is currently unclear how the bacterium is able to induce this localized depolarization.

Conclusion

It is becoming increasingly clear that co-evolution with eukaryotic hosts has enabled successful opportunistic pathogens such as *P. aeruginosa* to develop sophisticated adaptations to host innate and adaptive immune responses. This allows the pathogen to evade immune detection partially though resistance to antimicrobial agents. *P. aeruginosa* is tremendously adept at adapting to the multiple innate immune effectors encountered in the airway, and tremendous genetic and metabolic flexibility facilitate their ability to colonize, persist, and occasionally cause invasive infection and sepsis. Modifications of virulence expression impede recognition by

host immune receptors and direct interactions with innate immune cell function prevent efficient phagocytosis and killing. Preventative strategies must take into account these complex interactions with the host and the involvement of epithelial and endothelial cells as well as the phagocytes and immune cells that target these organisms.

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Chapter 2
Pseudomonas entomophila: A Versatile Bacterium with Entomopathogenic Properties

Guennaelle Dieppois, Onya Opota, Jorge Lalucat and Bruno Lemaitre

Abstract *Pseudomonas entomophila* is unique among Pseudomonas species in being able to activate a systemic immune response in both *Drosophila* larvae and adults. It has been subsequently shown that oral infections with high doses of this bacterium are highly pathogenic to Drosophila and cause massive destruction of the Drosophila gut epithelium. Besides *Drosophila, P. entomophila* was able to kill other insects from at least three different orders, suggesting that it has a potentially wide host range and making it a promising model for the study of host pathogen interactions and for the development of bio-control agents against insect pests. In order to unravel the features contributing to *P. entomophila's* pathogenic properties, its complete genome was sequenced and genetic screens were performed to identify virulence factors encoded by this bacterium. The aim of this chapter is to review the current knowledge we have on this bacterium with a particular focus on the pathogenesis it induces, its virulence effectors and their genetic regulation.

Keywords Pseudomonas · Drosophila · gut · virulence · NRPS · regulation · entomopathogenic

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An Introduction to P. entomophila

Pseudomonas entomophila is unique among *Pseudomonas* species in being able to naturally infect and kill insects upon ingestion. Since it was first identified in 2005, *P. entomophila* has become one of the most important models for the study of insect-microbe interactions. It was originally isolated from a single *Drosophila melanogaster* female collected in Calvaire (Guadeloupe) in a screen to identify bacterial pathogens of *Drosophila*, and was named strain L48T. Upon ingestion, this strain was able to activate a systemic immune response in both *Drosophila melanogaster* larvae and adults (Vodovar et al. 2005). It has been subsequently shown that oral infections with high doses of this bacterium are highly pathogenic to *Drosophila* and cause massive destruction of the *Drosophila* gut epithelium. Since the strain L48T belonged to the *Pseudomonas* genus and exhibited entomopathogenic properties, it was named *Pseudomonas entomophila*. Besides *Drosophila*, *P. entomophila* was able to kill other insects from at least three different orders, suggesting that it has a potentially wide host range and making it a promising model for the study of host pathogen interactions and for the development of bio-control agents against insect pests. To unravel the features contributing to *P. entomophila*'s pathogenic properties, its complete genome was sequenced (Vodovar et al. 2006). In parallel, studies were performed to identify virulence factors encoded by this bacterium. This approach, combined with mechanistic studies on the host immune response and pathology, has provided considerable insights into the interaction of this bacterium with its insect host. The aim of this chapter is to review the current knowledge we have on this bacterium with a particular focus on the pathogenesis it induces, its virulence effectors and their genetic regulation.

Insights from Phenotypic and Genomics Analyses

Phenotypic and Genomic Features

The first studies of the entomopathogenic strain L48^T showed beyond doubt that it is closely related to the saprophytic soil bacterium *P. putida*. Recent work has finally allowed it to be designated officially as a novel *Pseudomonas* species (Mulet et al. 2012). Its closest relative appears to be *P. mosselii*, a poorly characterized species in the *P. putida* group that is a clinical isolate (Dabboussi et al. 2002).

Phenotypic characterization revealed that *P. entomophila* cells are Gram negative rods that are motile by means of one polar flagellum. This bacterium is strictly aerobic, catalase and oxidase positive. Figure 2.1 *P. entomophila* produces a fluorescent pigment but no pyocianin. Interestingly, the colonies exhibit a strong hemolytic activity on blood agar plates and a significant protease activity Figure 2.1 on skim milk plates that contain casein. It is also positive in the gelatinase test on the API 20NE strips. Although it is able to tolerate a wide range of temperatures

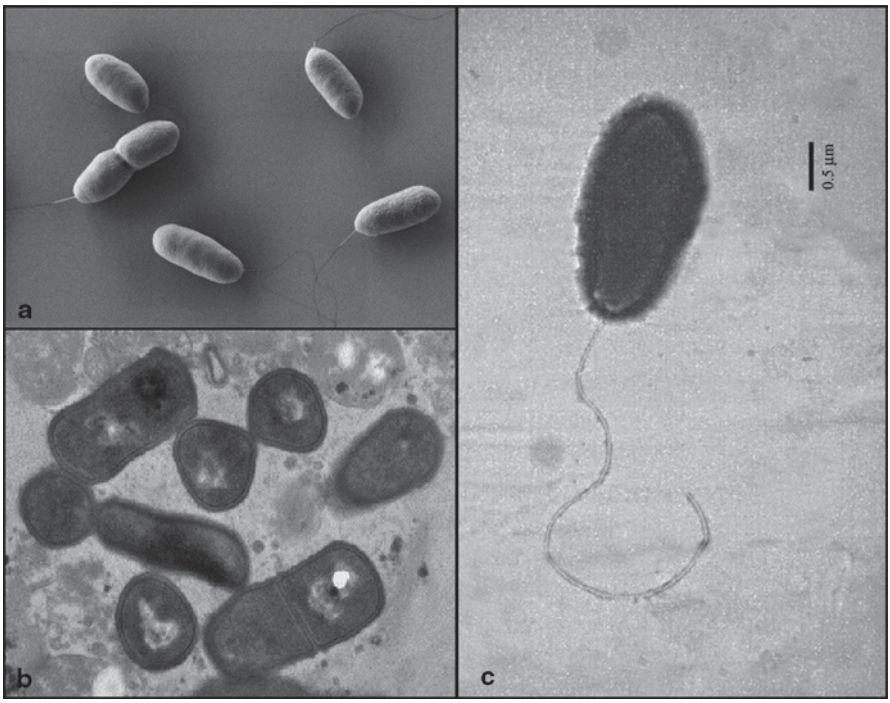

Fig. 2.1 Electron microscopy images of *Pseudomonas entomophila* L48. **a** Scanning electron microscopy picture of a *Pseudomonas entomophila* L48 bacterial culture (EPFL imaging platform B. Lemaitre laboratory). **b** Transmission electron microscopy picture of *Pseudomonas entomophila* L48 in the lumen of a *Drosophila* gut 12 h after infection (CCME Orsay B. Lemaitre laboratory). **c** Transmission electron microscopy of a negatively stained cell of *P. entomophila* L48. (Mulet et al. 2012, Supplementary data)

(4 to 42 °C) and pHs (pH 3 to pH10), its optimal temperature growth is around 30 °C and its optimal pH between 5 and 9 (Mulet et al. 2012; Guennaëlle Dieppois, unpublished results).

The unique *P. entomophila* chromosome sequence was published in 2006 (Vodovar et al. 2006). It contains 5,888,780 nucleotides with a GC content of 64.2 %. It is of an intermediate size compared to the other 20 sequenced *Pseudomonas* genomes but is smaller than the genome of the opportunistic pathogen *P. aeruginosa*. Comparisons between the genome of *P. entomophila* and those of five other *Pseudomonas* species including *P. putida* identified a set of 1002 genes that were unique to *P. entomophila*. In agreement with the close relationship between *P. entomophila* and *P. putida*, 70.2 % of *P. entomophila* genes have orthologs in *P. putida*. However, the genome of *P. entomophila* has been remodeled to lesser extent by mobile elements such as bacteriophages than that of *P. putida*. This observation could be explained by the fact that the *P. entomophila* genome contains only six putatively active transposase-like proteins. Furthermore, unlike the genome of *P. putida*, the genome of *P. entomophila* is devoid of type II introns.

P. entomophila is a Versatile Bacterium

The complete genome sequence of *P. entomophila* provides interesting insights into this organism's lifestyle. Similar to other *Pseudomonas* species, the predicted properties of *P. entomophila* indicate that this strain contains a large set of genes involved in the adaptation to multiple carbon sources. This suggests that *P. entomophila* is a metabolically versatile bacterium capable to survive in the soil, rhizosphere and water. Notably, the *P. entomophila* genome contains several genes that encode proteins with hydrolytic activities such as chitinases, lipases, proteases and uncharacterized hydrolases that might be involved in the degradation of polymers found in the soil. In addition, the *P. entomophila* genome harbors determinants for the catabolism of various aromatic compounds and long-chain carbohydrates making it potentially useful for bioremediation. Finally, *P. entomophila* encodes more than 535 transporters and a high numbers of regulators suggesting that it is able to adapt to substantial substrate variations. Corroborating these findings, additional *P. entomophila* strains have recently been isolated from the rhizosphere (Kamala-Kannan et al. 2010) and soil (Shahbaz-Mohammadi and Omidinia 2011) including contaminated soils (Yergeau et al. 2012). However, in contrast to phytopathogenic strains such as *P. syringae*, the genome of *P. entomophila* is devoid of genes encoding enzymes capable of degrading plant cell walls. This is consistent with the observation that this species is not pathogenic for plants (Vodovar et al. 2006). On the contrary, *P. entomophila* may even benefit plants by promoting resistance to various stresses (Kamala-Kannan et al. 2010). Moreover, Vallet-Gely and colleagues determined that *P. entomophila* was able to protect plants from pathogenic fungi and to promote their growth as *P. fluorescens* does (Vallet-Gely et al. 2010a). This was demonstrated by the ability of cucumber plants to grow in the presence of root pathogenic fungus *Pythium ultimum* only when *P. entomophila* or alternatively the well-characterized *P. protegens* (formaly *fluorescens*) biocontrol strain CHA0 was added into the soil.

P. entomophila Iron Acquisition

Like all other organisms, iron is an essential nutrient for *Pseudomonas* species. Fluorescent *Pseudomonas* have adapted to the poor solubility of Fe^{3+} in their aerobic environment by evolving high affinity iron uptake systems. These systems rely on the uptake of heme or iron siderophore complexes by Ton-B outer membrane receptors that recognize the iron loaded complexes. The *P. entomophila* genome possesses gene clusters encoding proteins required for pyoverdine siderophore biosynthesis and uptake (Vodovar et al. 2006) and for the synthesis of another siderophore related to acinetobactin (Yamamoto et al. 1994). In addition, the *P. entomophila* genome harbors *hasR* and *hxuC*, the heme uptake-related genes that are typically found in the opportunistic pathogen *P. aeruginosa* (Ochsner et al. 2000; Bodilis et al. 2009) but not in *P. putida*. More strikingly, the *P. entomophila* genome carries a considerable number of putative TonB-dependent receptor genes and a high number of 'orphan' TonB-dependent receptor genes that are not found on the

genomes of *P. putida* strains (Bodilis et al. 2009; Matthijs et al. 2009). In support of these data, *P. entomophila* is able to utilize a large variety of heterologous pyoverdines siderophores (Matthijs et al. 2009). The wide variety of iron uptake systems in *P. entomophila* shows that this bacterium is well equipped to survive extreme iron limitation and argues for the importance of iron acquisition in the ecology of this bacterium. This feature may be extremely important not only for its lifestyle in soil but also for its survival within its insect hosts where iron is likely to be rarer. Moreover, for a number of bacterial pathogens including *P. aeruginosa*, iron deprivation is important in triggering the synthesis of many virulence factors (Prince et al. 1993). In agreement with an essential role of *P. entomophila* iron uptake systems in the infection process, we have recently identified a Ton B receptor gene as an important factor for the virulence of this bacterium (G. D. unpublished data).

P. entomophila as a Source of Secondary Metabolites

Among the factors that may be fundamental for interaction with insects, the genome of *P. entomophila* harbors five gene clusters associated with production of secondary metabolites. One gene cluster, coding for a non-ribosomal peptidesynthase (NRPS) is responsible for hydrogen cyanide (HCN) production. The other four clusters code for NRPSs predicted to synthetize at least three different lipopeptides and a polyketide of unknown function (Vodovar et al. 2006).

It has indeed been confirmed that *P. entomophila* can synthesize HCN, placing it among the small number of cyanogenic bacteria (Ryall et al. 2009). Although HCN production by *P. entomophila* is regulated by oxygen availability, it does not seem contribute to the pathogenicity towards *Drosophila* (Vallet-Gely et al. 2010b). The role of the other NRPS gene clusters in *P. entomophila* virulence has also been investigated (Vallet-Gely et al. 2010b). Only one of them (*pseen*0131, *pseen0132*, *pseen0133*,) appears to play a crucial role. It has subsequently been named PVF for *P*seudomonas *V*irulence *F*actor (See below for further details on PVF).

The nature of a product that is synthetized by another NRPS (*pseen3332, pseen3044, pseen*3045) has also been deciphered. It is involved in the production of a new cyclic lipopeptide of 14 amino acids and 3 C10-OH fatty acids. This lipopeptide is responsible for both the hemolytic and surfactant activity observed in the supernatant of *P. entomophila* and has, thus, been called Entolysin (Vallet-Gely et al. 2010a) (see later).

Although, HCN and most of the secondary metabolites encoded by *P. entomophila* do not seem to be essential for its virulence, their production may have other substantial roles. For example in other *Pseudomonas* species, these molecules are involved in bio-control (Haas 2005), in the killing of nematodes and in suppressing microbial competitors in the soil (de Bruijn et al. 2007; Li et al. 2013; Neidig et al. 2011). Of note, it has recently been observed that *P. entomophila* is clearly pathogenic to *C. elegans* (Olivier Zugasti personal communication) and was able to outcompete several other species, such as *Mycobacterium marinum* and the fungus *Candida albicans* (Onya Opota personal communication).

Putative Virulence Factors Against Insects

Several genes in the genome of *P. entomophila* have been associated with its entomopathogenicity. For example, the presence of genes that encode for TccC-type insecticidal toxin are particularly striking since they are only found in entomopathogenic bacteria such as *Photorhabus luminescens* and *Xenorhabus nematophila* (Hinchliffe et al. 2010) and absent from other *Pseudomonas* genomes (Vodovar et al. 2006). Moreover, like *P. syringae*, the *P. entomophila* genome encodes other proteins more distantly related to TccC and TcdB-type insecticidal proteins. The function of these proteins in the virulence towards insect has not yet been tested.

Proteases are also thought to contribute to the virulence of bacterial species. Interestingly, *P. entomophila* encodes three serine proteases (*pseen3027, pseen3028, pseen4433*) and one alkaline protease (*pseen1550*) absent from *P. putida*. The latter is a homolog of AprA which has been shown to be involved in virulence in other bacteria by protecting against the immune response and degrading of host tissues (Hong and Ghebrehiwet 1992; Leduc et al. 2007; Miyoshi and Shinoda 2000; Parmely et al. 1990; Travis et al. 1995). Notably, AprA has been shown to be the most abundant protein in *P. entomophila* supernatant (Liehl et al. 2006). Illustrating its importance, the visible protease activity of *P. entomophila* on skim milk plates is completely abolished in an *aprA* mutant. How AprA contributes to *P. entomophila* pathogenesis will be more specifically addressed in another section of this chapter.

P. entomophila also carries a number of genes coding for cell surface associated factors that usually contribute to pathogenesis by allowing adhesion to the host surface and thus, an effective colonization. Among them, we find genes coding for filamentous haemagglutinin, a surface adhesion protein and the amyloid curli fiber. In agreement with a role of these genes in *P. entomophila* virulence, they often cluster with genes coding for type I secretion systems (T1SS). T1SS, T3SS, T4SS, and T6SS are actively involved in the virulence of pathogenic bacteria as they promote the direct delivery of exo-proteins into the extracellular medium (T1SS) or into the host cell (T3SS, T4SS, and T6SS).

Interestingly, *P. entomophila* is the first *Pseudomonas* strain that is pathogenic in a multicellular organism and yet is devoid of a T3SS secretion system. This bacterium only possesses a single locus containing the conserved core genes of T6SS proteins as well as several T6SS homolog proteins (VgrG and Hcp) dispersed in the genome. Notably, we found that components of the T6SS (Vgr, Rhs and Hcp proteins) were among the most abundant proteins excreted in the supernatant of this bacterium (Opota et al. 2011). Moreover, this system might actively participate in *P. entomophila* virulence since T6SS mutants were found in a screen for factors contributing to *P. entomophila* pathogenesis (G. D. unpublished). Owing to the lack of the common T3SS and T4SS, the single T6SS could play an essential role in the insect–bacterium interactions (Sarris and Scoulica 2011).

Overall, the analysis of the *P. entomophila* genome and its general phenotypic properties indicate that this bacterium has the potential to infect and colonize insect niches. How this is useful for the bacteria's life cycle remains unclear, as it

has never been found associated with other insects in the wild beyond its initial isolation. However, the use of this bacterium as a model to study insect pathogen interaction has helped to understand the interconnected nature of the mechanisms underlying pathogenesis and the host immune pathways.

P. entomophila has so far been shown to be pathogenic for three orders of insects from Diptera (Anopheles gambiae D. melanogaster), Lepidoptera (e.g. *Bombyx mori, Galleria mellonella*) and Coleoptera (e.g. *Sitophilus oryzae*). It is also lethal to the ameobae *Dictyostelium discoideum* (Vallet-Gely et al. 2010a) and leads to the slow killing of *C.elegans* (O Zugasti personal communication). *P. entomophila* pathogenesis has been mostly studied in *Drosophila* adults and larvae. In a model of septic injury (i.e. direct pricking of a culture pellet into the body cavity), this pathogen kills flies in a day. When *P. entomophila* is ingested at high doses by adults (OD_{600} 50–100), the flies succumb to infection within 2–3 days. Survival increases if the bacteria are less concentrated (OD_{600} between 5 and 25) although the mortality is still significantly greater compared to non-infected flies after 4 days. However, below OD_{600} 1 ingestion of *P. entomophila* is apparently not lethal for the flies (Buchon et al. 2009a) indicating that only a large dose can overcome the *Drosophila* immune system and kill flies (Figs. 2.1 and 2.2). Of note, none of the other Pseudomonas species exhibit the same level of virulence towards *Drosophila* when ingested at high dose, demonstrating specific pathogenic properties of *P. entomophila*. Considering these observations, the overall pathogenesis of *P. entomophila* must depend on the following properties (i) the ability to enter and persist in the gut which is related to its capacity to survive both physicochemical conditions and the immune defenses of this organ (ii) the excretion of toxic substances that disrupt the host physiology.

Survival of P. entomophila in the Harsh Conditions of the Drosophila Gut

The mechanisms by which ingestion of high doses of *P. entomophila* can kill *Drosophila* have been well characterized. We have observed that after starvation *Drosophila* does not particularly avoid *P. entomophila* and ingests high doses easily when the bacteria pellet is mixed with sucrose (G.D. unpublished). Similar conditions might occur in the wild since bacteria are found at high concentrations in decaying fruits.

Interestingly one of the most overt phenotype observed after the ingestion of relevant doses of *P. entomophila* is an immediate blockage of food uptake (Liehl et al. 2006; Vodovar et al. 2005). This results in a visible accumulation of ingested bacteria in the fly crop. This food-uptake blockage phenotype is also associated with other entomopathogenic bacteria such as *Serratia entomophila* and *Yersinia pestis* (Vallet-Gely et al. 2008). How this phenomenon occurs and contributes to pathogenesis is still unclear. It has not yet been established whether *P. entomophila* directly induces this phenomenon or if food uptake blockage is a host response to intestinal damage associated with the infection process.

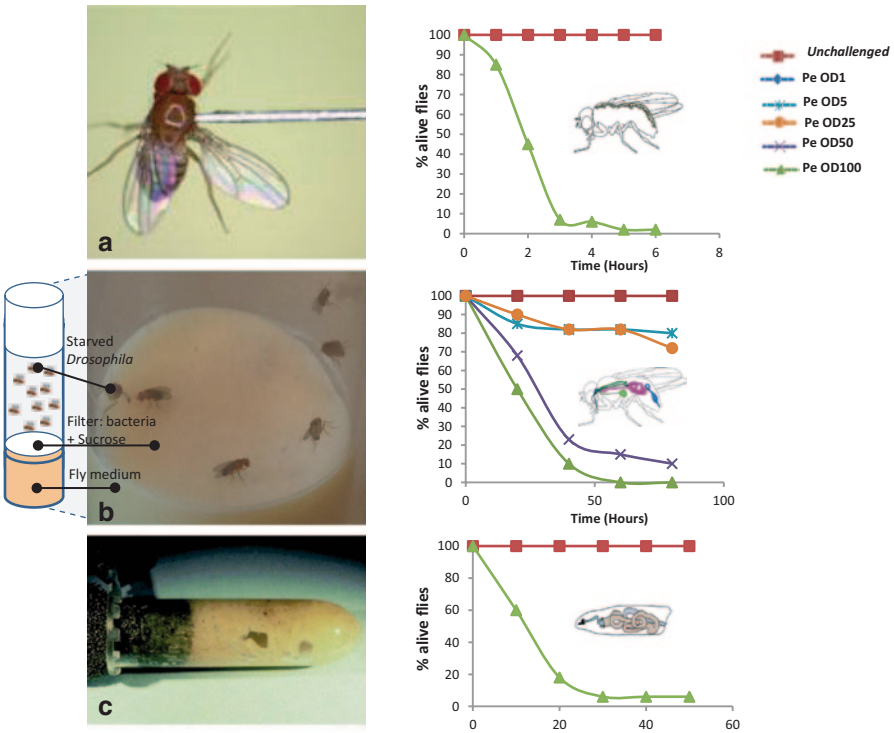

Fig. 2.2 Methods developed to infect *Drosophila* with *P. entomophila*. **a** Systemic infection. Bacterial injection is achieved by pricking adult flies in the thorax with a needle that has been dipped into a concentrated bacterial solution or by microinjecting a precise dose of microbes into the body cavity. Through this type of infection, all microbes induce a strong immune response that is specific for that type of microbe as they are in direct contact with immune system sensors in the body cavity. *P. entomophila* kills flies within a few hours only. **b** Natural infection of *Drosophila* adults. To mimic a natural infection by feeding, the adult flies, preferentially females of 3–5 days are starved prior to the infection. They are then transferred to in fly vials containing 200 ul of a bacterial pellet usually at OD_{600} 100 mixed with 1 % sucrose put on a filter on top of the fly medium in the vial. The fly medium contains antibiotics that rapidly inactivate bacteria. Flies are thus infected by a single high dose of live bacteria. After ingestion, the flies die within 2–3 days with a pellet at OD_{600} 100. They survive longer when the pellet is less concentrated. Their survival is not affected when they ingest bacteria at OD_{600} below 5 (5.10^9 cells/ml). **c** Natural infection of *Drosophila* larvae. Third instar larvae are mixed with a solution of crushed banana containing bacteria at OD_{600} 100 for one hour before being transferred back to a normal fly vial. Larvae are more susceptible than adults to *P. entomophila* infection and are killed in 1 day. These two types of natural infection are inducing the local immune response in the gut and the systemic immune response. Only few strains are able to induce with these types of infection immune response reflecting that it can efficiently mimic the specifics interactions between *Drosophila* and its pathogens

However, living and dividing *P. entomophila* cells have been observed by both fluorescence and electron microscopy in the lumen in different regions of the gut, confirming that this bacterium is able to travel along the gut and to resists the gut conditions, at least for 16 h.

The *Drosophila* midgut is considered a hostile environment for most ingested bacteria due to its very low pH (pH = 2–3 in the middle midgut) (Shanbhag and Tripathi 2009) and the production of a large number of lysozymes (Daffre et al. 1994). Nevertheless, in vitro studies indicate that *P. entomophila* can grow even at a low pH of 3 (Mulet et al. 2012) and is highly resistant to lysozymes activity (2 mg/ml) (Opota and Dieppois, personal communication). Interestingly, *P. entomophila* is also resistant to high concentrations of H_2O_2 in vitro (up to 1 mM; Dieppois, unpublished). These observations could explain why *P. entomophila* is able to resist those first line defenses, including the microbicidal activities of reactive oxygen species that are immediately produced by in the gut by the NADPH Duox, in response to microbial infections (Chakrabarti et al. 2012; Ha et al. 2005a, b, 2009a, b).

P. entomophila Counteracts the Production of Antimicrobial Peptides

Another important line of defense is the production of antimicrobial peptides such as Diptericin, Attacin and Drosocin. They are secreted by the gut epithelium upon oral infection gram-negative bacteria. This response is mediated by the Imd signaling pathway which is activated by diaminopimelic acid (DAP)-type peptidoglycan found in bacilli and gram-negative bacteria (Kaneko et al. 2004; Leulier et al. 2003; Stenbak et al. 2004). A microarray analysis showed that *P. entomophila* infection strongly stimulates the expression of antimicrobial peptide genes in the gut (Chakrabarti et al. 2012). Thus, this bacterium is specifically recognized by the local immune system. Moreover, our laboratory previously showed that overexpression of the Imd pathway in the gut prior to infection confers resistance to *P. entomophila* (Liehl et al. 2006) (Chakrabarti, unpublished results), indicating that this bacterium is to some extent sensitive to the activity of antimicrobial peptides. We recently understood that the ability of *P. entomophila* to survive the *Drosophila* innate immune response is dependent on its capacity to inflict damages to the gut epithelium.

Histological studies on larvae and adults show that *P. entomophila* infection is associated with epithelial cell damage such as enterocytes displaying abnormal microvilli or undergoing cell death. Electron microscopy pictures show that the peritrophic matrix (PM), a chitinous matrix that lines the midgut epithelium of insect is still present after 12 h of infection, suggesting that *P. entomophila* is unable to cross this protective barrier and resides within the lumen at least in the first hours of infection (Kuraishi et al. 2011; Vodovar et al. 2005). Consistent with these observations, *P. entomophila* is not detected in the haemocoel of flies. Hence, this bacterium is able to damage the gut without physically contacting the gut epithelial layer. The severity of intestinal damage is consistent with data showing that the repair and stress pathways are strongly activated upon *P. entomophila* ingestion (Chakrabarti et al. 2012).

It has been shown that *P. entomophila* survives the activation of the immune response because the infection rapidly reduces the translation level in the gut

epithelium cells as an adaptive stress response to damages inflicted by the bacterium (Chakrabarti et al. 2012). While antibacterial genes are induced at the transcriptional level in the gut upon infection with *P. entomophila*, the effective production of antimicrobial peptides is blocked due to this general inhibition of translation that affects the translation of all newly synthesized transcripts.

P. entomophila Ingestion Leads to Death by Rupture of the Gut

Another consequence of this translational arrest is the inhibition of the epithelium renewal program which is necessary to repair the damage caused by the infection (Amcheslavsky et al. 2009; Buchon et al. 2009b). Studies using non-lethal bacteria pathogens have shown that recovery from infection only occurs when bacterial clearance by the immune system is coordinated with a repair of tissue damages. The current model is that translational inhibition is the primary cause of fly death by preventing both immune and repair pathways. As a consequence, the bacteria are not eliminated and flies succumb as their gut shrink and rupture.

The reduction of translation is a direct consequence of the excessive activation of stress pathways caused by the cellular damage inflicted to the intestine by *P. entomophila*. Two factors contribute to these cellular damages. *P. entomophila* produces toxins that cause epithelium damage (Opota et al. 2011). However, the main factors damaging the gut epithelium are the reactive oxygen species (ROS) produced by the host itself. Several studies in *Drosophila* have demonstrated the importance of intestinal ROS production surviving oral infection (Ha et al. 2005b, 2009b). However, the level of ROS produced by the gut is significantly higher upon *P. entomophila* ingestion than with other pathogenic but non-lethal bacteria such as *Erwinia carrotovora* subsp. *Carrotovora* strain *15* (Chakrabarti et al. 2012). In the case of *P. entomophila* infection, this oxidative burst not only fails to eliminate the bacterium but is also highly deleterious to the gut. By both creating irreversible damages and inducing the translational inhibition that prevents repair and immune response, the high level of ROS that results from ingesting *P. entomophila* is the cornerstone of this bacterium's pathogenesis.

The Toxins of *P. entomophila*

As mentioned above, the *P. entomophila* genome contains a large set of genes encoding putative virulence factors (Vodovar et al. 2006). A genome wide transposon mutagenesis subsequently led to the identification of some of the genes involved in virulence. With the exception of the genes coding for the NRPS synthesizing PVF, no genes predicted to be virulence effectors were identified in the initial screens (Vallet-Gely et al. 2010b; Vodovar et al. 2006). In contrast, mutations in several genes encoding regulators significantly attenuated virulence. Furthermore, it later turned out that PVF was also a regulatory factor instead of

an effector (Vallet-Gely et al. 2010b) (see later). The fact that several general regulators have a major effect on virulence suggested that *P. entomophila* virulence is multifactorial (Figs. 2.3 and 2.4). Studies on entomopathogenic bacteria such as *Bacillus thuringiensis*, *Bacillus sphaericus* or *Serratia entomophila* have shown that the production of secreted toxins is a common strategy to interfere with insect gut homeostasis (Bravo et al. 2007, 2011; Charles et al. 1996; Hurst et al. 2000, 2007; Soberon et al. 2007). This prompted us to analyze the proteins secreted by *P. entomophila* and led to the identification of three virulence factors: the protease AprA, the pore-forming toxins Monalysin and Entolysin.

The Metalloprotease AprA

In many γ-proteobacteria, the GacS-GasA two-component system is a major regulator controlling the expression of secondary metabolites, protein secretion systems, and virulence determinants. Interestingly, *P. entomophila* derivatives lacking the GacS-GacA system are completely avirulent (Vodovar et al. 2006). This suggested that *P. entomophila* secretes some virulence factors under the control of the GacS/GacA two-component system. Accordingly, a concentrated *P. entomophila* supernatant is highly toxic to adult flies when directly injected into the hemocoel while it has a detrimental effect on *Drosophila* larvae (but not adult) upon ingestion (Liehl et al. 2006). Proteomic analysis revealed that *P. entomophila* but not the *gacA* mutant secretes a major protein of 51 kDa, an alkaline metalloprotease of the AprA family. These proteases belongs to the metzincin RTX proteins family which are metalloendopeptidases secreted via a T1SS secretion system (Lally et al. 1999). Similar to the other bacterial species, the *aprA* genomic region also contains *aprI*,a gene coding for the specific inhibitor of alkaline protease, as well as three genes that encode a T1SS components (*aprD, E, F*) (Bardoel et al. 2012), which are required for AprA secretion in other species (Duong et al. 1996). Since AprI is predicted to be a periplasmic protein in other bacteria, it might function as a chaperon to prevent degradation of self-protein by AprA prior its secretion.

The *P. entomophila aprA* mutant does not exhibit any protease activity in *in vitro* assays (Liehl et al. 2006). Initial results from our laboratory showed that *aprA* mutant was less virulent than the wild-type after oral infection of larvae and adult flies, with a lower persistence. The observation that AprA mutants persist as the wild-type in immune deficient (Imd) mutant flies raised the hypothesis that AprA could degrade antimicrobial peptides induced by the Imd pathway in the gut. Accordingly, *aprA* mutants persist less well in flies over-expressing an antibacterial peptide. A role of AprA in the protection against AMPs is consistent with other studies showing that homologues of AprA have the ability to degrade AMPs *in vitro* (Miyoshi and Shinoda 2000; Travis et al. 1995). Contrasting with the initial report, recent studies of our laboratory did not reproduce the attenuated virulence as *aprA* mutants kill flies in a manner that is similar to wild-type bacteria (Fig. 2.2b of (Liehl et al. 2006)). Thus, although *P. entomophila* AprA can counteract the action

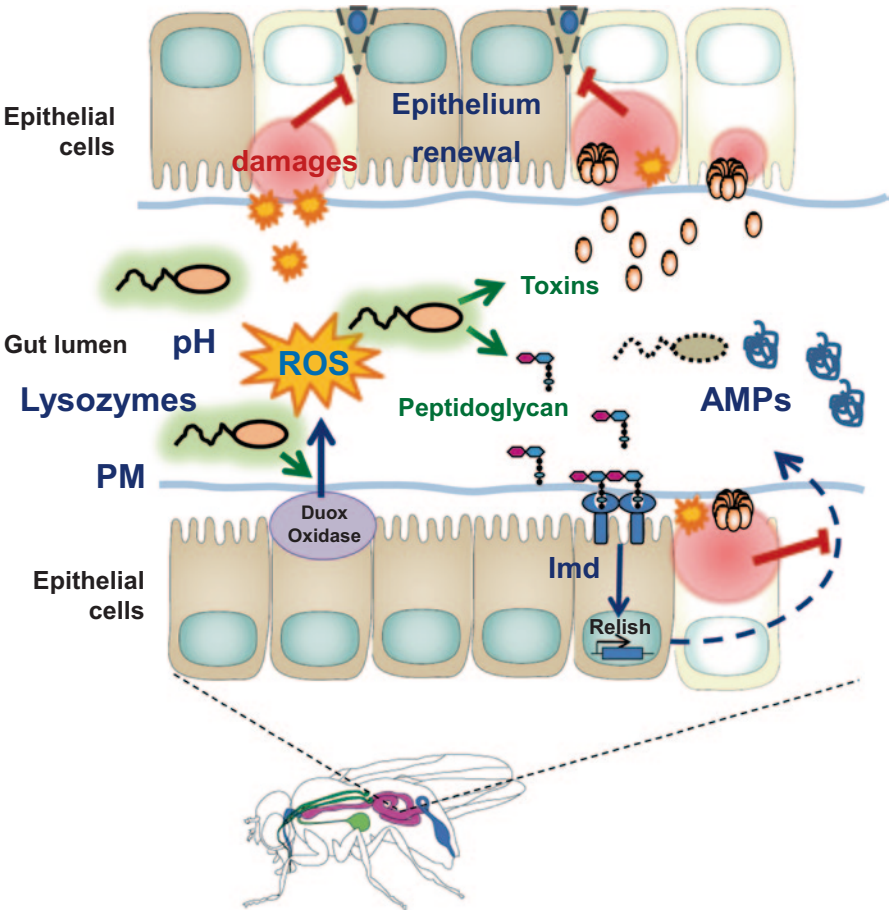

Fig. 2.3 Mechanisms of *P. entomophila* pathogenesis. In *blue*: The gut epithelium defenses: A naïve gut is protected by its low pH, production of digestive enzymes such as lysozymes and the periptrophic matix (PM) lining the epithelium. Upon infection, the NAPDH oxidase Duox is activated by the uracil released by the bacteria and produces reactive oxygen species (ROS). In addition, peptidoglycans fragments liberated from the bacteria are detected by extracellular and intracellular receptors that activate the Imd pathway and the synthesis of antimicrobial peptides (AMPs). In order to replace the gut epithelium that could have been damaged by bacterial toxins or the oxidative burst, the repair pathways are activated and promotes the replication and the differentiation of stem cells (Epithelium renewal). In *green*: *P. entomophila* properties. *P. entomophila* is able to resists to the gut conditions and to oxidative stress (ROS). It induces a particularly high level of ROS production and directly produces toxins. It releases peptidoglycan fragments that are recognized by the Imd pathway. In *red*: Consequences of a *P. entomophila* infection. The high level of ROS and the toxins produced by the bacterium are creating important damages in the epithelium cells. It over-activates the stress pathways that inhibits the general translation and thus blocks both epithelium repair and the effective production of AMPs. The fly succumbs to a disruption of their gut

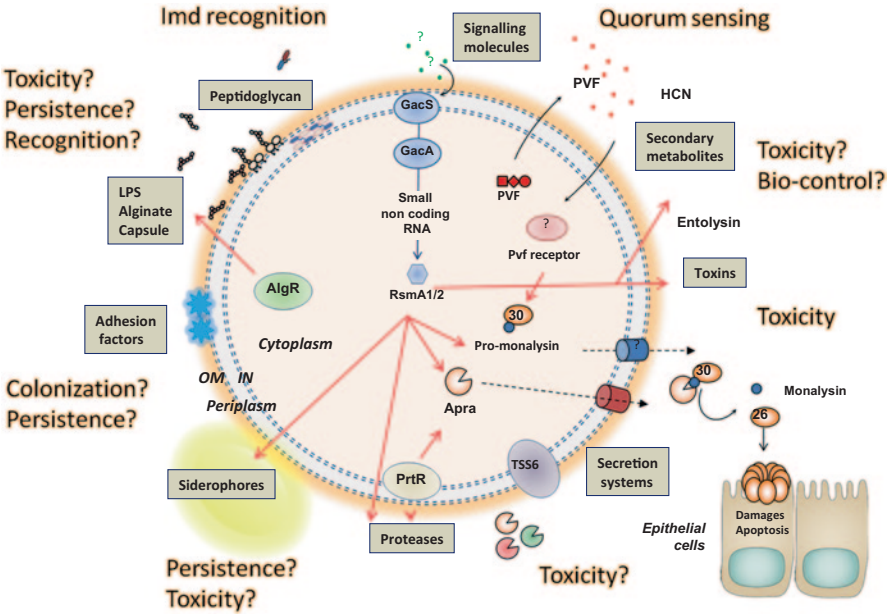

Fig. 2.4 *P. entomophila* virulence is multifactorial. The Gac, AlgR and PrtR systems and PVF signal transduction system plays a central role in the regulation of virulence factor expression. Among the secreted factors involved in virulence are proteases, toxins secondary metabolites and siderophores. They might be involved directly in the toxicity or promote the persistence or colonization within the gut. The secretion systems of these different factors are not all known. Some other cellular factors might be also very important for the virulence of *P. entomophila*such as adhesion protein, LPS or components of the capsule. Peptidoglycan is directly recognized be the receptors of the Imd pathways

of antimicrobial peptides, the precise contribution of this factor to *P. entomophila* virulence towards Drosophila should be revaluated.

However, there are several lines of evidence of more direct roles of AprA in promoting *P. entomophila* killing of *Drosophila*. First, pure AprA protease has a toxic activity when injected directly into the body cavity of adult flies or fed to larvae (Liehl et al. 2006). Moreover, besides its possible direct effect on cytotoxicity, AprA has also been recently shown to contribute to the maturation of the Monalysin pore forming toxin (Opota et al. 2011) (see later). Altogether, these studies indicate that AprA is a virulence factor that plays a role in many facets of *P. entomophila* pathogenesis. On one hand it can improve persistence by counteracting the effect of the immune response effectors. On the other, it might promote the capacity of *P. entomophila* to damage the gut epithelium.

The Pore-Forming Toxin Monalysin

Monalysin was initially identified as a secreted protein of 30 KDa present in the culture supernatants of wild type strains but not the *gac* mutants. Mutation in the Monalysin gene (*pseen3174*) caused an attenuated virulence phenotype associated with a reduction of intestinal damage (Opota et al. 2011). Biochemical characterization revealed that Monalysin is a new pore-forming toxin, which is consistent with its contribution to the cellular damage induced by *P. entomophila*. With the exception of two uncharacterized orthologs found in *P. putida*, Monalysin does not display any significant homology to other proteins.

Pore-forming toxins (PFT) are among the most prevalent virulence effectors found in pathogenic bacteria (Iacovache et al. 2008). These proteins form oligomers that can insert into the cell wall of target cells inducing the formation of lytic pores. PFTs can insert into the membrane of target cells by two main mechanisms: the first involves insertion of amphipathic alpha-helices, while the second involves insertion of ß-hairpins. The Monalysin sequence contains an internal domain consisting of amphipathic patches flanked by serine and threonine rich sequence, which is an hallmark of the membrane-spanning region of ß-barrel pore-forming toxins (Opota et al. 2011). Accordingly, Monalysin displays all the structural characteristics of members of the ß-PFT group, which includes Aerolysin, (produced by several *Aeromonas* species) and ε-toxin from *Clostridium perfringens*, and MTX-3 from *Bacillus sphaericus* (Bischofberger et al. 2009).

To prevent oligomerization of the toxins within the bacterium, PFTs are usually synthesized as immature pro-toxins that require a proteolytic cleavage to become fully active. The processing of exotoxins can be performed by proteases produced by the bacterium itself or by enzymes of the host digestive tract such as trypsin or chymotrypsin (Bischofberger et al. 2009; Gonzalez et al. 2008; Opota et al. 2011). Monalysin, is produced as a soluble 30.2 kDa pro-toxin, pro-Monalysin. After secretion, Pro-Monalysin is processed by proteolytic cleavage at the N-terminus to a mature form of 26.5 kDa. In a supernatant of wild-type *P. entomophila* the mature form of Monalysin is predominant, while the immature form is predominant in the supernatant of *aprA* mutant. This suggests a model in which the metalloprotease AprA participates in the maturation of pro-Monalysin to Monalysin.

The removal of the N-terminal fragment allows the formation of SDS resistant oligomers able to insert within the membrane of eukaryotic cells leading to cell death (Buchon et al. 2009a; Opota et al. 2011; Vallet-Gely et al. 2010b). This maturation step thus, enhances the cytotoxic activity of Monalysin. To date the exact size of the pore formed by the aggregation of Monalyin monomers remains unknown.

Monalysin displays a strong cytotoxicity towards cell lines derived from *Drosophila*, lepidoptera *Spodoptera frugiperda* and even mammals. The *Drosophila* cells treated with purified Monalysin showed DNA fragmentation and condensation phenotypes that are characteristic of apoptosis (Opota et al. 2011). Many pore-forming toxins act via a specific receptor, such as the GPI anchor (Aerolysin), present on the surface of the target cells (Darboux et al. 2001; Opota et al. 2008). The receptor

targeted by Monalysin remains to be identified. However, the broad range of activity of Monalysin indicates that it could be a receptor present in diverse cell lines.

Injection of Monalysin in the body cavity of flies killed them within few hours, which is consistent with its cellular toxicity *in vitro*. Nevertheless, the recombinant protein fed alone to flies was not lethal.

The Dual Lipopeptide Entolysin

P. entomophila has a strong diffusible hemolytic activity which is easily visualized on blood agar plates (Vallet-Gely et al. 2010a; Vodovar et al. 2006). To identify the genes responsible for this hemolytic activity, a transposon library of *P. entomophila* was individually screened for their ability to lyse blood cells. One of the hemolytic deficient mutants was affected in a gene, which was named *etlA* for Entolysin A. *etlA* encodes a NRPS similar to *P. putida* NRPS protein PsoB. PsoB is involved in the synthesis of the cyclic lipopeptide, Putisolvin that exhibits surfactant activity (Dubern et al. 2008).

In addition to *psoB*, the synthesis of Putisolvin requires two other NRPS genes, *psoA* and *PsoC*. Analogs of these genes are also present in the *P. entomophila* genome and were named *eltB* and *etlC*. As expected, mutations in each of the *P. enthomophila* Entolysin genes (*etlA, etlB and etlC*) induce a loss of hemolytic activity and surfactant production. Consistent with other studies showing a link between surfactant production and swarming motility (Berti et al. 2007; Daniels et al. 2006; Deziel et al. 2003; Kearns and Losick 2003), these *P. entomophila* mutant strains were also unable to swarm (Vallet-Gely et al. 2010a).

The Entolysin product responsible for hemolytic and surfactant properties was isolated from *P. entomophila* supernatant (Vallet-Gely et al. 2010a). It is a cyclic lipopeptide composed of 14 amino acids that form a ring with three C10-OH fatty acids. Unusually, the cyclization of this lipopeptide occurs between the C-terminal carboxyl group and the first amino acids. *etlA, etlB and etl*C genes are predicted to encode three NRPS of two, eight, and four modules that incorporate one monomer each. Since Entolysin is composed of 14 AA, this corresponds to the number of modules and suggests that these three genes might be the only ones that are directly involved in the biosynthesis of the lipopeptide. In addition, the *etl* genes are organized in two distinct gene clusters, which comprise a LuxR-type transcriptional regulator (EtlR) and an analog of a macrolide ABC transporter (MacAB) (Kobayashi et al. 2001), that are also involved in Entolysin production.

Surprisingly, Entolysin deficient *P. entomophila* strains were able to infect and kill adult *Drosophila* as well as the wild type bacterium. It is possible that *etl* mutants are still virulent because other factors, such as Monalysin or AprA, are still active. Interestingly, recent results from our laboratory, showed that *Drosophila* third instar larvae fed with a concentrated supernatant of *P. entomophila* that lacks Entolysin survived better when compared to these fed with a wild-type supernant (G.D. unplublished data). This data indicates that at high concentration Entolysin contributes to the toxicity of *P. entomophila*.

Gene Regulatory Network Controling P. entomophila Virulence

The virulence of P. *entomophila* is multifactorial and includes the production of toxins as well as factors that protect the host from the immune system. As in other pathogenic bacteria, expression of virulence factors is energetically expensive and thus, is tightly regulated. Like other Pseudomonas species, P. *entomophila* possesses an extraordinary number of regulatory systems, which give it a sophisticated ability to dynamically regulate metabolism and processes that are important in interacting with its host. The following section summarizes what is known about virulence factor regulation in P. *entomophila***.**

The GacS/GacA Two Component System

The most critical regulation factor controlling virulence in *P. entomophila* is the GacS/A two-component system. Indeed, *P. entomophila gacA* or *gacS* mutants are completely avirulent against *Drosophila*, *Dyctiostelium* and *C. elegans*. The sensor kinase GacS and the response regulator GacA are members of a two-component system present in many other Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae, Erwinia carotovora*, and *Legionella pneumophila* (Heeb and Haas 2001). The GacS sensor kinase autophosphorylates and activates the GacA response regulator by phosphorylation in a cell population density-dependent manner but in response to an as yet unidentified signals or stimuli. In most pathogenic bacteria, *gacS/gacA* mutants show a reduced production of virulence factors and are less virulent than the wild type (Rahme et al. 2000). The GacS/GacA two-component system positively regulates (the name gac for *g*lobal *ac*tivator) the expression of virulence factors, secondary metabolites, biofilm formation and quorum sensing in early stationary phase (Balasubramanian et al. 2011; Gao and Stock 2010; Mascher et al. 2006; Mitrophanov and Groisman 2008; Raghavan and Groisman 2010; Rodrigue et al. 2000; Sonnleitner et al. 2006). In *P. entomophila* GacA and/or GacS control the expression of Monalysin, AprA, hydrogen cyanide, and Entolysin (Liehl et al. 2006; Opota et al. 2011; Vallet-Gely et al. 2010a). The GacA/GacS system may control many more secreted factors since the supernatant of a *gacA* mutant lacks most of the protein produced by a wild type strain, including components of a T6SS (Liehl et al. 2006; Opota et al. 2011). Regulation by GacS/GacA occurs via transcriptional activation of small noncoding RNAs genes that sequester the small RNA-binding proteins RsmA and RsmE that normally repress translation. In *P. entomophila,* the GacS sensor and the GacA transcriptional regulator act via the two small RNAs, rsmY and rsmZ, that titrate the two small RNA-binding proteins RsmA1 and RsmA2 (Vallet-Gely et al. 2010a, b). *P. entomophila* exhibits phenotypic variation resulting from spontaneous *GacS* or *GacA* mutation, as commonly seen

in many other *Pseudomonas*. In addition, *gac* mutants display a yellow fluorescent phenotype, probably resulting from an accumulation of the fluorescent pigments inside the bacterium. They also have a growth advantage compared with wild type bacteria and can easily predominate when *P. entomophila* is grown in rich medium. Overall, the key role of the GacA/GacS two-component system in *P. entomophila* pathogenicity suggests that the passage into the stationary phase (when virulence factors are produced) is a pre-requisite for *P. entomophila* virulence. Accordingly, concentrated cultures of *P. entomophila* at stationary phase are much more virulent than when the cells are collected during the exponential phase (G.D unpublished data).

AlgR and PrtR Regulators

P. entomophila carrying mutations in the regulator genes *prtR* or *plgR* display reduced pathogenesis against *Drosophila* relative to wild type bacteria, without affecting their ability to induce an immune response (Vodovar et al. 2006; Liehl, unpublished results). This shows that these two regulators play a key role in controlling the expression of important toxic factors.

As in *P. fluorescens* (Burger et al. 2000), PrtR regulates the production of the AprA protease in *P. entomophila* (Liehl et al. 2006). However, in contrast to *aprA* mutants, *prtR* mutants are almost avirulent. PrtR might therefore regulate other factors implicated in pathogenesis. Consistent with this hypothesis, PrtR is predicted to be a transmembrane activator that clusters with a sigma factor, PrtI, required for the expression of several extracellular products in other bacteria (Hughes and Mathee 1998; Missiakas and Raina 1998).

In *P. aeruginosa*, *algR* mutants are less virulent and cleared more rapidly than wild type in a mouse model of infection (Lizewski et al. 2002). In this bacterium, the AlgR response regulator and the atypical sensor FimS (= AlgZ) form a two-component system that regulates alginate production (Deretic and Konyecsni 1989; Yu et al. 1997). AlgR also regulates several other phenotypes associated with virulence, such as lipopolysaccharide and hydrogen cyanide production, motility and biofilm formation, as well as the Rhl quorum sensing system (Carterson et al. 2004; Lizewski et al. 2002, 2004; Morici et al. 2007; Overhage et al. 2007; Whitchurch et al. 1996, 2002). In *P. entomophila*, how AlgR controls virulence and whether alginate synthesis is involved are not yet known. Nevertheless, the drastic attenuation of virulence caused by an *algR* mutation highlights an important role in *P. entomophila* virulence program.

Altogether, these studies indicate that GacA/GacS is a master regulator of the interaction of this bacterium with its *Drosophila* host and that PrtR and AlgR regulators play more specific roles in the infection process.

The Pseudomonas Virulence Factor PVF

P. entomophila genes that encode NRPS were systematically disrupted in order to investigate the role of secondary metabolites in the virulence of *P. entomophila*. While the disruption of Entololysin genes only weakly affects virulence (see above), disruption of another NRPS single transcription unit containing 4 genes, showed a much reduced pathogenicity. These genes were thus named *pvf* (*pvfA, pvfB, pvfC* and *pvfD*) for *Pseudomonas v*irulence *f*actors. Consistent with its reduced ability to kill flies, infection with a *pvf* mutant induces less cell damage, persists less in the *Drosophila* gut and fails to trigger induction of immune responses relative to wild type bacteria (Vallet-Gely et al. 2010b; Vodovar et al. 2005, 2006). Strikingly, *pvf* mutants also show a reduced expression of several genes including Monalysin and the hcnABC genes involved in cyanide production (Opota et al. 2011; Vallet-Gely et al. 2010b). These pleiotropic effects suggest that *pvf* genes might produce a signaling molecule that works in an uncharacterized regulatory system.

In agreement with this hypothesis, supernatant extracts of a wild type culture that had reached the stationary phase restore both gene expression and virulence when supplemented to *pvf* mutant cultures. Interestingly, a similar rescue was obtained with extracts from a *gacA* mutant, suggesting that the PVF is synthesized independently of the GacS/GacA system.

So far, the nature of the PVF product has not been accurately predicted by bioinformatics or elucidated by Mass Spectophotometry. However, the *pvf* genes are also found in several strains of *P. fluorescens*, *P. syringae*, *P. phaseolicola*, *P. aeruginosa* and four strains of the *Burkholderia cenocepacia* complex. In some of these bacteria, the *pvf* cluster is composed of four genes with the same genomic organization as in *P. entomophila*. In other strains, such as the multidrug resistant clinical isolate *P. aeruginosa* PA7, the *pvf* cluster comprises an additional gene referred to as *pvfE*. *PvfE* is predicted to encode a protein containing a cupin domain characterized by a ß-barrel fold (Dunwell et al. 2004). Interestingly, in *Streptomyces,* cupin domain proteins are involved in secondary metabolite synthesis (Dunwell et al. 2000). Whether the PVF molecule synthesized in the bacteria listed above can induce the PVF pathway in *P. entomophila,* in *trans,* remains unknown and could be tested using interspecies complementation assays. Alternatively, we can also imagine that *P. entomophila* PVF is used to communicate with other bacteria.

Many bacteria rely on Quorum Sensing (QS) systems to interact with each other. In the opportunistic human pathogen *P. aeruginosa* several QS systems control the expression of the virulence factor genes: Las, Rhl and the Quinolone signal PQS (Haussler 2010; Williams and Camara 2009). These QS systems are based on the production of small signal molecules, which diffuse freely through the cell envelope. Their concentration increases exponentially with bacterial concentration eventually reaching a threshold that stimulates specific receptors that integrate the signal and change gene regulation (Bassler 1999; Ng and Bassler 2009; Schaefer et al. 1996). Interestingly, the QS systems that are known in in *Pseudomonas*, Las, Rhl and PQS, are not present in the *P. entomophila* genome (Vallet-Gely et al. 2010b).

It is likely that cell-cell communication in *P. entomophila* relies on other signaling systems and PVF is a good candidate as it fulfills all the criteria of a QS signaling molecule. Future studies should address whether PVF represents a new class of signaling molecule.

While the mechanism of post-transcriptional regulation by the Gac two-component system is well characterized, virtually nothing is known about how PVF controls gene expression. Several pieces of evidence suggest a complex interplay between the GacS/GacA and the PVF systems in regulating *P. entomophila* virulence as PVF is not regulated by Gac and some genes, such as the gene of Monalysin, are co-regulated by the PVF and Gac systems.

Conclusion

P. entomophila was first identified as a *Pseudomonas* strain that has the capacity to kill *Drosophila* and to induce a systemic immune response upon ingestion. This initial report already showed that this bacterium exhibits specific entomopathogenic properties since it was the sole *Pseudomonas* strain that could rapidly kill flies upon ingestion of high doses. Subsequent genomic and genetic analyses have also revealed that this bacterium possesses an arsenal of genes suitable for infecting insect guts, and that this bacterium exhibits high metabolic versatility. Consistent with these findings, several new isolates of *P. entomophila* have recently been collected from the soil and in association with plants.

Rapid killing of *Drosophila* is only observed when high doses of *P. entomophila* are used. This raises the question of whether *P. entomophila* is pathogenic beyond laboratory conditions. A number of features suggest that *P. entomophila* is indeed entomopathogenic in the wild. First, very high bacterial concentrations are likely to be found in the rotting fruits, the main food source of fruit flies. Second, damage to the intestinal epithelium are induced by *P. entomophila* even at low doses (Buchon et al. 2009b). Third, the genetic studies performed so far on *P. entomophila* have shown it to be highly relevant to other model entomopathogenic bacteria. Nevertheless, *P. entomophila* should not be considered as a pathogen that is commonly associated with *Drosophila* since this bacterium has not been found in the few survey of wild caught *Drosophila* (Broderick and Lemaitre 2012; Chandler et al. 2011; Juneja and Lazzaro 2009). Moreover, it cannot persist without causing severe damage in the intestinal epithelium, as it is sensitive to the production of AMPs. Unless it does not induce such a strong immune response at low doses *P. entomophila*, might not able to establish a persistent interaction with its host. Altogether these observations suggest that *P. entomophila* is not a long-term associate of *Drosophila* and that insects do not represent an essential ecological niche for this bacterium.

From this perspective, *P. entomophila* should perhaps be viewed as an opportunistic pathogen that is able to infect fruit flies when fortuitously ingested. It's Entomopathogenic properties would then have developed and would present an advantage for this versatile and opportunist bacteria and enable it to resist and use insects

(and likely other invertebrates found in its environment). Indeed, *P. entomophila* shares many characteristics with other opportunistic bacteria, such as *S. marcescens* and *P. aeruginosa* that are also often associated with fruit flies.

In any case, the *P. entomophila/Drosophila* interaction provides us with an interesting model to study the insect gut response and entomopathogenic factors. For instance, pore-forming toxins and metalloproteases are also part of the equipment of other known entomopathogenic bacteria, including *Bacillus thuringiensis* and *Photorhabdus luminescens* (Nielsen-LeRoux et al. 2012). Several pieces of evidence show that the *Drosophila* gut response to *P. entomophila* is likely to be a general feature of insect gut responses to pathogens. These responses include protection by the peritrophic matrix, ROS and AMP production, gut repair through increased stem cell activity, as well as impairment of the host response through inhibition of translation, which leads to pathogenesis (Ferrandon 2013). The fact that both *Drosophila* and *P. entomophila* are easy to manipulate genetically is a key asset in the study of host-pathogen interaction.

Understanding the spatio-temporal deployment of *P. entomophila* virulence, together with the *Drosophila* intestinal response should help to define the sequence of this infectious process. On the bacterial side, future studies should identify additional virulence factors and their mode of regulation. A key question is now to understand why *P. entomophila* triggers a higher level of ROS than other pathogenic bacteria. Since ROS production by Duox is induced by uracil released from bacteria (Ha et al. 2005a; Lee et al. 2013), we might predict that *P. entomophila* produces more uracil than other bacteria or contains another uncharacterized factor that is also able to activate ROS production.

Finally, in addition to its entomopathogenic properties, *P. entomophila* is also pathogenic to other invertebrates and fungi. These strong bio-control abilities also enable it to protect plants from pathogenic fungi. Thus, interest in *P. entomophila* may be significant in the future. Eight years since its first identification, *P. entomophila* has not only found its place among other *Pseudomonas* but may also be considered as a valuable model organism to decipher multiple facets of bacterial properties and interactions with other species.

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Chapter 3
Pseudomonas Activation of the Inflammasome

Ami Joy Hughes and Alan R. Hauser

Abstract Mammals have evolved a multi-layered defense system to prevent infection and rapidly kill microbes. The ability to circumvent each of these defensive barriers distinguishes successful pathogens from other microbes. *Pseudomonas aeruginosa* is no exception. It has developed an armamentarium of virulence determinants that allow its persistence and spread within a host. One aspect of *P. aeruginosa* pathogenesis for which our understanding is rapidly evolving is the interaction of this bacterium with a particular element of the host innate immune response: the inflammasome. Here we will discuss the emerging models of how *P. aeruginosa* suppresses, subverts, evades and even exploits the inflammasome.

Keywords *Pseudomonas aeruginosa* · inflammasome · caspase-1 · type III secretion · flagellin · RhsT · pili

Introduction

The inflammasome is a multimeric protein complex that is an important component of the innate immune response to many endogenous and exogenous danger signals (Davis et al. 2011; Strowig et al. 2012). It is activated by a broad spectrum of microbial pathogens including *Pseudomonas aeruginosa*, *Salmonella enterica, Shigella flexneri, Bacillus anthracis, Listeria monocytogenes* and *Legionella pneumophila.* The interactions between *P. aeruginosa* and the inflammasome will be the focus of this chapter.

While some studies have shown that inflammasome activation by *P. aeruginosa* is beneficial to the host (Franchi et al. 2007; Sutterwala et al. 2007; Descamps et al. 2012; Patankar et al. 2013), increasing evidence suggests that this bacterium can also cause an excessive and pathologic inflammasome-mediated response that promotes bacterial persistence (Schultz et al. 2002; Schultz et al. 2003; Thakur et al. 2004a; Kung et al. 2012; Cohen and Prince 2013). Thus, *P. aeruginosa* has developed mechanisms to subvert inflammasome signaling, turning an important

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host defensive shield into a bacterial offensive weapon. A better understanding of these processes and their roles in pathogenesis is vital and may identify targets for therapeutic intervention. Such novel interventions are critically needed to combat *P. aeruginosa* infections, which are increasingly difficult to treat with conventional antibiotics (Aloush et al. 2006; Gales et al. 2001).

Overview of Inflammasome Assembly and Activation

During infection, the inflammasome functions as a sensor that recognizes the presence of pathogenic bacteria and initiates a proinflammatory response. The current model of inflammasome assembly proposes that a pattern recognition receptor (PRR) in the cytosol of a host immune cell senses a pathogen associated molecular pattern (PAMP) and triggers an activation cascade (Fig. 3.1) (Zhao et al. 2011; Kofoed and Vance 2011). At the core of the active inflammasome is a NOD-like receptor (NLR). NLR proteins include three structural domains with a pyrin or caspase recruitment domain (CARD) at the amino terminus, a central nucleotide oligomerization domain (NOD) and a leucine rich repeat (LRR) at the carboxy terminus. Through these domains, NLRs directly or indirectly bind additional proteins to form the inflammasome complex. Many inflammasomes are known to be activated during bacterial infection (Strowig et al. 2012). Here we will provide a brief overview of inflammasome function. We use the NLRC4 inflammasome to illustrate the workings of these multi-protein complexes, since NLRC4 is activated in several *P. aeruginosa* infection models including peritonitis, keratitis and pneumonia. A more detailed description of each step in the inflammasome pathway will be provided in subsequent sections.

Briefly, the first step in inflammasome activation occurs when the cell becomes primed by sensing the presence of bacteria through surface-exposed Toll-like receptors (TLRs). This sets in motion a signaling cascade that causes expression of immature forms of proinflammatory cytokines. The NLRC4 inflammasome itself becomes activated upon recognition of microbial PAMPs in the cytosol such as *P. aeruginosa* flagellin and components of the type 3 secretion apparatus. PAMP recognition is mediated by NLR apoptosis-inhibitory proteins (NAIPs). NAIPs then bind to NLRC4 proteins, which in turn form a complex with the inactive form of the cysteine protease pro-caspase-1. This interaction may utilize the adapter molecule apoptosis-associated speck-like protein containing CARD (ASC). Once assembled, the regulatory pro-domain of caspase-1 is autocatalytically cleaved. Mature caspase-1 cleaves the regulatory pro-domains from cytokines interleukin-1β (IL-1β) and IL-18. These mature cytokines are then secreted through a poorly characterized mechanism. This process is dependent on a decrease in intracellular K^+(Arlehamn et al. 2010). Cytokine release initiates a broader innate immune response involving recruitment of phagocytes to the site of infection (Sahoo et al. 2011). Another consequence of NLRC4 activation is death of the host cell by pyroptosis, a proinflammatory form of regulated cell death (Miao et al. 2010a; Bergsbaken et al. 2011).

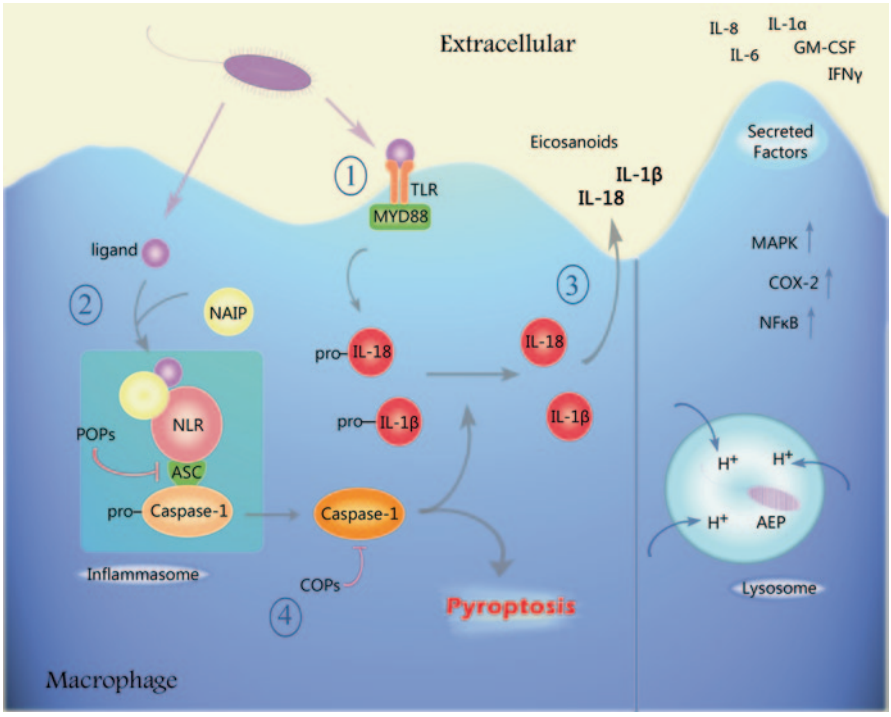

Fig. 3.1 Overview of the Inflammasome Pathway—Inflammasome activation can be divided into four broad steps: *1* Priming. Inflammasome assembly is initiated by recognition of a pathogen associated molecular pattern (PAMP) at the cell membrane that primes transcription of immature cytokines. *2* Ligand recognition in the cytosol. PAMP recognition in the cytosol results in oligomerization of the inflammasome complex and subsequent autoproteolysis of caspase-1. *3* Inflammasome effector functions. Active caspase-1 initiates downstream effector pathways including processing of proinflammatory cytokines, production of eicosanoids and cell death by pyroptosis. Active cytokines propagate the signal cascade by inducing transcription of genes regulated by MAPK, COX-2 and NF-κB and eventual release of secreted factors. IL-1β induces acidification of the phagolysosome. *4* Regulation of the inflammasome response. Inflammasome assembly and response is regulated by several mechanisms, including modulation by POPs and COPs. (*Arrows* in the illustration do not represent direct interactions between factors but rather the direction of signaling pathways important in inflammasome activation. Comprehensive molecular details of these pathways can be found in recent reviews (Davis et al. 2011; Lee et al. 2004; Balamayooran et al. 2010))

Each of these processes contributes to the host immune response to *P. aeruginosa* infection. Inflammasome activation is tightly regulated and endogenous factors can dampen this activation under specific circumstances.

An alternate model of inflammasome assembly suggests that NLRC4 can directly interact with caspase-1 by virtue of its N-terminal CARD domain (Broz et al. 2010), obviating the need for ASC. In this model, caspase-dependent cell death can occur in the absence of ASC and caspase cleavage. Interaction with ASC, however, is required for autoprocessing of the regulatory pro-domain from caspase-1 and

efficient cytokine maturation (Broz et al. 2010). This may explain certain observations that cell death after *P. aeruginosa* intoxication was dependent on NLRC4 but independent of ASC (Franchi et al. 2007) or caspase-1 catalytic activity (Motani et al. 2011). While this second model remains controversial, future studies will no doubt clarify the molecular events that trigger assembly of specific inflammasome components and activation of the complex.

In summary, microbial PAMPs are sensed in the cytosol by the inflammasome. This initiates a robust immune response to clear the infecting bacteria. The processes of the inflammasome response can be divided into the following steps, which are illustrated in Fig. 3.1: (1) priming, (2) ligand recognition, (3) functions of effector pathways and (4) activity regulation. Our understanding of each of these steps in the context of *P. aeruginosa* infection is discussed in the following sections.

Priming Inflammasome Activation

Inflammasome activation occurs in a multi-step process that requires two independent signaling events (Fig. 3.1). The first signal activates a TLR on the cell surface that in turn activates a signaling cascade culminating in transcription of specific genes to yield immature cytokines (Bauernfeind et al. 2009; Puren et al. 1999). TLR engagement, however, is not sufficient to induce caspase-1 autoproteolysis or secretion of the mature forms of IL-1β or IL-18 cytokines (Descamps et al. 2012; Bauernfeind et al. 2009; Puren et al. 1999). The second signal (discussed in the next section) activates a NLR in the cell cytoplasm. NLR activation by itself cannot promote the synthesis of pro-IL-1β or pro-IL-18 but is required for caspase-1-dependent cytokine processing and release (Franchi et al. 2007; Descamps et al. 2012; Bauernfeind et al. 2009; Puren et al. 1999). Thus, TLR engagement primes macrophages for inflammasome activation by producing pools of immature cytokines, which are subsequently processed upon stimulation of intracellular NLRs.

The processes of priming and activation may be linked in certain contexts such that a separate priming step may not be observable or required. For example, in the context of infection, a cell is assaulted with multiple bacterial PAMPs (lipopolysaccharide [LPS], flagellin, lipoproteins, etc.) at the same time, and priming may occur concomitantly with inflammasome ligand recognition. Pre-stimulation to induce immature cytokine transcription may not be required *in vivo* or during infection of macrophages *in vitro* with live bacteria (Sutterwala et al. 2007; Kung et al. 2012). Additionally, there is evidence that pro-IL-18 is constitutively expressed in human peripheral blood mononuclear cells (PMBCs). However, the second NLR signal is still required for maturation and secretion of IL-18 (Puren et al. 1999). This suggests that the model for IL-18 maturation varies slightly from the two-signal activation of inflammasomes discussed previously. A similar variation of the two-signal activation model occurs in the gut and allows the host to discriminate between non-pathogenic and pathogenic bacteria. In the context of the gut, resident intestinal macrophages are anergic to TLR stimulation by LPS, CpG DNA and lipoproteins

from constant engagement by commensal bacteria (Franchi et al. 2012). Whereas intestinal macrophages failed to produce TNF or IL-6 in response to TLR stimulation, these cells constitutively produced immature pro-IL-1β. As a result, they produced mature capase-1 and IL-1β when exposed to pathogenic bacteria but not commensal bacteria (Franchi et al. 2012). This response was dependent on NLRC4, demonstrating a role for the inflammasome in the immune response to pathogenic bacteria in the gut (Franchi et al. 2012). In this way, the host appropriately activates the inflammasome when pathogenic bacteria are detected but fails to mount an immune response against normal gut flora.

Ligand Recognition

Ligand recognition is the paramount step that leads to assembly and activation of the inflammasome complex. Four *P. aeruginosa* PAMPs are known to activate the inflammasome (Fig. 3.2). They are (1) flagellin, the major component of flagella (Franchi et al. 2007; Miao et al. 2010b), (2) the type III secretion system (T3SS) (Miao et al. 2010b; Miao et al. 2008), (3) pilin, the major component of type IV pili (Arlehamn et al. 2011) and (4) RhsT, a novel virulence determinant (Kung et al. 2012). While the specific inflammasomes activated by type IV pilin and RhsT have yet to be identified, both flagellin and the T3SS activate the NLRC4 inflammasome.

Flagellin and T3SS Ligand Specificity—Activation of the NLRC4 inflammasome requires sensing of the ligands in the cytosol of the target cell. Yet how the ligands, particularly flagellin, gain access to the cytosol has long been a perplexing question. Early studies examining the activation of the inflammasome noted a dependence of flagellin recognition on functional T3SS (Arlehamn et al. 2010; Mariathasan et al. 2004; Miao et al. 2006; Wangdi et al. 2010). This was initially interpreted as T3SS-dependent delivery of flagellin into the cytosol. Indeed, studies with *S. enterica* established a direct link between secretion of flagellin and functional T3SS (Sun et al. 2007). However, it has since been shown that flagellin and T3SS are distinct activators of the inflammasome (Zhao et al. 2011; Kofoed and Vance 2011; Arlehamn et al. 2010; Miao et al. 2010b). Consistent with this finding, non-flagellated strains of *P. aeruginosa* are capable of activating the inflammasome (Sutterwala et al. 2007; Descamps et al. 2012; Arlehamn et al. 2010; Wangdi et al. 2010). Thus, the manner by which these ligands access the cytosol for detection by the inflammasome remains unclear, but it is now understood that flagellin and T3SS independently activate the inflammasome.

Until recently the nature of ligand recognition (specific or non-specific, direct or indirect) by NLRC4 was the source of some debate. It had been known for some time that murine NAIP5 played an important role in flagellin recognition by NLRC4 (Lightfield et al. 2008). For example, in the absence of NAIP5, *L. pneumophila* no longer activated the NLRC4 inflammasome (Lightfield et al. 2008). During *P. aeruginosa* infection, however, inflammasome-dependent cell death (Lightfield et al. 2008) and cytokine processing (Lightfield et al. 2008) were not abrogated in

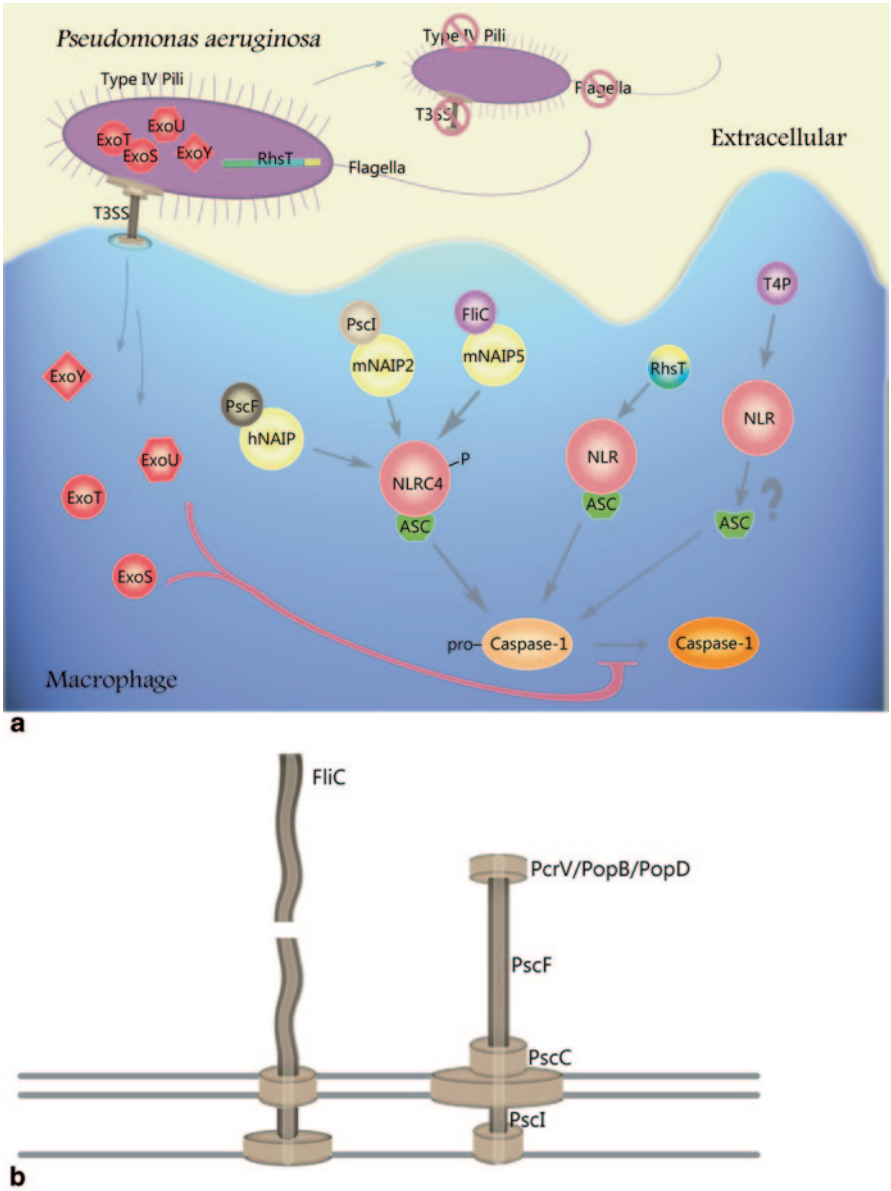

Fig. 3.2 *P. aeruginosa* Interactions with the Inflammasome—A. PscF, PscI and FliC are ligands of NLRC4 through interactions with human NAIP (hNAIP) or murine NAIP2 and 5 (mNAIP), respectively. RhsT and type IV pili (T4P) activate the inflammasome through unknown mechanisms. T3SS effectors ExoU and ExoS can inhibit caspase-1 maturation. T3SS, flagellin and pilin may not be expressed during chronic infections. B. Schematic of flagella (*left*) and T3S apparatus (*right*).

NAIP5-deficient murine macrophages. Early studies of flagellin-dependent activation of the inflammasome during *P. aeruginosa* infection were likely confounded by T3SS-dependent activation of the inflammasome. (Note that *L. pneumophila* does not have a T3SS.) The specific nature of ligand recognition was examined using a reconstituted inflammasome expression system in non-immune cells. In this way, it was demonstrated that flagellin (designated FliC in *P. aeruginosa*) is directly recognized by murine NAIP5 (Zhao et al. 2011; Kofoed and Vance 2011). *L. pneumophila* flagellin interacted with NAIP6 in addition to NAIP5. Components of the T3SS apparatus are also recognized by different NAIPs. It was discovered that an inner rod component of the T3SS apparatus (designated PscI in *P. aeruginosa*) is recognized by murine NAIP2 (Zhao et al. 2011; Kofoed and Vance 2011). Similarly, NAIP1 recognizes the T3SS needle proteins of bacteria such as *S. enterica,* enterhemorrhagic *E. coli, S. flexneri and Burkholderia* spp. (Yang et al. 2013). It remains unclear whether this is true for the needle protein (designated PscF) of *P. aeruginosa.* Upon ligand recognition, the NAIP proteins subsequently oligomerize with NLRC4 in the inflammasome complex (Zhao et al. 2011; Kofoed and Vance 2011; Yang et al. 2013). Incorporating this information into the current model of NLRC4 activation suggests that ligand recognition is mediated by distinct NAIPs that directly bind specific ligands and in turn interact with NLRC4 to initiate assembly of the inflammasome complex (Figs. 3.1 and 3.2).

An unanticipated aspect of flagellin-mediated recognition of *P. aeruginosa* is that motility, rather than the mere presence of flagella, is associated with increased inflammasome activation. The *motAB* and/or *motCD* genes are required for flagellar rotation and bacterial motility but do not affect flagellar architecture. Previous studies demonstrated that disruption of these genes, which rendered *P. aeruginosa* strains non-motile, conferred resistance to phagocytosis (Amiel et al. 2010; Lovewell et al. 2011). Non-motile strains also failed to fully induce inflammasome-dependent maturation of pro-caspase-1 (Patankar et al. 2013). Similar observations were made with *S. enterica;* during infection, non-motile strains elicited reduced levels of IL-1β secretion and pyroptosis-like cytotoxicity compared to motile strains (Miao et al. 2006). Collectively, the data suggest that flagellar motility, not just specific ligand recognition, plays a role in activating the inflammasome, although the mechanisms by which this occurs remain unclear.

Sequence-Dependent and -Independent Ligand Recognition—Specific recognition of NAIP/NLRC4-ligands is dependent on critical residues in the carboxy termini of these ligands. NAIP5-dependent recognition requires leucine residues in the C-terminus of flagellin (Zhao et al. 2011; Miao et al. 2010b; Lightfield et al. 2008). Interestingly, this region of flagellin is distinct from the region necessary for TLR5 signaling (Lightfield et al. 2008), indicating that the NLRC4 and TLR5 pathways rely upon detection of different PAMPs within the same protein. Alignment of the *S. enterica* T3SS inner rod protein PrgJ and flagellin revealed sequence similarity in the C-termini at these critical residues (Miao et al. 2010b). Likewise, these residues are conserved between PrgJ and the homologous *P. aeruginosa* T3SS inner rod protein PscI. This region of PscI is required for T3SS-dependent activation of NLRC4 in murine macrophages (Miao et al. 2010b). While PscI is necessary for

inflammasome recognition, mutations in genes encoding other *P. aeruginosa* T3SS apparatus proteins also abolish inflammasome activation. These include genes encoding the outer membrane channel protein PscC (Franchi et al. 2007), the basal structure component PscJ (Wangdi et al. 2010) and the translocon proteins PcrV, PopB and PopD (Arlehamn et al. 2010; Wangdi et al. 2010). In the absence of PscC or PscJ, *P. aeruginosa* fails to assemble a functional T3SS apparatus. In contrast, null mutants of PcrV, PopB or PopD assemble a functional T3SS apparatus but fail to inject effector proteins into host cells because they cannot form the channel in the host cell plasma membrane through which these effectors are transported. Presumably, these T3SS mutants do not elicit an inflammasome response because they fail to deliver PscI (or possibly PscF) to the host cytosol. Collectively, these findings demonstrate that NLRC4 ligand recognition is sequence specific and distinct from other known receptor-mediated signaling pathways.

Differences in Murine and Human Ligand Recognition—Most of the current knowledge regarding inflammasome ligand recognition is based on studies in murine cells. Key differences, however, have been identified using human macrophages. Whereas mice produce multiple NAIP proteins, humans have only a single variant (Zhao et al. 2011). Intriguingly, human NAIP failed to recognize flagellin under the conditions tested. Furthermore, human NAIP recognized the T3SS needle protein PscF rather than the inner rod protein PscI (Fig. 3.2), which is recognized by murine NAIP2 (Zhao et al. 2011). As with murine NAIP2/5 and their ligands, human NAIP recognition of the needle protein was dependent on amino acids in the C terminus (Zhao et al. 2011), although the required amino acids are distinct from those recognized in the rod protein (Miao et al. 2010b). Further studies are needed to fully characterize the differences between human and murine NLRC4 inflammasomes and to clarify the role of flagellin in human inflammasome activation. Even though human and murine NAIPs differ, the central theme of ligand recognition appears to be consistent in both cell types. Ligand recognition is mediated by NAIP(s), which interact with NLRC4 to initiate assembly of the inflammasome complex.

Other Inflammasome Activators—Although much is known about inflammasome activation by flagellin and the T3SS, the specifics of how type IV pili or RhsT are recognized by inflammasomes remains unclear (Fig. 3.2). Type IV pili are long protein polymers of pilin that mediate twitching motility and adhesion (Burrows 2012). Type IV pilin was shown to activate the inflammasome by liposomal transfection of bone marrow derived macrophages (BMDMs). Delivery of pilin to these cells resulted in cleavage of caspase-1 and secretion of mature IL-1β (Arlehamn et al. 2011). Intriguingly, IL-1β release was abrogated in BMDMs from caspase-1$^{-/-}$ mice but not NLPR3$^{-/-}$, NLRC4$^{-/-}$ or ASC$^{-/-}$ macrophages (Arlehamn et al. 2011). Additionally, NLRC4$^{-/-}$, ASC$^{-/-}$ and caspase-1$^{-/-}$ BMDMs were only marginally protected from cell death by pyroptosis following transfection (Arlehamn et al. 2011). Thus the activity of inflammasomes containing NLRP3, NLRC4, or ASC cannot completely explain the activation of caspase-1 upon exposure to type IV pilin.

Similarly, the novel virulence determinant RhsT also activates the inflammasome. RhsT is a member of the rearrangement hot spot (Rhs) protein family. Rhs proteins have a characteristic tripartite structure that includes an N-terminal domain

shared among members of the same subfamily, a highly-conserved YD-repeat central domain and a hypervariable C-terminal tip (Jackson et al. 2009). Many questions remain regarding the secretion pathways used by these large proteins and their general function. While Rhs proteins in *D. dadantii* and a related protein in *B. subtilis* targeted competing bacteria (Poole et al. 2011; Koskiniemi et al. 2013), RhsT in *P. aeruginosa* targeted mammalian cells and enhanced virulence (Kung et al. 2012). RhsT caused inflammasome-dependent macrophage cell death and release of mature IL-1β and IL-18 (Kung et al. 2012). Cell death and cytokine release were abrogated by the caspase-1 inhibitor YVAD or the absence of ASC (Kung et al. 2012). The dependence of cell death on ASC is not characteristic of NLRC4 inflammasome activation (Franchi et al. 2007; Motani et al. 2011), and RhsT does not possess the C-terminal signature sequence of FliC and PscI (unpublished sequence analysis). These observations suggest that an inflammasome other than NLRC4 is activated by RhsT. As with type IV pilin, further studies are required to clarify the nature of inflammasome recognition of RhsT.

Inflammasome Effector Functions

The canonical effector of the NLRC4 inflammasome is caspase-1. Upon maturation, caspase-1 promotes the immune response to bacterial infection by inducing multiple pathways (Fig. 3.1). Caspase-1-dependent release of mature IL-1β and IL-18 is a characteristic outcome of inflammasome activation and is responsible for a number of downstream inflammatory events. Recently the production of eicosanoids, a class of lipid mediators, was shown to also play a role in the inflammasome-mediated immune response. Finally, inflammasome activation induces the pyroptotic cell death pathway. Each of these effector pathways contributes to resolution of bacterial infection in the appropriate context.

Propagation of Cytokine Signaling—Interleukin 1 family cytokines IL-1β and IL-18 are translated as the inactive precursors pro-IL-1β and pro-IL-18, respectively, that require cleavage by caspase-1. Cleavage of the regulatory pro-domain results in release of active cytokines. Mature IL-1β and IL-18 are then recognized by cell surface receptors and induce the NF-κB or MAPK signaling pathways, respectively, to produce IL-6, IL-8 and IL-1α (Lee et al. 2004). IL-1β release has pleiotropic effects that together promote a successful immune response. In the context of *P. aeruginosa* infection, Descamps and colleagues have shown that TLR5 engagement results in secretion of IL-1β, which is required for acidification of the phagosome after bacterial uptake by macrophages. Subsequent activation of the lysosomal cysteine protease AEP is necessary for bacterial killing (Fig. 3.1). These authors speculate that inflammasome activation mediated the production of IL-1β that they observed (Descamps et al. 2012). (Of note, inflammasome-dependent processes are not the only manner by which mature IL-1β is generated. For example, cleavage of pro-IL-1β by the serine protease elastase of neutrophils also generates mature IL-1β (Karmakar et al. 2012).) IL-18 stimulates T lymphocytes to produce

IFNγ and bolsters the neutrophil response by promoting production of GM-CSF (Sahoo et al. 2011). In this way, assembly of the inflammasome complex results in a cascade of events that ultimately produces a robust cytokine response and activates multiple signaling pathways.

Production of Lipid Mediators—Macrophages produce eicosanoids, a family of biologically active lipids, through the action of cyclooxygenase (COX) and lipoxygenase (LOX). COX-dependent eicosanoids include prostaglandin D_2, prostaglandin E_2 (PGE2), prostaglandin I_2 (PGI2) and thromboxane A_2 (TXA2). They modulate the function of immune cells and result in a fever response (Hirata and Narumiya 2012). Leukotrienes, such as the neutrophil chemoattractant leukotriene B_4 (LTB4), are LOX-dependent (Funk 2001). It has been known for some time that eicosanoid signaling and IL-1β signaling are linked (Hirata and Narumiya 2012; Funk 2001). Likewise, IL-18 also promotes the eicosanoid response indirectly through IL-1α production, although this response is slower than the IL-1β response (Lee et al. 2004). The NLRC4 inflammasome stimulated the rapid and robust production of both LOX-dependent and COX-dependent eicosanoids in response to stimulation with flagellin (von Moltke et al. 2012). This "eicosanoid storm" was dependent on caspase-1 but independent of IL-1β or IL-18, indicating an alternate target of caspase-1. Interestingly, activation of either NLRC4 or NLRP1b inflammasomes resulted in eicosanoid release. Thus the eicosanoid response may be universally upregulated in a caspase-1 dependent manner and not specific to the activation of any one inflammasome. The rapid induction of the eicosanoid response was associated with the enhanced severity of infection, both *in vitro* following stimulation with purified flagellin and *in vivo* following challenge with *B. anthracis* (von Moltke et al. 2012). Whether inflammasome-mediated eicosanoid production plays a protective role under other conditions requires further investigation. This study was conducted with the *L. pneumophila* flagellin, and it remains to be demonstrated whether homologous *P. aeruginosa* flagellin causes a similar phenomenon. It will be interesting to learn whether eicosanoid release is clinically relevant to the inflammasome-mediated response to *P. aeruginosa* infection.

Proinflammatory Cell Death—Pyroptosis is a form of programmed cell death that is intrinsically different from apoptosis (Fink and Cookson 2005). Apoptosis is dependent on caspase-3, caspase-6 and caspase-8 and is marked by release of membrane blebs that are phagocytosed by neighboring cells (Bergsbaken et al. 2011; Fink and Cookson 2005). In contrast, pyroptosis is dependent on caspase-1 and characteristically leads to the formation of membrane pores that result in cell swelling and lysis from osmotic stress (Bergsbaken et al. 2011). Whereas apoptosis tends to be accompanied by minimal or no inflammation, pyroptosis is a proinflammatory process by virtue of IL-1β and IL-18 release. At first glance, a signaling cascade that leads to the death of an important cell of the immune system (macrophages) would appear to favor bacterial pathogens rather than the host. But studies using *S. enterica, L. pneumophila* and *B. thailandensis* have demonstrated that caspase-1 dependent pyroptosis is an important mechanism for eradicating intracellular bacteria by exposing them to neutrophils in the extracellular environment (Miao et al. 2010a). Whether the same process is important for *P. aeruginosa*, which is primar-

ily an extracellular pathogen but does access the intracellular compartment to a small degree (Fleiszig et al. 1995), is unclear. Furthermore, lysosomes fuse with the plasma membrane during pyroptosis, releasing antimicrobial factors into the extracellular milieu (Bergsbaken et al. 2011). In this way, pyroptotic cell death actively contributes to the inflammasome response to microbial infections by exposing intracellular bacteria to the extracellular environment and expelling intracellular antimicrobial components into the extracellular milieu.

A recent report indicates that programmed cell death pathways induced by PAMPs may be more varied than previously thought. Lage and colleagues showed that purified flagellin from *S. enterica* was able to induce macrophage cell death even in the absence of ASC, NLRC4, or caspase-1 (Lage et al. 2013). Furthermore, *in vitro* infection with live bacteria resulted in increased cell death that was correlated with reduced bacterial persistence (Lage et al. 2013). Collectively, this suggests that flagellin is activating a cell death mechanism that is independent of the NLRC4 inflammasome pathway but that is required to restrict *Salmonella* during infection. Further investigation revealed that lysosomal proteases cathepsin B and cathepsin D played a role in this form of cell death. Interestingly, inhibition of cathepsin-B reduced flagellin-dependent cell death but was also associated with decreased IL-1β (Lage et al. 2013). This suggests that the caspase-1-dependent inflammasome cell death pathway and the cathepsin B-dependent cell death pathway may be related. Additional studies are required to determine whether flagellin activates the cathepsin B cell death pathway in the context of *P. aeruginosa* infection in a similar manner to *S. enterica*.

Regulation of the Inflammasome Response

Appropriate regulation is necessary for inflammasomes to maintain productive control of infections without causing off-target disease pathologies (Strowig et al. 2012). Several regulatory strategies have been discussed thus far, such as the two-signal activation model and production of inactive pro-caspase-1, pro-IL-1β and pro-IL-18 that require post-translational processing for release of mature proteins. Cytokine levels are also regulated by Toll IL-1R 8 (TIR8), which reduces levels of IL-1β and IL-18 (Huang et al. 2006; Veliz Rodriguez 2012) and prevents excessive tissue damage. These mechanisms modulate the inflammasome response to stimuli. There is mounting evidence that the inflammasome is also regulated at the level of complex formation.

Assembly of the NLRC4 inflammasome complex requires phosphorylation of NLRC4 itself (Fig. 3.2). Murine NLRC4 is phosphorylated by kinase PKCδ at serine residue 533 (Qu et al. 2012). In the absence of S533 phosphorylation, NLRC4 was unable to oligomerize with ASC or caspase-1 (Qu et al. 2012). Furthermore, phosphorylation was required for unimpaired cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 after infection with *S. enterica* (Qu et al. 2012). While NLRC4 phosphorylation has not been studied in the context of *P. aeruginosa* infection, it will be

interesting to examine whether the hyper-inflammatory response observed during certain *P. aeruginosa* infections (Kung et al. 2012; Cohen and Prince 2013) can be attributed to bacterial interference with cellular processes that would otherwise attenuate inflammasome activation.

Immunomodulatory proteins also regulate inflammasome complex formation by competing for ASC or pro-caspase-1 binding. CARD-only proteins (COPs) and pyrin-only proteins (POPs) are small proteins that sequester inflammasome components, preventing association with the complex (Fig. 3.1). COPs sequester caspase-1 from the inflammasome complex through interaction with the CARD domain of caspase-1 (Stehlik and Dorfleutner 2007). POPs interact with ASC, preventing association with NLRs (Stehlik and Dorfleutner 2007; Dorfleutner et al. 2007; Bedoya et al. 2007) and thereby inhibiting downstream events associated with the inflammasome cascade. Interestingly, POP1 prevents NF-κB signaling but enhances caspase-1 cleavage of pro-IL-1β (Stehlik et al. 2003). In contrast, POP2 disrupts both NF-κB signaling and caspase-1 activity (Dorfleutner et al. 2007; Bedoya et al. 2007; Atianand and Harton 2011). Although five COPs and two POPs have been identified in humans, they are lacking in mice, preventing mechanistic studies of these proteins in regulation of inflammasome activity during mouse models of infection (Stehlik and Dorfleutner 2007). Additionally, POPs have not been implicated in regulation of NLRC4. Thus, inflammasome activation is regulated by a complex network of protein interactions, but the implications of these mechanisms to infections by *P. aeruginosa* are unknown.

Non-Canonical Inflammasome Pathway

The model of inflammasome activation became more complex when it was recently discovered that murine caspase-11 interacts with the canonical inflammasome activation pathway. Under certain infection conditions, cleavage of pro-caspase-1 and subsequent maturation of pro-IL-1β and pro-IL-18 are dependent on caspase-11 (Kayagaki et al. 2011). Additionally, caspase-11 resulted in secretion of IL-1α and cell death by pyroptosis, independent of its interaction with caspase-1. Thus, caspase-11 intersects with the NLRC4 inflammasome activation cascade at the level of pro-caspase-1. These results indicate a previously unappreciated role for caspase-11 in inflammasome activation. Because many of the published inflammasome studies on the role of caspase-1 used caspase-1$^{-/-}$ mice that were unknowingly also deficient in caspase-11 (Kayagaki et al. 2011), the contribution of caspase-11 to the observed phenotypes of those studies cannot be excluded**.** Notably, caspase-11 dependent cleavage of caspase-1 was not observed in response to *P. aeruginosa* (Cohen and Prince 2013; Kayagaki et al. 2011). Regardless, the contribution of caspase-11 to the host response against *P. aeruginosa* must be explored further to understand the unique role of each inflammasome component in the context of various infection conditions. Furthermore, since humans do not make a protein identical to murine caspase-11 (Bedoya et al. 2007), the relevance of the non-canonical inflammasome pathway in humans remains to be determined.

General *P. aeruginosa* Strategies to Counter the Inflammasome-Mediated Immune Response

Inflammasomes have certainly evolved to protect mammals from microbes. Therefore it is not surprising that the inflammasome functions to clear *P. aeruginosa* in certain infection models. It is also anticipated, however, that successful pathogens have evolved countermeasures to subvert or even co-opt inflammasomes. In support of the last possibility, inflammasome activation is associated with improved bacterial persistence and worse infection outcomes in some model systems. In these infections, the bacteria exploit the inflammasome response in such a way that it is ineffective in resolving the infection and detrimental to the host by inducing pathological inflammation. *P. aeruginosa* uses a multi-faceted approach to counter the inflammasome immune response, including the dampening of caspase-1 effector function, the down-regulation of inflammasome ligands and the exploitation of inflammasome-induced inflammation (Table 3.1). The following sections will review each of these strategies.

T3SS Effectors Dampen Inflammasome Activation

T3SSs are major virulence determinants that inject effector proteins through a needle-like apparatus into the cytosol of the host cell in a manner that is dependent on cell-cell contact. Their importance in pathogenesis is underscored by the evolution of inflammasomes to detect components of the T3SS secretion apparatus. The *P. aeruginosa* T3SS secretes four known effectors: ExoU, ExoT, ExoS and ExoY (Hauser 2009). The gene encoding ExoU is present in about 30 % of *P. aeruginosa* clinical isolates and is generally exclusive of the gene encoding ExoS (Feltman et al. 2001). Although ExoT and ExoS share 76 % amino acid similarity, the distribution of the *exoT* and *exoS* genes among clinical isolates is quite different. Whereas the *exoT* gene is present in nearly all strains, only about 70 % contain the *exoS* gene (Feltman et al. 2001). Approximately 90 % of strains contain the *exoY* gene (Feltman et al. 2001), although the contribution of ExoY to infection remains unclear. While

Table 3.1 —Summary of strategies employed by *P. aeruginosa* to negate the inflammasome-mediated immune response.

Strategy	Effector	Mechanism
Dampen activation	ExoU ExoS	Inhibit caspase-1 maturation
Evade detection	Flagellin T3SS Pili	Absence of PAMPs
Exacerbate activation	RhsT Flagellin	Unknown

components of the T3SS apparatus itself are activators of the NLRC4 inflammasome, the effector proteins ExoU and ExoS are capable of blocking the activity of the inflammasome.

ExoU Inhibits Caspase-1 Maturation—ExoU has phospholipase A2 activity that requires ubiquitin and phosphatidylinositol 4,5-bisphosphate (PIP2) as coactivators (Anderson et al. 2011; Tyson and Hauser 2013). ExoU translocation results in rapid lysis of the target cell but also dampens the inflammasome. Caspase-1 maturation was inhibited during infection of murine macrophages with ExoU-secreting *P. aeruginosa* (Sutterwala et al. 2007) (Fig. 3.2). Inhibition of caspase-1 was dependent on the phospholipase activity of ExoU, although the mechanism by which this occurred is unknown (Sutterwala et al. 2007). Cleavage of pro-casapase-1, pro-IL-1β and pro-IL-18 occurred when cells were infected with isogenic strains containing a disrupted *exoU* gene (Sutterwala et al. 2007; Wangdi et al. 2010). ExoU secretion caused rapid lysis of target cells but cell death was still observed when the *exoU* gene was disrupted. This latter form of cell death was presumably pyroptotic as it was dependent on NLRC4 and was associated with the release of LDH from the cell, indicating membrane damage (Sutterwala et al. 2007). In this manner, ExoU has the dual functions of causing lysis of target cells and inhibition of caspase-1 maturation. Furthermore, in a mouse model of peritonitis, mice were able to clear ExoU-deficient bacteria in an NLRC4-dependent manner (Sutterwala et al. 2007). Thus, ExoU dampening of the inflammasome response appears to be biologically relevant to bacterial persistence.

ExoS Inhibits Caspase-1 Maturation—Like ExoU, ExoS also suppresses the inflammasome by inhibiting maturation of caspase-1 (Galle et al. 2008) (Fig. 3.2). ExoS is a bi-functional T3SS effector with a GTPase activating protein (GAP) domain and an ADP-ribosyl transferase (ADPRT) domain (Hauser 2009). Of the two functional domains, the ADPRT domain was required for ExoS inhibition of caspase-1 (Galle et al. 2008). Similar inhibition of the inflammasome occurred with a related T3SS effector of *Yersinia enterolitica,* YopE (Schotte et al. 2004; Brodsky et al. 2010). However, unlike ExoS, YopE-dependent caspase-inhibition required the protein's GAP activity (Schotte et al. 2004). This difference may be attributed to the fact that the effectors are similar but not identical. YopE has GAP activity but no ADPRT activity and the two effectors have different host cell substrates (Schotte et al. 2004). Even though inhibition is achieved by different functional activities, in both cases the outcome is an alteration of the cell death phenotype with a reduction in cell death by pyroptosis (Galle et al. 2008; Schotte et al. 2004). ExoS causes an apoptotic-like form of cell death related to caspase-3 activation (Shafikhani et al. 2008). In the absence of ExoS, infection resulted in pyroptosis characteristic of inflammasome activation (Galle et al. 2008). ExoT also shifted cell death towards apoptosis in a manner that was dependent on its ADPRT activity (Shafikhani et al. 2008). Thus, ExoS (and perhaps ExoT) dampens the inflammasome response through inhibition of caspase-1 maturation and shifts the cell death phenotype to a mechanism that does not propagate the immune response.

Several questions remain regarding the role of ExoS in dampening the inflammasome response. An independent study that examined the roles of ExoS, ExoT and

ExoY in inflammasome activation did not observe differences in caspase-1 maturation, IL-1β release, or cell death attributed to these effectors (Franchi et al. 2007). In this study, BALB/c mice, which are known to be more resistant to *P. aeruginosa* infection, were used (Stotland et al. 2000). Indeed, although the bacteria elicited an NLRC4-dependent inflammasome response during acute infection *in vivo,* the infection was ultimately cleared regardless of inflammasome activation (Franchi et al. 2007). In contrast, inflammasome inhibition by ExoS was observed using DBA2 mice (Galle et al. 2008), which are more susceptible to *P. aeruginosa* infection (Stotland et al. 2000). In these latter experiments, the outcome of infection was not reported, although a similar model of acute pneumonia was used (Galle et al. 2008). Therefore, the apparent discrepancy between these reported observations may be due to experimental differences. Further studies are required to clarify the role of ExoS in inflammasome inhibition and the consequence for infection outcomes.

Evasion of Inflammasome Activation by Loss of PAMPs

While dampening the immune response is important during acute infection, bacteria must also neutralize host defense systems during chronic infection. Characterization of isolates from cystic fibrosis (CF) patients chronically infected with *P. aeruginosa* demonstrated that these isolates exhibited marked decreases in functional T3SS (Jain et al. 2004) and production of flagella and pili (Lee et al. 2005; Luzar et al. 1985; Mahenthiralingam et al. 1994). Whereas 90% of *P. aeruginosa* environmental isolates were capable of type III secretion, only 49% of isolates cultured from newly infected children with CF were secretion-positive (Jain et al. 2004). This percentage was further reduced to only 4% of isolates from chronically infected adults, demonstrating a clear loss of functional type III secretion following longer periods of infection (Jain et al. 2004). Similarly, *P. aeruginosa* isolates from chronically-infected CF patients exhibited a loss of pili and flagella (Lee et al. 2005; Luzar et al. 1985; Mahenthiralingam et al. 1994). The frequency of non-flagellated strains during chronic CF infection was particularly high compared to isolates from the environment or acute infections (Luzar et al. 1985; Mahenthiralingam et al. 1994). Despite the loss of motility, non-flagellated strains were associated with worse clinical outcomes in CF patients (Luzar et al. 1985). The reasons why type III secretion, flagella and pili are lost during chronic infections are unclear but may involve the inability of patients to clear *P. aeruginosa* in the absence of robust inflammasome activation (Fig. 3.2).

Several observations support a model whereby loss of T3SS favors *P. aeruginosa* persistence in chronic respiratory infections. Pulmonary infection of mice with a T3SS-negative strain caused reduced cytokine responses and neutrophil recruitment relative to a T3SS-positive strain (Wangdi et al. 2010). Additionally, T3SS-negative bacteria were not cleared from the lungs as quickly as isogenic strains that assembled the T3SS apparatus. Finally, caspase-1 played an integral role in the

differential response to T3SS-competent versus T3SS-null strains (Wangdi et al. 2010). Thus, the ability of *P. aeruginosa* to persist during chronic infections may be at least partially attributable to evasion of the inflammasome-mediated immune response by loss of functional T3SSs (Fig. 3.2).

The absence of flagella during chronic infection may also allow *P. aeruginosa* bacteria to evade immune detection. *P. aeruginosa* possesses a single polar flagellum that is required for swimming motility, colonization and full virulence during infection. Flagella mediate bacterial penetration across the epithelial barrier and binding to the basolateral cell surface (Parker and Prince 2013; Bucior et al. 2012). However flagella also engage TLR5, causing release of chemokine and cytokine signals (Parker and Prince 2013). TLR5 and MYD88 signaling were essential for bacterial clearance in certain *in vivo* infection models (Descamps et al. 2012). In contrast, non-flagellated isogenic strains of *P. aeruginosa* failed to stimulate TLR5/MYD88 signaling or secrete IL-1β, indicating the absence of inflammasome activation (Descamps et al. 2012; Amiel et al. 2010). As a consequence, non-flagellated bacteria persisted in higher numbers than flagellated bacteria (Descamps et al. 2012; Cohen and Prince 2013). Therefore, loss of flagella may represent a means to avoid immune activation and promote bacterial persistence during chronic infection (Fig. 3.2).

Infection-Induced Pathologic Inflammation

In contrast to evading and dampening the inflammasome response, *P. aeruginosa* also has the ability to amplify and take advantage of inflammasome-induced inflammation in certain contexts. Mounting evidence suggests that *P. aeruginosa* induces pathological inflammation and persists better under these conditions (Schultz et al. 2002; Cohen and Prince 2013). Contrary to studies demonstrating that IL-1β is necessary for bacterial clearance (Descamps et al. 2012), other reports suggest that this cytokine can also cause excessive recruitment of neutrophils (Thakur et al. 2004) and induce tissue damage (Schultz et al. 2002; Thakur et al. 2004) under certain conditions. Similar tissue damage has been observed with IL-18 (Schultz et al. 2003). Thus, inflammasome activation by *P. aeruginosa* has the potential to adversely impact outcomes.

Flagella have been implicated in the pathological inflammation observed in certain infection models. The production of flagella was associated with inflammasome activation and increased tissue damage in a mouse model of acute pneumonia (Cohen and Prince 2013). The magnitude of tissue damage was dramatically reduced by depletion of alveolar macrophages or chemical inhibition of caspase-1 by YVAD (Cohen and Prince 2013). Interestingly, NLRC4$^{-/-}$ mice were better able to clear flagellated *P. aeruginosa* than their wild-type counterparts. Thus, *P. aeruginosa* is able to thrive in the midst of flagellin-mediated inflammasome activation.

RhsT has also been associated with increased bacterial persistence and pathological inflammation (Kung et al. 2012; Battle et al. 2009). In a mouse model of acute

pneumonia, expression of *rhsT* was associated with enhanced bacterial persistence despite increased recruitment of phagocytic cells and higher amounts of IL-1β and IL-18. An isogenic strain with a deleted *rhsT* gene was more readily cleared and resulted in improved host survival (Kung et al. 2012). Thus, RhsT causes inflammasome activation and results in increased inflammation, tissue damage and mortality. It is currently unknown, however, whether the excessive inflammasome activation is responsible for the more severe infection manifestations or whether RhsT has other activities that are responsible for these phenotypes.

In summary, *P. aeruginosa* has developed a number of mechanisms to hinder or confound the inflammasome-mediated immune response (Table 3.1). At first glance, some of these mechanisms appear to be contradictory and result in both enhancement and inhibition of an inflammatory response. Data supporting a definitive explanation for these differences are not currently available. It is conceivable, however, that different mechanisms are employed by different *P. aeruginosa* strains or in different infection contexts. For example, ExoU- and ExoS-mediated dampening of the inflammasome may occur during the first hours of infection when bacteria are few and vulnerable to sterilizing effects of inflammation (Sutterwala et al. 2007; Wangdi et al. 2010; Galle et al. 2008). After an acute infection has been established and large numbers of bacteria are present, bacteria may switch to a strategy of inducing an overly exuberant immune response (Schultz et al. 2002; Cohen and Prince 2013). *P. aeruginosa* may exploit off target tissue damage from pathologic inflammation by a number of mechanisms. For example, tissue damage may promote dissemination. During chronic infections, which require bacteria to persist for months and years, *P. aeruginosa* may adopt a stealth strategy by eliminating some PAMPs all together (Descamps et al. 2012; Cohen and Prince 2013; Wangdi et al. 2010). Collectively, these strategies represent a multipronged approach by *P. aeruginosa* to escape the inflammasome-mediated immune response and promote infection.

Inflammasome Activation During Specific Types of Infection

P. aeruginosa causes infections of a number of different body sites, including the peritoneal cavity, the eye and the lung. To accomplish this, it must overcome several host defenses, such as epithelial barriers containing tight junctions between cells to block bacterial invasion, the mucociliary transport system of the airways, the antimicrobial properties of tears in the eye and the innate immune response (reviewed in (Williams et al. 2010)). During infection, cytokines and chemokines are released from macrophages and epithelial cells to initiate the immune response. In addition to the inflammasome response, other cytokines such as IL-6, IL-8 and TNF-α are also secreted during *P. aeruginosa* infection (Williams et al. 2010). Neutrophils are recruited to the site of infection to promote bacterial clearance through phagocytosis and production of defensins, elastases and reactive oxygen species (Williams

et al. 2010). Thus, inflammasome activation is one part of a robust and sophisticated host defense system against bacterial infection. Perhaps not surprisingly, the consequences of *P. aeruginosa* inflammasome interactions are themselves complex and appear to vary in different models of infection. In some models, inflammasome activation is required for bacterial clearance (Franchi et al. 2007; Sutterwala et al. 2007; Patankar et al. 2013) while other models indicate that such activation leads to worse infection outcomes (Schultz et al. 2002; Schultz et al. 2003; Thakur et al. 2004a; Kung et al. 2012; Cohen and Prince 2013; Thakur et al. 2004b). Whether inflammasome activation is protective or detrimental to the host may be related to the route of infection, the duration of infection, the magnitude of bacterial load, or other experimental differences between the various infection models employed. Understanding the consequences of inflammasome activation in the context of each infection model has important implications.

Peritonitis

Peritonitis is an infection of the abdominal cavity that is a common complication of ascites, peritoneal dialysis, or rupture of the intestines (Szeto et al. 2001; Siva et al. 2009; Chadha et al. 2010). Although many different bacteria can cause peritonitis, infection by *P. aeruginosa* is especially severe (Siva et al. 2009; Chadha et al. 2010). A mouse model of peritonitis is convenient because bacteria are easily injected directly into the peritoneum.

Inflammasome activation is essential for clearance of *P. aeruginosa* during peritonitis (Sutterwala et al. 2007; Patankar et al. 2013). Infection of peritoneal macrophages *in vitro* with *P. aeruginosa* caused increased caspase-1-dependent release of mature IL-1β (Sutterwala et al. 2007; Patankar et al. 2013) and increased cell death (Sutterwala et al. 2007). Inflammasome activation was dependent on recognition of either the T3SS apparatus or flagellin (Sutterwala et al. 2007; Patankar et al. 2013). *P. aeruginosa* modulated the magnitude of inflammasome activation by translocation of ExoU into macrophages (Sutterwala et al. 2007) or by loss of flagellin and/or flagellar motility (Patankar et al. 2013). These results were confirmed *in vivo* as well. Although host survival outcomes were not explicitly reported, inflammasome-competent mice were better able to clear bacteria after intraperitoneal challenge with *P. aeruginosa* (Sutterwala et al. 2007; Patankar et al. 2013).

Keratitis

Keratitis is an infection of the cornea that can lead to blindness. *P. aeruginosa* is the most common pathogen cultured from contact lens wearers with keratitis (Stapleton and Carnt 2012). The ability to persist in biofilms on the lens surface undoubtedly contributes to the frequency with which *P. aeruginosa* causes keratitis, but epidemiological evidence also supports a role for specific virulence factors. For example,

strains that secrete the T3SS effector ExoU are especially common in *P. aeruginosa* contact lens keratitis (Stapleton and Carnt 2012). The pathology of keratitis is associated with robust inflammation that results in opacification of the cornea, damage to the epithelium and stroma, as well as corneal perforation (Thakur et al. 2004a; Karmakar et al. 2012; Rudner et al. 2000; Sun et al. 2010).

As mentioned, inflammation plays a prominent role in the pathogenesis of *P. aeruginosa* keratitis. Proinflammatory cytokines are produced from multiple pathways during these infections. IL-1β is produced from macrophages following TLR-MYD88-dependent signaling (Sun et al. 2010), which presumably feeds into the inflammasome-dependent pathway for maturation of pro-IL-1β and pro-IL-18 by caspase-1 (Thakur et al. 2004a; Thakur et al. 2004b). Mature IL-1β is also produced from neutrophils through cleavage by neutrophil elastase (Karmakar et al. 2012). IL-1β from the TLR/MYD88 pathway or the neutrophil elastase pathway is necessary for restriction of bacterial numbers at early time points during infection (generally, less than 48 h) but also results in corneal opacification (Karmakar et al. 2012; Sun et al. 2010). Conversely, prolonged high levels of IL-1β are associated with worse outcomes over longer infection courses (generally, over several days) (Rudner et al. 2000). Bacterial loads and clinical scores for corneal damage were improved in caspase-1 deficient mice despite decreased neutrophil recruitment, suggesting a role for the inflammasome in causing pathological inflammation (Thakur et al. 2004a). Similarly, chemical inhibition of caspase-1 at 18 h post-infection also resulted in improved clinical scores and reduced corneal perforation (Thakur et al. 2004b). Of therapeutic relevance, these outcomes were further improved by the addition of antibiotics (Thakur et al. 2004b). Thus, persistent inflammasome activation during *P. aeruginosa* keratitis results in worse infection outcomes that can be rescued by inhibition of caspase-1-dependent release of proinflammatory cytokines.

Acute Pneumonia

In a National Healthcare Safety Network study, *P. aeruginosa* was the fifth most common cause of overall nosocomial infections and the second most common cause of ventilator-associated pneumonia during the period from 2009–2010 (Sievert et al. 2013). Multiple murine infection models of acute pneumonia, including intranasal and intratracheal inoculation models, have been used to investigate the pathology associated with *P. aeruginosa* pneumonia.

Whether inflammasome activation is beneficial or detrimental to the host during acute pneumonia caused by *P. aeruginosa* remains controversial. Some studies have reported that inflammasome activation was required for more rapid clearance of *P. aeruginosa*; bacteria infecting mice deficient in components of the inflammasome cascade persisted in greater numbers during infection (Franchi et al. 2007; Wangdi et al. 2010). In contrast, other studies that employed the same infection models reported detrimental outcomes associated with inflammasome activation. Inflammasome activation was associated with increased bacterial persistence (Schultz et al.

2002; Schultz et al. 2003; Kung et al 2012; Cohen and Prince 2013; Veliz Rodriguez et al. 2012) and increased tissue damage (Schultz et al. 2002; Schultz et al. 2003; Kung et al 2012; Cohen and Prince 2013). Bacterial clearance was improved and tissue damage was reduced when levels of IL-1β or IL-18 were inhibited (Schultz et al. 2002; Schultz et al. 2003; Veliz Rodriguez et al. 2012). Furthermore, a recent study found that infection outcomes were improved following depletion of alveolar macrophages or chemical inhibition of caspase-1 (Cohen and Prince 2013). Thus, studies differ as to whether inflammasome activation is beneficial or detrimental for the host in acute pneumonia.

Variations in experimental design may explain the differing conclusions regarding the consequences of inflammasome activation during acute pneumonia. In particular, different strains of *P. aeruginosa* were used in the various studies. Whereas some studies used strain PAK, which secretes the T3SS effector ExoS (Franchi et al. 2007; Cohen and Prince 2013), others used the strain PA103, which secretes ExoU and is non-flagellated (Schultz et al. 2002; Schultz et al. 2003; Wangdi et al. 2010). A third strain, PSE9, which expresses the novel virulence determinant RhsT, was used in one study (Kung et al. 2012). Additionally, although similar routes of infection were used, the size of the bacterial inoculum differed greatly between studies. Higher doses of bacteria resulted in early mortality, preventing measurements at later time points. This precludes comparisons of longer term infections between studies using lower and higher inocula. Furthermore, the protective or detrimental role of the inflammasome may differ between mild and severe infections. Collectively, experimental differences regarding bacterial strain, inoculum size and duration of infection may have influenced the conclusions of each study. Understanding the implications of inflammasome activation during acute pneumonia, therefore, must be carefully considered within the context of the experimental design.

Conclusions

The inflammasome is an integral component of the host innate immune response against microbial infections. Multiple virulence determinants of *P. aeruginosa* are known to activate the inflammasome, including flagellin, components of the T3SS, type IV pilin and RhsT. Upon sensing these microbial PAMPs, the inflammasome initiates an activation cascade resulting in autoproteolytic cleavage of pro-caspase-1 and subsequent maturation of proinflammatory cytokines IL-1β and IL-18, production of eicosanoids and initiation of a pyroptotic cell death cascade. These events have evolved to facilitate the host immune response to *P. aeruginosa* and aid in bacterial clearance. However, *P. aeruginosa* has developed several strategies to circumvent the inflammasome response. Maturation of caspase-1 can be inhibited by the T3SS effectors ExoU or ExoS, effectively dampening the activity of the inflammasome. During chronic infection, flagellin, T3SS proteins and pili may not be produced, allowing bacteria to evade immune detection. Conversely, inflammasome activation can be exploited to induce pathologic inflammation that is ineffective at

clearing the bacteria. Ultimately, whether inflammasome activation is beneficial for the host to promote clearance or to the bacterium to promote persistence may be related to the context of the infection and to the virulence determinants expressed by the particular strain of *P. aeruginosa*. Thus, the interplay between the bacterium and the host inflammasome-mediated immune response is a complex balance of factors. Further understanding of this balance will inform future efforts to develop novel therapeutics for *P. aeruginosa* infection.

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Chapter 4
P. aeruginosa Type VI Secretion Machinery: Another Deadly Syringe

María-Guillermina Casabona, Sylvie Elsen, Valentina Cogoni and Ina Attrée

Abstract As a ubiquitous, wide-spread bacterium, *Pseudomonas aeruginosa* thrives in complex environments, forming often multi-species communities called biofilms. Competition for nutrients and space in these conditions may be crucial for bacterial fitness and survival. In this context bacteria employ different strategies to slow down the growth or kill the neighbour or its host. One of these strategies makes use of the complex macromolecular nanomachine that is embedded in the bacterial envelope and serves to export effector proteins from the attacking bacterium towards the prey. This secretion system, being the most recently discovered in Gram-negative bacteria, has been named Type VI Secretion System (T6SS). It is widespread amongst Gram-negative bacteria and is built up of structures homologous to bacteriophage T4-like tail tube and sheath, on one hand, and of two proteins similar to DotU and IcmF, components of the membrane-anchoring complex of the Type IV Secretion System (T4SS), on the other hand. The main function of T6SSs is killing of a target prokaryote or eukaryote cell, but they have been proposed also to be implicated in communication between the members of the multispecies community. Thus, the T6SS constitutes an original membrane-spanning complex that allows Gram-negative bacteria to communicate with other cells in many contexts and with diverse outputs. This book chapter deals with genetic organization of its components, regulatory circuits controlling its expression and activity, as well as with function and structure of the apparatus itself with emphasises on *P. aeruginosa* systems. More advanced findings on the T6SS machinery in other bacterial species, such as in *Escherichia coli* and *Vibrio cholera*, but that may be analogous to *P. aeruginosa*, have been included.

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Introduction

As a ubiquitous, wide-spread bacterium, *Pseudomonas aeruginosa* thrives in complex environments, forming often multi-species communities called biofilms. Competition for nutrients and space in these conditions may be crucial for bacterial fitness and survival. In this context bacteria employ different strategies to slow down the growth or kill the neighbour or its host. One of these strategies makes use of the complex macromolecular nanomachine that is embedded in the bacterial envelope and serves to export effector proteins from the attacking bacterium towards the prey. This secretion system, being the most recently discovered in Gram-negative bacteria, has been named Type VI Secretion System (T6SS). It is widespread amongst Gram-negative bacteria and is built up of structures homologous to bacteriophage T4-like tail tube and sheath, on one hand, and of two proteins similar to DotU and IcmF, components of the membrane-anchoring complex of the Type IV Secretion System (T4SS), on the other hand. The main function of T6SSs is killing of a target prokaryote or eukaryote cell, but they have been proposed also to be implicated in communication between the members of the multispecies community. Thus, the T6SS constitutes an original membrane-spanning complex that allows Gram-negative bacteria to communicate with other cells in many contexts and with diverse outputs. This book chapter deals with genetic organization of its components, regulatory circuits controlling its expression and activity, as well as with function and structure of the apparatus itself with emphasises on *P. aeruginosa* systems. More advanced findings on the T6SS machinery in other bacterial species, such as in *Escherichia coli* and *Vibrio cholera*, but that may be analogous to *P. aeruginosa*, have been included Fig. 4.1.

T6SS in the Microbial World and in *P. aeruginosa*

First large-scale genome screening gained insights into the phylogenetic distribution, gene content, organization and evolution of the T6SSs (Boyer 2009; Das and Chaudhuri 2003). Using as bait sixteen conserved T6-genes, 176 loci from 92 different bacteria were identified as containing at least five bait genes. These results were in accordance with another study that determined putative T6SS-encoding gene clusters in over one fourth of sequenced Gram-negative bacteria (Bingle et al. 2008). Interestingly, T6SSs are largely confined to proteobacteria, even though they also occur in planctomycetes and acidobacteria (Boyer et al. 2009; Bingle et al. 2008; Shrivastava and Mande 2008; Persson et al. 2009). Originally this secretion system was thought to have a role essentially in pathogenesis; however this point of view changed since the list of T6SS-encoding microbes, along with known human

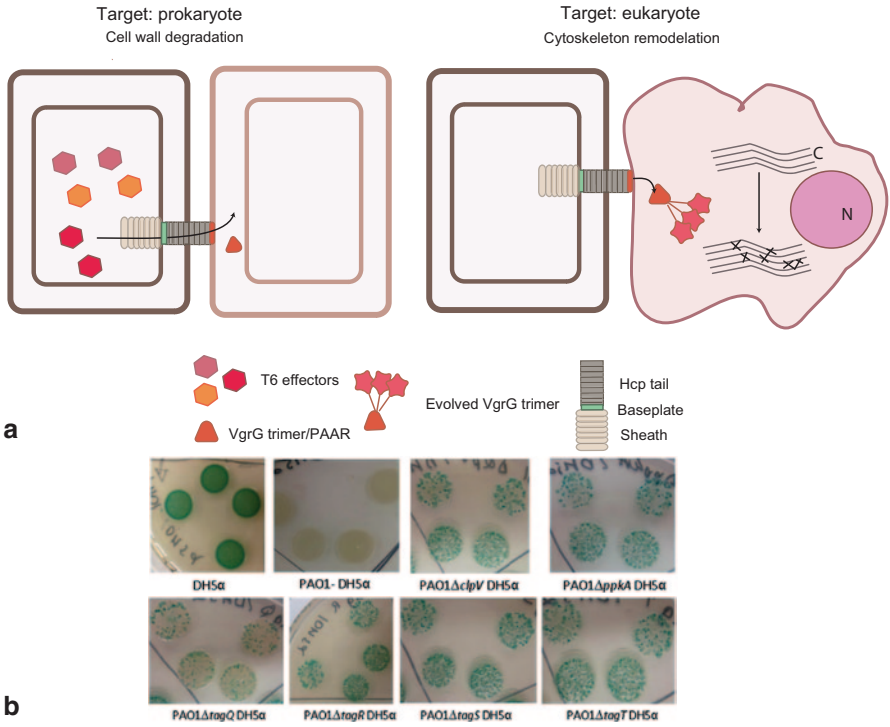

Fig. 4.1 **a** T6SSs target both prokaryotic (*left*) and eukaryotic (*right*) cells. The machinery, composed of the Hcp tube, baseplate and sheath structure, is reminiscent of bacteriophage injection structure. T6SS effectors are injected within the target cell as individual proteins or by being fused to other proteins of the machinery. *N* nucleus, *C* actin cytoskeleton. **b** Plate assays showing the killing activity of the *P. aeruginosa* T6SS towards DH5α *Escherichia coli*. The method and mutants are described in (Casabona et al. 2013; Hachani 2013). Briefly, genetically modified *E. coli* (in *blue*) are challenged by different *P. aeruginosa* strains. After 4 h of incubation on solid medium, the competition is scrapped off the plate and serial dilutions are made and spotted on selective medium. *E. coli* survivals will appear blue and *P. aeruginosa* survivals, *white*

pathogens, included symbionts, pathobionts, non-pathogenic bacteria, commensals and mutualists (Boyer et al. 2009; Jani and Cotter 2010).

The presence of multiple copies of T6SS loci was found in one third of the genomes (Boyer et al. 2009). It has been proposed that different loci in one genome have distinct evolutionary history, which means that they have been likely acquired by horizontal transfer and not by duplication (Bingle et al. 2008; Shrivastava and Mande 2008). T6SS gene loci can be grouped into five different phylogenetic clusters (Boyer et al. 2009; Barret et al. 2011). Genome of *P. aeruginosa* PAO1 harbours three T6SS-encoding clusters. These clusters were named Hcp Secretion Islands (HSI-I, -II and -III) upon the Haemolysin-coregulated protein (Hcp) that was the first secreted "core" protein identified (Mougous et al. 2006). Outside of the three HSI clusters and dispersed on the genome, *P. aeruginosa* harbours genes encoding VgrG proteins, Hcp and other components functionally related with T6SS machineries (Fig. 4.2).

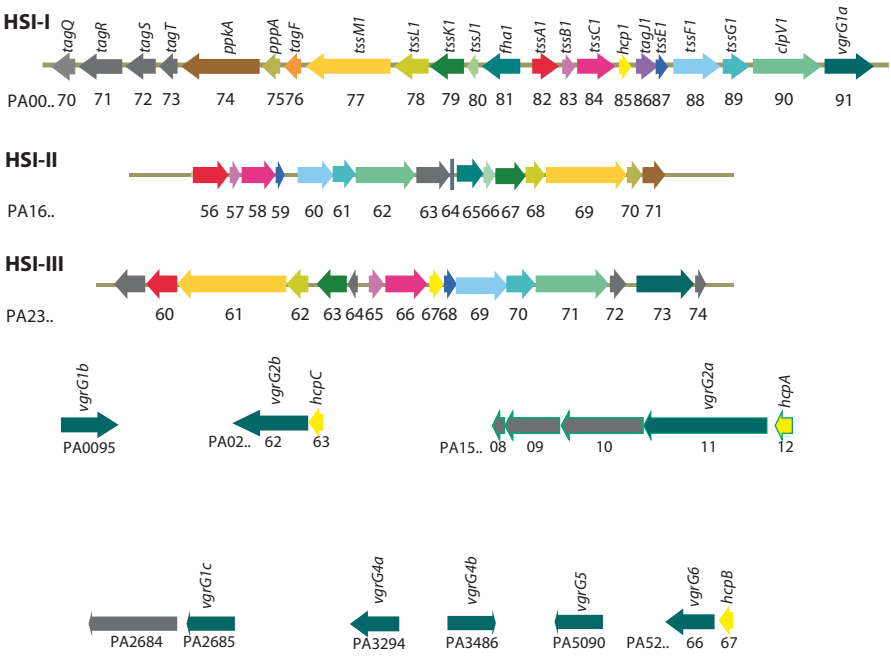

Fig. 4.2 Genetic organization of T6SS-encoding genes. Three clusters HSI-I, -II and –III carry the genes encoding the components of secretion machineries H1-, H2- and H3-T6SS, respectively, and some regulatory elements. Some VgrG and Hcp proteins are encoded out of the loci. The genes are annotated by PA numbers (pseudomonas genome project; http://www.pseudomonas.com) and using nomenclature from (Hachani et al. 2011; Shalom et al. 2007). Colour codes are respected between three clusters for homologous and related genes

These genetic elements of up to 20 kb encode 13 conserved proteins, and frequently additional regulatory elements. Based on the nomenclature proposed by (Shalom et al. 2007), the genes that code for the conserved core components of the T6SS have been named *tss*, for *t*ype *s*ix *s*ecretion genes. Interestingly, and further supporting the idea of specialized T6SSs with distinct functions, some clusters contain genes that are not conserved and encode accessory components of T6SS. In *P. aeruginosa*, the accessory genes were named *tag* (for *t*ype six *a*ssociated *g*enes) and code for proteins with diverse functions, indispensable for the T6 assembly, function and/or regulation.

Despite a conserved gene organization and general predicted structure of the apparatus, the three *P. aeruginosa* T6SSs seem to be highly host-specific and regulated in a different manner. Indeed, a recent study demonstrated that TssB/TssC proteins, which form the tail sheath of the secretion apparatus, encoded by different clusters are not exchangeable, further suggesting that there is no redundancy between multiple T6SSs within one organism (Lossi et al. 2013). In addition, the major regulatory circuit controlling the expression of HSI-I is the same for other virulence factors associated with chronic infection. H1-T6SS may play a role in chronic infections,

as sputa of cystic fibrosis patients harbour both the Hcp1 protein and antibodies against Hcp1 (Mougous et al. 2006). On the contrary, results obtained from animal and nematode models indicate that H2- and H3-T6SS seem to be employed during more aggressive types of infection (Lesic et al. 2009).

Type VI Secretion System effectors

Although some T6SS-dependent phenotypes are revealed by using eukaryotic cells, many have been characterized as having anti-bacterial activity, either intra- or interspecies, where the T6SS is decisive in the outcome of the bacterium when challenged with a competitor (Schwarz et al. 2010a, b). This can be of high importance in the establishment and persistence of a given pathogen during an infection within the host. T6SS may have evolved as "anti-virulence" mechanisms, where the secretion system would have a role in the regulation of cell density within the host, being a key factor in disease progression and microbe persistence by limiting colonization. Other phenotypes that have been associated to T6SS are stress sensing, biofilm formation and persistence in mixed biofilms. Together, T6SSs are involved in diverse social behaviours and act as mediators in inter-bacterial interactions and virulence (Schwarz et al. 2010b).

One of the best studied examples of anti-bacterial T6SS is the *P. aeruginosa* H1-T6SS that acts as device injecting effector proteins directly into target cells. The first H1-T6SS effectors were elegantly identified using proteomic approach on *P. aeruginosa* H1-T6SS regulatory mutants (Mougous et al. 2006). These effectors, named Tse1, Tse2, and Tse3 for Type-6-Secretion-Exported, are encoded outside of the HSI-I locus but rely on the machinery to be injected directly into the recipient after physical contact between the two cell types. Tse1 and Tse3 are lytic enzymes able to degrade peptidoglycan by amidase and muramidase activity, respectively. The third effector, Tse2, is toxic in the cytoplasm of recipient cells by a still unknown mechanism. These effectors play a key role in the outcome of *P. aeruginosa* in interspecies bacterial interactions in niches competition. *P. aeruginosa* avoids self-intoxication by synthesizing cognate immunity proteins, named Tsi1, Tsi2 and Tsi3 (Type-6-Secretion-Immunity), which bind to the secreted effectors to neutralize their detrimental activities. In addition of *P. aeruginosa* toxin/antitoxin Tse/Tsi couples (Hood et al. 2010; LeRoux et al. 2012; Russell et al. 2011), the same classes of proteins have been identified in *Burkholderia thailandensis, Pseudomonas fluorescens, Salmonella typhimurium* (Russell et al. 2012) and *Serratia marcescens* (English et al. 2012). Further structural analysis of Tse1 showed that this protein belongs to the N1pC/P60 papain-like cysteine peptidases where the catalytic diad is formed by Cys30 and His91. However, unlike the other members of the N1pC/P60 superfamily, Tse1 is a single domain protein without any putative localization domain (Chou et al. 2012). Inhibition of Tse1 by Tsi1 occurs by formation of a hydrogen bond between Tsi1 and the His91 of the Tse catalytic diad which consequently inhibits the enzyme activity (Benz et al. 2012).

A second class of T6SS effectors encompasses a diverse superfamily of bacterial phospholipase enzymes named Tle, for *T*ype VI *L*ipase *E*ffectors (Russell et al. 2013). Although some of these phospholipases, such as PldA (Tle5), have been implicated in *P. aeruginosa* virulence against eukaryotic hosts (Wilderman et al. 2001), it seems that they have also a specific function in intra- and interspecies bacterial interactions. Genetic and phenotypic studies demonstrated that the H2-T6SS is required for Tle5 toxic activity against *Pseudomonas putida* in mixed population. Indeed, purified PldA/Tle5 protein catalyses the release of choline from phosphatidylcholine, the activity being strictly dependent on the conserved catalytic domain in the protein. Furthermore, Tle5 exhibits high in vivo specificity to phosphatidylethanolamine, the major constituent of bacterial membranes. As for Tse molecules, Tle proteins possess their cognate immune partners that prevent self-killing (Russell et al. 2013).

The third class of T6S effectors is composed of VgrGs (Valine-Glycine Repeat protein G). Some of them, in addition of having essential role in T6S function, harbour catalytic domains with detrimental activities for the victim cell, such as actin remodelling and peptidoglycan disruption (Hachani et al. 2011; Ma and Mekalanos 2010; Durand et al. 2012a; Brooks et al. 2013). Dong et al. (2013) recently identified two *V. cholerae* effectors that possess both anti-bacterial and anti-eukaryotic activity. TseL harbours lipase domain, suggesting that it may target membrane-associated lipids and VasX has affinity for phospholipids in vitro (Miyata et al. 2011). Interestingly, TseL interacts with VgrG3 and its secretion depends on this interaction. Thus, this class of effectors is likely to be secreted in a complex with VgrGs.

The fourth class of potential T6SS effectors is represented by Rhs proteins. This family of YD-peptide repeat proteins is encoded frequently in the vicinity of T6SS loci or VgrG-encoding genes. Some of Rhs proteins have been shown to be *a bona fide* virulence factors, while others were involved in inter-bacterial competition. *P. aeruginosa* RhsT, harboured by the PA9 strain, acts by inducing inflammasome-mediated cell mortality (Kung et al. 2012); however its link with T6SS is still to be established.

Finally, the recent work by Leiman and Mekalanos groups further diversifies the list of T6SS effectors by discovering the so-called PAAR (proline-alanine-alanine-arginine) proteins that associate with VgrG on the tip of the injecting device (Shneider et al. 2013). This class of proteins are described later.

Architecture of the Type VI Secretion Apparatus

The T6S machinery is composed of two major sub-structures (Fig. 4.3), one sharing homology with bacteriophage T4 tail, and the other consisting of membrane-spanning peptidoglycan-bound complex that forms the anchoring complex.

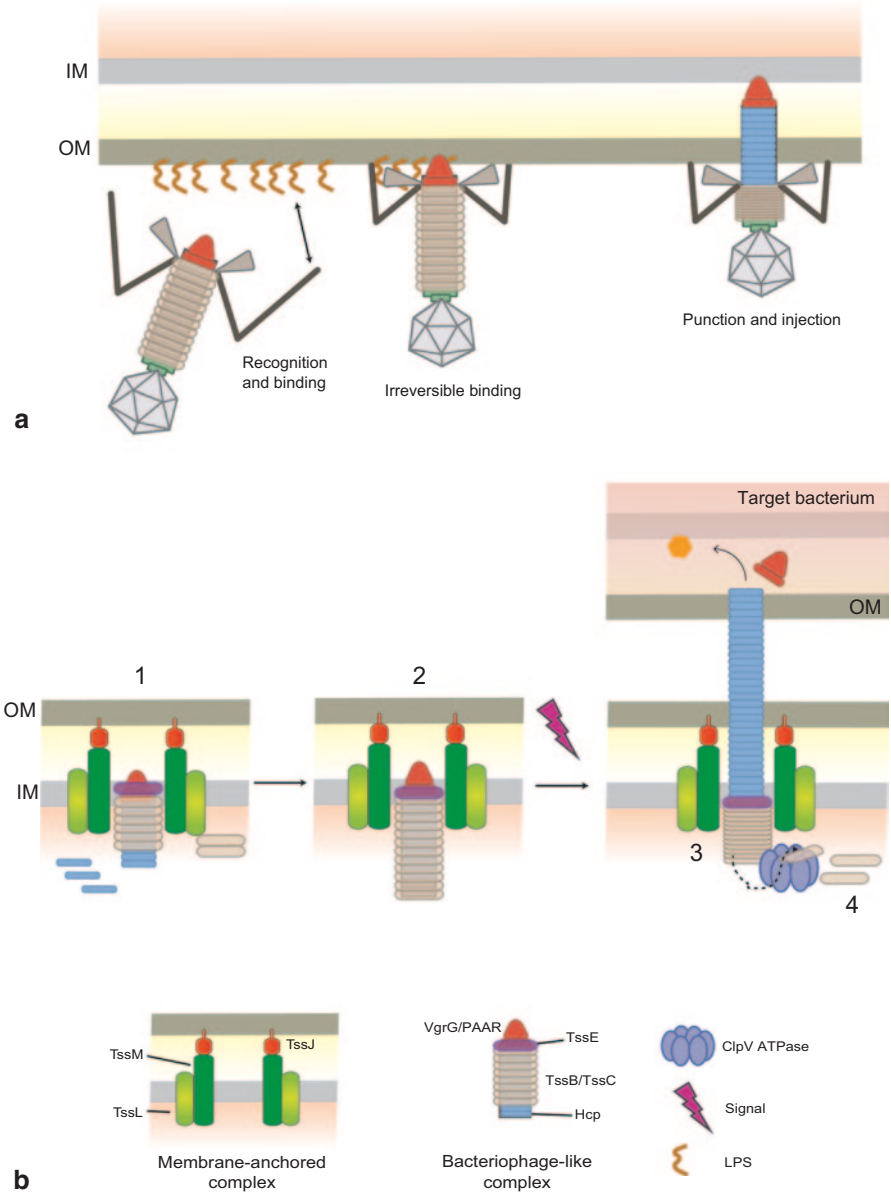

Fig. 4.3 Schematic representation of bacteriophage T4 action (**a**) and homology with T6SS machines (**b**). The assembly and attack by T6S may be divided in 4 steps: *1* membrane anchored and bacteriophage-like complexes are built up on defined regions within the bacterial envelope; *2* bacteriophage-like complex is extended, perpendicular to the IM, by addition of TssB/C subunits: *3* upon a signal, the TssB/C tubule contracts leading to the export of effectors; *4* the tubule associated AAA + ATPase, ClpV, participates in recycling of TssB and TssC subunits. The legend of the figure with protein names is given below. Colour code is respected between the bacteriophage and T6SS. *OM* outer membrane, *IM* inner membrane

Bacteriophage T4 Tail and Needle-like Structure

The marker of T6S functionality is presence of two conserved proteins, Hcp and VgrG, in the bacterial culture supernatants. Early structural studies revealed the striking similarities between these two proteins and proteins of bacteriophages. In addition to Hcp and VgrG, other bacteriophage-like subunits represent core components of the nanomachinery (Fig. 4.3).

The Tail Sheath

The bacteriophage T4 sheath consists of more than a hundred oligomerized gp18 subunits arranged as rings that form a hollow tube, able to accommodate the bacteriophage tail (Leiman et al. 2004). TssB and TssC subunits have been reported as forming a hollow tubular cogwheel-like complex of several hundreds of angström (Å) long. The internal diameter of the TssB/TssC complex is approximately 100 Å, big enough to accommodate the Hcp tube, estimated to have an external diameter of 85 Å (Lossi et al. 2013; Bonemann et al. 2009; Basler et al. 2012). Although TssB/TssC do not share sequence similarity with the bacteriophage sheath-forming subunit gp18, the modelling of a cross section of TssB/TssC tubules indicated that they shared same structural organization (Cascales and Cambillau 2012). Hcp co-fractionates with TssB and TssC homologues in *Francisella novicida*, further supporting the hypothesis that TssB and TssC form a tube-like structure able to engulf the Hcp filament (de Bruin et al. 2011). Direct interaction between TssB and TssC has been shown to be mediated by the N-terminus of TssC and was found highly specific as no cross interactions occurred between multiple T6SSs within one organism (Lossi et al. 2013). Assembled cytoplasmic structures appear in a perpendicular fashion to the cell envelope (Bonemann et al. 2009). It has been proposed that the formation of the TssB/TssC complex might be TssC-driven, since the TssB homologue in *V. cholerae* (VipA) does not form tubules in vitro in the absence of its partner, and in *P. aeruginosa* the C-terminus of TssC is required for tubule formation. Furthermore, TssC homologue (VipB) is needed for the interaction of the complex with ClpV in *V. cholerae*, a conserved T6S-ATPase (Lossi et al. 2013; Bonemann et al. 2009). In an effort to elucidate which proteins associate with TssB/TssC sheaths in *V. cholerae*, mass spectrometry analysis for protein identification was carried out on purified TssB/TssC structures. Four T6S proteins were identified: ClpV, VCA0109, VCA0111 and VCA0114 (Basler et al. 2012). ClpV had already been shown as directly interacting with TssB (Bonemann et al. 2009; Pietrosiuk et al. 2011). VCA0109 is a gp25 homologue, a protein that probably forms part of the baseplate and that is localized in the cytoplasm of *P. aeruginosa*. Finally, VCA0111 and VCA0114 (TssG and TssK, respectively) are conserved T6SS components, whose exact function is still unknown (see also below).

Recently, it has been shown that TagJ1 and TssB1 form a subcomplex of the sheath by using bacterial two-hybrid assays and NMR peptide mapping (Lossi et al. 2012). This interaction is mediated by the N-terminus of TssB1, conserved in other microorganisms encoding TagJ, such as *S. marcescens* (Lossi et al. 2012). Interestingly, TagJ1 is present in H1-T6SS in *P. aeruginosa* and absent in both H2- and H3-T6SS. Moreover, the interaction between TagJ1 and TssB1 is specific, since the authors demonstrated that TagJ1 does not form any complex with TssB2. The exact role of this subcomplex remains to be elucidated.

Hcp: Not Only the Tail Tube

Hcp is a ~ 17 kDa protein that arranges in hexameric rings with an internal diameter of approximately 40 Å (Mougous et al. 2006; Jobichen et al. 2010). Hcp is able to form structured non-helical, nanotube-like assemblies in vitro, which can be stabilized by the introduction of disulfide bonds (Ballister et al. 2008). Hcp shares homology with the protein gp19 that forms the tail tube of phage T4 (Pell et al. 2009; Leiman et al. 2009), further supporting the idea that Hcp may form a tubular structure that could traverse the cell envelope for substrate secretion. First structural studies on T6S effectors revealed that the size of the fully folded Tse1 is compatible with the internal diameter of the Hcp hexamer, thus proposing the Hcp as an effector conduit (Benz et al. 2012). The observation that *P. aeruginosa* Hcp1 stabilizes Tse effectors posttranslationally prompted Silverman et al. to examine direct interaction between Hcp1 and Tse2 (Silverman et al. 2013). These analyses allowed proposing the Hcp as a multipurpose protein playing a role of both a chaperon and a substrate receptor. Furthermore, as Hcp is known to be a T6S-secreted component, Hcp1-Tse complex is likely to traffic together across the bacterial envelope. However, the final destination of the complex, the way of the effectors' release and the final fate of the Hcp protein are unknown.

VgrG and PAARs: Not Only a Cell-Puncturing Complex

VgrG proteins, found in the bacterial extracellular milieu, are the second marker of the T6SS functionality. Some bacterial genomes, such as *P. aeruginosa*, encode up to ten different VgrGs (Boyer et al. 2009; Hachani et al. 2011; Pukatzki et al. 2007). Based on structural analysis of VgrGs and their similarity to the T4 puncturing device, it has been proposed that they are localized at the distal tip of the Hcp tube and act as a membrane-puncturing device. However, the direct interaction between these two proteins has not been shown. The N-ter domain of the VgrG protein encoded by *E. coli* CFT073 showed that VgrG architecture is comparable to the bacteriophage spike subdomain, notably the gp5-gp27 complex, despite their low sequence identity (Leiman et al. 2009).

As is the case for VgrGs of different species, *P. aeruginosa* VgrGs (VgrG1a and VgrG1c) multimerize in tripartite structure and their export is Hcp-dependent

(Hachani et al. 2011; Pukatzki et al. 2007; Zheng and Leung 2007). VgrG spike is decorated with the protein containing proline-alanine-alanine-arginine (PAAR) repeats. The PAAR protein folds into a symmetrical cone-shaped structure with a sharp tip and a triangular base which sits on the VgrGs surface (Shneider et al. 2013). Extensive bioinformatic analysis showed that PAAR proteins carry C- or N-terminus extensions which are diverse in size and predicted function, comprising peptidase, nuclease or lipase activities. However, most of extensions have no predicted activity. Interestingly, some Rhs proteins harbour PAAR domains and may also act as a tip carrying diverse functional groups (Shneider et al. 2013).

In *P. aeruginosa*, the H2-T6SS targets eukaryotic cells by modifying PI3K/Akt-dependent bacterial entry into epithelial cells (Sana et al. 2012). VgrGs and Rhs proteins, co-regulated by the H2-T6SS locus, have been suggested to be direct actors of this phenotype.

The Baseplate Assembly

The T4 bacteriophage baseplate, a macromolecular complex composed of at least 130 proteins of 14 different families, is required for initiation of tail tube and sheath assembly. It is assembled around the "hub", a central three-fold-symmetric cylindrical structure (Rossmann et al. 2004). In T4, gp25 forms a wedge-shaped complex with gp6 and gp53 that is localized around the centre of the baseplate and has a key role in the connection of its central and peripheral parts (Kostyuchenko et al. 2003; Yap et al. 2010).

TssE shares approximately 40% sequence similarity with gp25 of T4 bacteriophage (Leiman et al. 2009; Lossi et al. 2011). Moreover, it shows also remarkable similarity with gp25 secondary and tertiary structures as revealed by prediction tools. *P. aeruginosa* TssE is localized in the cytoplasm and has no lysozyme activity, unlike T4 gp25 protein family (Lossi et al. 2011). It is conceivable to propose that TssE might form part of the baseplate of the T6SS, that would assemble in the inner leaflet of the IM of the bacterium prior to secretion, and that the baseplate would be the linker structure between the tail and spike complex and the membrane-anchored complex.

Several questions remain unanswered. Does TssE form part of a larger IM baseplate-like structure? Does TssE interact with TssM and/or TssL in the inner leaflet of the IM? How is TssE recruited, if so, to the IM machinery complex?

Interestingly, it has been observed by electron microscopy that the T6S machinery presents a bell-shaped platform that connects the sheath to the IM (Basler et al. 2012). TssB/TssC elongated tube assembly is TssE-dependent in *V. cholerae* and they can be co-purified. Gp25 interacts with gp5-gp27 trimer in T4 bacteriophages, suggesting that TssE may have a similar role in the T6SS (Basler et al. 2012, Basler and Mekalanos 2012; Kapitein et al. 2013).

Membrane-Associated Components

The T6S machinery is anchored to the envelope of Gram-negative bacteria by several membrane-associated proteins forming a complex bound to peptidoglycan. This membrane subcomplex has been isolated by Cascales and co-workers from *E. coli* (Aschtgen et al. 2010a), and is composed at least by TssJ, TssM and TssL (Fig. 4.3). All these proteins are indispensable for T6SS activity.

TssJ is an OM lipoprotein able to homodimerize (Aschtgen et al. 2008; Rao et al. 2011b). Its X-ray crystal structure showed its arrangement in parallel β-sheets connected by a protruding loop that in *E. coli* is required for interaction with the periplasmic domain of TssM (Rao et al. 2011b; Felisberto-Rodrigues et al. 2011; Robb et al. 2013). The structural conservation of this loop across all species suggests that the interaction with TssM may also be conserved (Robb et al. 2013).

TssL is an IcmH-like integral IM protein (Aschtgen et al. 2012). IcmH (DotU) is needed for the correct functioning of T4bSS in *Legionella pneumophila*, since knockout mutants are impeded in intracellular growth (Zusman et al. 2004). The C-terminus of TssL homologue in EAEC is oriented towards the periplasm (Aschtgen et al. 2012). Its N-terminus folds as a hook-like structure composed of two three-helix bundles and is necessary for TssL autoassociation (Durand et al. 2012b). Autoassociation has been proposed to be mediated by the trans-membrane domain of TssL, since overproduced cytoplasmic domains are monomeric in solution and they only interact transiently and with low affinity in bacterial two-hybrid assays. Mutation of crucial residues of the trans-membrane segments completely abrogates TssL-TssL interaction, further supporting the hypothesis. Autoassociation has been suggested to be crucial for TssL function, maybe by increasing local concentration of the protein and allowing recruitment of other T6S components (Durand et al. 2012b). The cytoplasmic domain of TssL behaves as a soluble globular protein and does not present hydrophobic patches. It does not present any apparent catalytic domain or catalytic residues, which further suggests that TssL is a structural component of the apparatus. TssL proteins can bear a peptidoglycan-binding (PGB) domain thought to stabilize the apparatus in the cell envelope by anchoring it to the cell wall. In the case of some T6SSs, TssL lacking the recognized PGB domain co-occurs with TagL, another integral IM protein able to bind peptidoglycan in vivo and in vitro (Aschtgen et al. 2010a, b). In *P. aeruginosa*, curiously, the HSI-II and HSI-III loci do not harbour any protein with identified PGB domains.

TssM is an integral IM protein with a large periplasmic domain (744 residues in EAEC (Felisberto-Rodrigues et al. 2011)). In *Agrobacterium tumefaciens* TssM has ATP-binding and hydrolysis activity, the latter being crucial for Hcp recruitment (Ma et al. 2009, 2012). On the other hand, TssM of *Edwardsiella tarda* does not require its NTP-binding domain for Hcp secretion (Zheng and Leung 2007). Moreover, some TssM do not possess any NTP-binding domains. These findings argue that TssM-associated ATPase activity is not widespread through T6SSs and that it might be related to specific needs of each bacterium. In *P. aeruginosa*, TssM1

harbours only one predicted transmembrane helix and no predicted NTP-binding domain.

TssM, in addition of interacting with TssJ, is able to form a complex with the C-terminal periplasmic domain of TssL. TssJ and TssL are both able to homodimerize, in the OM and in the IM, respectively. It is plausible that these three proteins form a membrane-spanning complex that is bound to peptidoglycan and has a ring shape-like structure. Of note, ring shape-like structures seem to be a common feature of different membrane-spanning machineries, such as T3SS and T4SS (Schraidt and Marlovits 2011; Fronzes et al. 2009).

Does the membrane-spanning complex function as a channel and support for the syringe-like complex? It has been suggested that the TssM-L-J complex can accommodate the phage-like injection machinery. The interaction of TssM via its periplasmic domain both with Hcp and TssB supports this model, where the membrane-spanning complex would stabilize the needle-like complex.

Recently, the IM proteome of *P. aeruginosa* PAO1 has been explored by mass spectrometry (Casabona et al. 2013). Among the detected proteins, 23 components encoded by the HSI-I locus have been identified. Notably, two predicted cytoplasmic proteins, TssE and TssK, were found associated with the IM, further suggesting their role in syringe formation. Interestingly, *E. coli* TssK homologue is a trimer able to interact with several components of the T6SS (TssM, TssA, TssB and Hcp), suggesting that it may be the missing glue between the two complexes (Zoued et al. 2013). Another protein, of yet unknown function, TagJ, forms a complex with TssB. Curiously TagJ is one of the "accessory" components found only in approximately 30% of T6SSs, which suggests the subtle adjustments in T6SS assemblies (Lossi et al. 2011).

Mechanism of Injection, a Dynamic Story

As discussed in the previous sections, bioinformatic, structural and biochemical analyses suggest that T6SSs are contractile injection systems reminiscent of tailed phages that employ a syringe-like macromolecular nanomachine to puncture host cell membrane.

T6SSs are highly dynamic structures that follow assembly/disassembly contact-dependent cycles that can be monitored by time-lapse fluorescence microscopy (LeRoux et al. 2012; Basler and Mekalanos 2012; Brunet et al. 2012). For example, *P. aeruginosa* cells can respond to T6SS activity in a neighbouring sister cell with an increase in their own T6SS dynamics. This phenomenon has been baptized bacterial dueling (Basler et al. 2013), and has also been documented for pathogenic *E. coli* EAEC cells. Interestingly, the propagation of T6SS activities was shown to increase over time, spreading through the bacterial lawn, suggesting the rapid genesis of the signal within cooperative microorganisms which could contribute to coordination in a spatial and temporal fashion of a bacterial community to eliminate competing bacteria.

Indeed, recent data obtained by time-lapse fluorescence microscopy showed that T6SS dueling also occurred between heterologous species (Basler et al. 2013). More precisely, the dueling response of *P. aeruginosa* was triggered by the T6SS activity of a heterologous microorganism which had attacked first whereas *P. aeruginosa* T6SS dynamic was absent in mixture containing species devoid of T6S activity. This ability to respond to a T6SS-mediated attack and efficiently kill the competitors was lost when the TagQRST-PpkA-Fha1-PppA regulatory cascade (see parts on regulation) was inactivated. This suggested that this cascade may form a regulatory module involved in direct sensing of envelope perturbations such as OM breach, peptidoglycan disruption/modification and/or IM perforation upon exogenous T6SS attack. Envelope perturbations will then be the point where the new T6SS will be assembled and "fired".

Finally, two reports describe inter-bacterial intoxication via T6SS in a quantitative manner in vivo (LeRoux et al. 2012; Brunet et al. 2012). By monitoring prey lysis over time, they demonstrated that cell-cell contact is needed for T6SS-mediated intoxication but it is not the cue for T6SS activation. Moreover, the T6SS is not an extended extracellular appendage, since immediate cell-contact (less than 200 nm) was needed for prey lysis. The authors proposed that T6SS-associated attacks may act as a cue to the donor that the susceptible cell is in its vicinity, conceivable given the broad distribution of T6SS among Gram-negative bacteria. In bacteriophages, host recognition occurs through a reversible interaction of the conserved tip of the long fibres with the OM protein OmpC or with LPS (Yu and Mizushima 1982). This triggers conformational changes in the short fibres that extend and bind to the outer core of LPS in an irreversible fashion. The binding is followed by contraction of the outer tail sheath, penetration of the bacterial membrane by the hollow inner tail tube (Leiman et al. 2004, 2009). Interestingly, structural studies revealed that the aromatic and positive residues on the surface of the fibre tips are most likely receptor-binding determinants with the receptor in the bacterial surface (Bartual et al. 2010). Up-to-date, homologues of these fibre tip components have not been found in T6SS.

In the case of bacteriophages, the syringe-like complex is used only once to inject DNA into the host. On the contrary, T6SS injection device can be reused. It has been suggested that ClpV, a conserved Hsp100/Clp family of AAA+ of the T6S, is responsible for the recycling of the TssB/TssC tubules. ClpV is able to disassemble and remodel the tubules in vitro and interacts directly with TssC in a specific manner. Finally, it has also been demonstrated that ClpV prevents the formation of non-productive TssB/TssC tubules formed spontaneously in the cell (Bonemann et al. 2009; Pietrosiuk et al. 2011; Kapitein et al. 2013). This would assure the presence of a pool of non-assembled TssB and TssC units, needed for T6S assembly upon cue sensing.

Regulation of Type VI Secretion Systems in *P. aeruginosa*

As for most of the complex secretion machineries embedded within bacterial membranes, the three T6SSs are highly regulated at different levels, including post-translational, transcriptional and post-transcriptional levels. Few signals and some molecular actors involved in these types of regulation are starting to be revealed and are summarized in Fig. 4.4.

Post-Translational Regulation

H1-T6SS activity depends on additional proteins. Seven proteins, PpkA, PppA, TagR, TagS, TagT, TagQ and Fha1, form part of the so-called threonine phosphorylation pathway (TPP) (Hsu et al. 2009), and TagF is a negative regulator acting independently of the TPP (Silverman et al. 2011).

The TPP pathway relies on a transmembrane serine-threonine kinase, PpkA, which dimerizes and autophosphorylates to then phosphorylate Fha1, a protein that contains a Forkhead-Associated domain. Once phosphorylated, Fha1 recruits

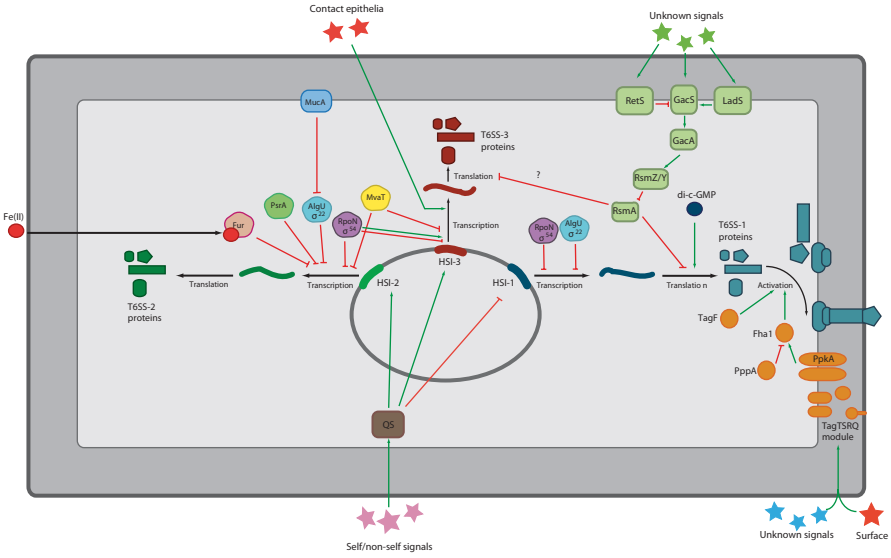

Fig. 4.4 Regulation of the *P. aeruginosa* HSI loci. The principal regulators and pathways involved in transcriptional, post-transcriptional and post-translational control are indicated. The positive effects are depicted by *green arrows*, the negative ones by *red bars*. For the regulation of orphan elements and the link between c-di-GMP and RsmZ/Y pathway, see the text. The direct binding of RsmA on HSI-I mRNAs was demonstrated by RNA mobility shift assays (Brencic and Lory 2009), that of LasR on *PA1656-59* locus (HSI-II) by electrophoretic mobility shift assay (Gilbert et al. 2009) and of MvaT on *PA1656* (HSI-II) and *PA2371-73* (HSI-III) by ChIP-on-Chip (Castang et al. 2008)

the ATPase ClpV1 to the IM and to TssB/C tubes, triggering both syringe assembly and effector export. The activity of PpkA is antagonized by its cognate phosphatase, PppA, which de-phosphorylates Fha1. TagR, a periplasmic protein that acts upstream of PpkA, is essential for the activation of the system. The modulation of the phosphorylation status of Fha1 on Thr362 determines the triggering of H1-T6SS activation (Hood et al. 2010; Hsu et al. 2009; Mougous et al. 2007). A recent study has described TagT, TagS and TagQ as additional players in the TPP by acting upstream of the PpkA/PppA phosphorylation checkpoint (Casabona et al. 2013). TagT and TagS form a membrane-bound ABC transporter with ATPase activity and TagQ is an OM lipoprotein that faces the periplasm. It was shown that TagR, under some conditions, associates to the OM in a TagQ-dependent manner. Thus, TagTSRQ form a novel signalling module in charge of sensing exogenous T6SS attacks, most probably by sensing cell envelope disruption or interacting with exogenous T6SS effectors. The sensing by TagTSRQ module promotes local Fha1 phosphorylation that leads to T6S machinery assembly and firing (Basler et al. 2013; Casabona et al. 2013).

Independent of the TPP, another posttranslational pathway has been described in which Fha1 phosphorylation is negatively regulated by TagF. The environmental cues that govern the two pathways are different, since surface growth induces TPP activation, but not the TagF-dependent pathway. Even though the mechanism by which TagF represses the H1-T6SS activation is still unknown, the authors have shown that both TPP and TagF pathways converge in the recruitment of ClpV1 (Silverman et al. 2011).

Transcriptional and Post-Transcriptional Regulations

HSI-I was discovered through microarray analysis revealing a new locus negatively controlled by RetS, a key component of the RetS/LadS/Gac/Rsm pathway known to orchestrate the chronic/acute virulence switch (Mougous et al. 2006; Goodman et al. 2004; Ventre et al. 2006; Coggan and Wolfgang 2012). Sensor kinases RetS and LadS control in opposite manner HSI-I expression that is inhibited by RetS and activated by LadS, thus promoting expression during chronic stage of infection (Mougous et al. 2006). The involvement of the other players of the cascade, namely GacA, RsmA and RsmYZ, was further confirmed by microarray analyses (Brencic et al. 2009). Furthermore, a direct binding of RsmA to the leader sequence of transcripts encoding PA0081 and PA0082 was shown (Brencic and Lory 2009). Interestingly genes located in the HSI-III are also under regulation of the pathway as transcript levels were downregulated in *gacA* and *rsmYZ* mutants (Brencic et al. 2009). In addition, H1-T6SS is inversely controlled with T3SS by c-di-GMP; indeed, an increased level of this second messenger, either triggered by *retS* mutation, *ladS* overexpression, or WspR adenylate cyclase activity, was shown to activate H1-T6SS activity and inhibit T3SS synthesis (Moscoso et al. 2011). This effect requires the RsmY and RsmZ small regulatory RNAs but the exact mechanism

connecting the two pathways is still unknown. The LadS/Gac/Rsm pathway, but not c-di-GMP, was recently shown to be required for the induction of both biofilm formation and HSI-I gene expression, along with the repression of T3SS genes, in response to sub-inhibitory concentration of kanamycin (Jones et al. 2013). This might be of importance for the survival within polymicrobial niche, even if no increase in bacterial killing was observed, probably linked to absence of Tse3 effector induction (Jones et al. 2013).

An inverse relationship between mucoidy and T6SS expression has been underlined in several studies. Mutation in *mucA*, encoding the anti-sigma factor of AlgU, leads to the release of the ExtraCytoplasmic Function (ECF) sigma factor; then AlgU is free to activate transcription of genes involved in alginate synthesis. This is the common mechanism for transition to mucoid phenotype (Damron and Goldberg 2012). Transcriptome analysis indicated that AlgU exerts a negative effect on HSI-I and HSI-II genes, as well as on *vgrG2a* (*PA1511*) and *hcpC-vgrG2b* (*PA0263–62*) (Tart et al. 2005). This suggests that mucoid conversion, the hallmark of chronic infection, should also decrease H1-T6SS activity, an idea contradicting the observed Hcp1 production in CF sputum (Mougous et al. 2006). In the same line, *mucA* mutants isolated from CF individuals were shown to exhibit reduced expression of virulence factors, such as T3SS but also H1-T6SS genes, compared to the non-mucoid strains (Rau et al. 2010). Furthermore, transcriptome and proteome analyses of two isogenic strains isolated from a CF patient pointed out a net difference in T6SS expression between the non-mucoid and mucoid strains (Rao et al. 2011a, 2008). Indeed, three proteins encoded within HSI-I, namely TssB1 (PA0083), TssC1 (PA0084) and Hcp1 (PA0085), were less abundant in the mucoid strain, and the transcriptome analysis indicated a decreased expression in the mucoid strain of most of HSI-I operons and few HSI-II genes, namely *tssA2* (*PA1656*), *tssC2* (*PA1658*) and *tssE2* (*PA1659*). The authors proposed that the non-mucoid bacteria during initial phases of infection could require T6SS to outcompete other bacteria, but then bacteria convert to mucoid phenotype and do not require any more their T6SS (Rao et al. 2011a).

Quorum sensing (QS) coordinates expression of the 3 HSI loci, as reported by numerous studies using approaches such as transcriptome analysis, RT-qPCR, or ChIP-on-chip ((Bernard et al. 2010); and references herein). Both HSL-based (Rhl and Las) and HAQ-based (MvfR) systems affect and control differentially the three loci, with HSI-I being negatively controlled by LasR and MvfR, while HSI-II and HSI-III are positively controlled by Las/Rhl and MvfR (Lesic et al. 2009; Sana et al. 2012; Schuster et al. 2003). A direct binding of LasR was even observed on *PA1656–59* region (HSI-II) (Gilbert et al. 2009). Hence, and as confirmed by transcriptional fusion analyses (Sana et al. 2013), H2-T6SS and H3-T6SS synthesis occurs at high cell density at the same timing as numerous other virulence determinants. The loci encoding these two T6SSs also belong to the MvaT regulon, as *PA1656* (HSI-II), *PA2371–73* (3 HSI-III-encoded genes) as well as the two T6SS related genes *PA1512–11 (hcpA-vgrG2a*), are direct targets of MvaT (Castang et al. 2008). MvaT is an H-NS family member functioning as a repressor of gene expression and proposed to be a transcriptional silencer of AT-rich foreign DNA

(Castang et al. 2008). HSI-II is also induced upon iron limitation, as the repressor Fur controls its expression, probably directly, 2 putative Fur boxes being located in the *tssA2* promoter (Sana et al. 2012). HSI-II locus (*PA1657–70*) as well as *hcpC* (*PA0263*), *vgrG2a* (*PA1511*) and *vgrG6* (*PA5266*), are repressed by TetR family repressor, PsrA, able to bind and respond to long-chain fatty acid signals, that controls fatty acid metabolism (Kang et al. 2008). PsrA also regulates expression of *Tfp* and *rpoS*, the latter being potentially of importance for stationary growth adaptations (see (Potvin et al. 2008), and references herein).

Based on transcriptional fusion and mutant analyses, a recent study has reported the control of HSI-II and HSI-III by the alternative sigma factor 54 (σ^{54}), known to be crucial for virulence in *P. aeruginosa* (Sana et al. 2013). Whereas σ^{54} (RpoN) activates one of the two HSI-III operons, *PA2364–59*, it affects negatively the HSI-II locus and the *PA2365–74* HSI-III operon probably through an indirect mechanism. The transcriptional activation by RNAP-σ^{54}, in general, requires bacterial Enhancer-Binding Proteins (EBPs) to provide energy through ATP-hydrolysis and direct molecular interaction. Both the identification of a consensus sequence for binding of σ^{54} in HSI-III locus by bioinformatics analyses (Bernard et al. 2010) and the positive control of RpoN exerted on *PA2364–59* suggest that this HSI-III operon might require such an EBP activator. Interestingly, two EBPs are potentially encoded within the two loci, the sigma factor activators Sfa2 (PA1663) and Sfa3 (PA2359) (Filloux et al. 2008). However, none of the two proteins was found involved in HSI-III operon expression, and Sfa2 participates with RpoN to the HSI-II repression (Sana et al. 2013). A crosstalk was previously reported between HSI-II and HSI-III, as HSI-II deletion triggered a reduced *hcp3* transcription (Lesic et al. 2009); as it does not rely on a positive effect of Sfa2 on HSI-III as it was suggested, the mechanism is still unknown.

A transcriptome study indicated a strong increase in PAO1 HSI-III (*PA2365–74* operon) gene expression as well as *hcpC* (*PA0263*) upregulation upon contact with respiratory epithelia but the underlying mechanism is not known (Chugani and Greenberg 2007).That might be relevant for the virulence in eukaryotes, as well as the observation that expression of HSI-III operons is maximal at 37 °C (Sana et al. 2013). However, lowering growth temperature from 37 to 25 °C strongly induced expression of *hcpB* (*PA5267*) encoding an orphan T6SS element in PAO1, as well as *vgrG2b* (*PA0262*) and *tssB1*, a HSI-I-encoded gene, but at a lower level (Termine and Michel 2009). The temperature decrease conducts also to upregulation of *hcpD* (*PA14_43070*) and *hcpB* (*PA14_69560*) in the PA14 isolate (Wurtzel et al. 2012). Furthermore, secretome analysis indicated that suboptimal temperature influences secretion of Hcp, whose production was increased at 25 °C. The temperature effect might give a competitive advantage over environmental bacteria and be of high importance for the persistence in hospitals (Termine and Michel 2009).

Deciphering all the intertwined regulatory pathways, the cross-talks, the sensed signals controlling expression of the T6SSs will help understanding their physiological roles and relevance. To complete the overall picture of T6SSs regulation, the reader can consult the following reviews (Bernard et al. 2010; Silverman et al. 2012; Leung et al. 2011; Miyata et al. 2013).

Concluding Remarks and Future Directions

T6SSs are the last protein machineries discovered in Gram-negative bacteria. Their structural and regulatory complexity, role in bacterial physiology and in host-bacteria interactions made it an extremely attractive field of investigations. *P. aeruginosa*, together with EPEC and *V. cholera*, became especially appropriate model for studying all of these aspects of T6SS biology. The homology with the T4 bacteriophage allowed the strong impact of structural studies and revealed tridimensional organization of T6SS sub-assemblies. However, the whole apparatus has not been yet isolated and visualized. In our point of view, some other challenges are confronting us. Although some membrane signalling modules have been identified, the environmental signals that trigger T6SS assembly and effector firing are still unknown. The first pieces of evidence for "membrane perturbation signals" came from the recent paper of Ho et al. (Ho et al. 2013), in which the authors identified mating pair formation system by T4SS as one of the natural membrane-disturbing agents that may induce T6SS response to delimit the acquisition of foreign DNA. The second challenge concerns the T6SS effectors; the list of T6SS-associated effectors is being rapidly increasing and up to date encompasses five effector families. Studies of how these effectors are recruited and recognized by the given syringe and what is the timing of their ejection will certainly require new technological and methodological developments. Finally, the activity of T6SSs being essential for bacterial fitness in different settings, including multi-species infections, may be considered in the future as target for novel anti-bacterial therapy.

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Chapter 5
Anaerobic Life Style of *Pseudomonas aeruginosa*

Masanori Toyofuku, George A. O'Toole and Nobuhiko Nomura

Abstract *Pseudomonas aeruginosa* is able to grow under anoxic conditions in the presence of nitrate or nitrite. This process is known as denitrification. Denitrification is not only important in bacterial ecology but also many studies indicate that it is associated with *P. aeruginosa* infection. While the regulation of denitrification is well studied, how the cells behave under denitrifying conditions is poorly understood. Recent studies show that under denitrifying conditions, many phenotypes change with respect to oxic conditions. This chapter shows how bacteria behave differently under denitrifying conditions compared to oxic conditions. For instance, cell-cell communication, membrane vesicle induction and biofilm structures are altered under denitrifying conditions. Although the underlying mechanisms of these observations are not fully understood, the presence of nitric oxide, which is produced during denitrification, seems to be one of the key molecules in the understanding the effect of anaerobiosis on *P. aeruginosa*.

Keywords Denitrification · nitric oxide · quorum-sensing · biofilm · membrane vesicle · pathogenicity

Introduction

Pseudomonas aeruginosa was previously considered as an obligatory aerobic bacterium, but it is now well acknowledged to have a versatile energy metabolism that allows the bacterium to survive under anoxic conditions. Under oxic conditions, at

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least five terminal oxidases with different affinity for oxygen are used according to the environmental conditions (Arai 2011). Denitrification is part of the biogeochemical nitrogen cycle, which reduces soluble nitrate and nitrite to gaseous nitrous oxide (N_2O) or dinitrogen (N_2), and releases these gases into the atmosphere. *P. aeruginosa* possesses the complete set of denitrification enzymes enabling the bacterium to reduce nitrate to N_2, and *P. aeruginosa* is one of the best-studied model organisms in denitrification. When nitrate or nitrite are available under low oxygen to anoxic conditions, *P. aeruginosa* generates energy through a respiratory process known as denitrification. Furthermore, when oxygen or nitrogen oxides are depleted, the bacterium is still able to survive through arginine and pyruvate fermentation (Vander Wauven et al. 1984; Eschbach 2004). More recently, it was shown that *P. aeruginosa* can use redox-active substances, phenazines, as electrons shuttle that promote survival (Dietrich et al. 2008; Wang et al. 2010). These versatile energy metabolisms would contribute to the ability of *P. aeruginosa* to inhabit a wide range of environments including the infected host.

Besides the ecological importance, denitrification is considered to be involved in the pathogenicity of *P. aeruginosa* (Hassett et al. 2009; Schobert and Jahn 2010). Hence, it is important to understand the anaerobic physiology of *P. aeruginosa,* both from the clinical to the ecological points of view.

Recent studies have revealed that *P. aeruginosa* has distinct gene expression patterns and behaves differently under anoxic versus oxic conditions (Wu et al. 2005; Platt et al. 2008; Filiatrault et al. 2005). This chapter illustrates the physiology of *P. aeruginosa* under anoxic conditions, particularly focusing on denitrification. To avoid confusion, in this chapter we will refer to the term anoxic for the conditions where oxygen is depleted and the term denitrifying conditions when oxygen is depleted and nitrate or nitrite is supplemented.

Regulation of Denitrification

Four sequential steps are involved in the denitrification, process where nitrate is reduced to nitrite, (nitric oxide) NO, N_2O and finally to N_2. Each step is catalyzed by a set of specific enzymes, namely, nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and N_2O reductase (NOS), respectively. The expression of the denitrifying enzymes are regulated in a sophisticated manner in response to low-oxygen tension and the presence of nitrate or nitrite. The master regulator of this regulatory cascade is ANR, which belongs to the CRP/FNR superfamily (Zimmermann et al. 1991). This oxygen-responsive regulator ANR contains a $[4Fe\text{-}4S]^{2+}$ cluster that is destroyed in the presence of oxygen (Yoon et al. 2007). The active ANR binds to a conserved DNA binding site termed the “ANR box” and regulates gene expression (Trunk et al. 2010). The ANR regulon contains approximately 170 transcription units including genes related to fermentation and denitrification, as well as a small RNA, PhrS, that regulates quorum-sensing (Trunk et al. 2010; Sonnleitner et al. 2011).

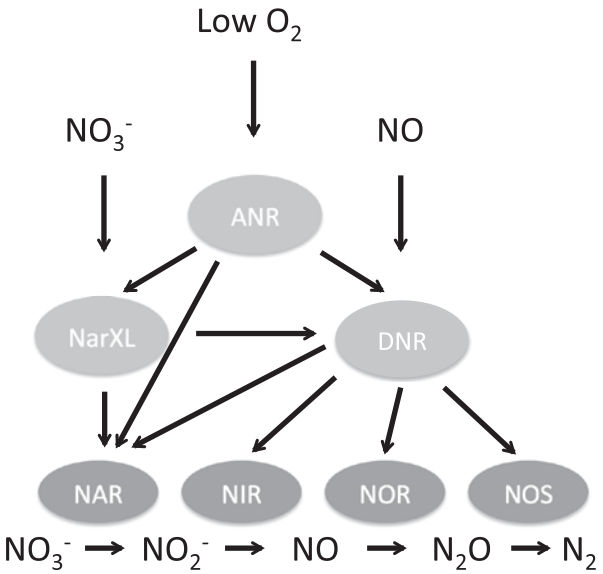

Fig. 5.1 Schematic representation of the denitrification regulation in *P. aeruginosa.* ANR is activated under anoxic or low oxygen concentrations, which increases the transcription of NarXL and DNR. DNR activates the expression of all four denitrifying genes in response to NO. NAR expression is also induced by ANR and NarL. In addition, NirQ is proposed to regulate the activity of NIR and NOR

Other transcriptional regulators involved in denitrification, such as NarXL and DNR, are also induced by ANR (Schreiber et al. 2007; Arai et al. 1997). The sensor kinase NarX detects nitrate and activates the response regulator NarL, which further regulates the transcription of *narK1, nirQ* and *dnr* (Schreiber et al. 2007). DNR also belongs to the CRP/FNR super family regulator and it is activated in response to NO. Unlike ANR, DNR only regulates the denitrification genes. Overall, ANR induces the expression of NarXL, DNR and NAR in response to low oxygen tension. Then, NarXL and DNR further activate the expression of denitrification genes. In this way, the regulators work in a hierarchical network that allows the denitrifying genes to be expressed in response to low-oxygen concentrations and nitrogen oxides (Fig. 5.1).

Cell-Cell Interactions Under Anoxic Conditions

Quorum-Sensing in P. aeruginosa

In *P. aeruginosa,* two chemically distinct types of quorum-sensing (QS) signals are well characterized and have been shown to regulate hundreds of genes including

virulence factors. One type of QS signals is *N*-acyl-L-homoserine lactone (AHL). AHLs are produced widely in Gram-negative bacteria, and *P. aeruginosa* produces two AHLs—*N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL). These AHL signals are bound by their cognate receptors, LasR and RhlR, that are activated by 3-oxo-C12-HSL and C4-HSL signals, respectively (Gambello and Iglewski 1991; Ochsner et al. 1994). The other type of the QS signal is the 2-heptyl-3-hydroxy-4-quinolone, that is referred to as the *Pseudomonas* quinolone signal (PQS) (Pesci et al. 1999). The direct precursor of PQS, 2-heptyl-4-quinolone (HHQ) and other chemically related 2-alkyl-4-qionolones (AQs) are produced from the product of the *pqsABCDE* operon. Such AQs are detected in the supernatant together with PQS (Déziel et al. 2004). HHQ is converted to PQS by PqsH, a monooxygenase encoded by a gene located at a locus distinct from the *pqsABCDE* operon (Gallagher et al. 2002). It has been shown that PQS and HHQ function as a signaling molecule by binding to PqsR. However, the binding affinity of HHQ to PqsR is much lower compared to PQS, suggesting that PQS is the major signal in *P. aeruginosa* (Xiao et al. 2006).

QS Under Anoxic Conditions

A number of studies indicate that under anoxic conditions, QS is attenuated. For instance, PqsH is a monooxygenase that requires oxygen for the conversion of PQS from HHQ. Therefore, under anoxic conditions, PQS synthesis is prevented (Schertzer et al. 2010; Toyofuku et al. 2010). Instead, HHQ accumulates in the supernatant (Schertzer et al. 2010), that may potentially be used as a QS molecule under these conditions. Still, HHQ is approximately 100-fold less potent in activating PqsR (Xiao et al. 2006) and whether HHQ is used as a signal under anoxic conditions has yet to be tested. PqsH produced under anoxic conditions is active, enabling the cells to produce PQS as soon as they are exposed to oxygen (Schertzer et al. 2010).

The AHL QS systems are also attenuated under anoxic conditions. Expression of *las* and *rhl* QS systems are altered under denitrifying conditions (Filiatrault et al. 2005; Wagner et al. 2003), and the production of AHLs is significantly lower under denitrifying conditions, compared to aerobic cultures in *P. aeruginosa* PAO1 (Lee et al. 2011). Because the QS systems regulates many virulence genes, the attenuation of QS systems under denitrifying conditions leads to the loss of cytotoxicity against A549 human airway epithelial cells in *P. aeruginosa* PAO1 (Lee et al. 2011). Still, the AHL signalling systems regulate gene expression to some extent under denitrifying conditions in *P. aeruginosa* PAO1, as has been observed in the regulation of denitrification (Toyofuku et al. 2007; Yoon et al. 2002).

Although *P. aeruginosa* PAO1 produces less QS molecules under anoxic conditions, the cells are still able to respond to these signals. When AHLs or PQS are exogenously added, these signalling molecules restore the transcription of the target genes (Lee et al. 2011; Toyofuku et al. 2008). It is important to note that natural en-

vironments are heterogeneous compared to laboratory conditions, and it is possible that the QS molecules produced under oxic conditions may affect the cells under anoxic conditions (Toyofuku et al. 2008).

The Affect of AHLs and PQS on Denitrification

While oxygen tension and the presence of nitrogen oxides act as the cue for induction of the denitrification process, QS is involved in the control of the denitrifying activity. In *P. aeruginosa,* all three QS molecules, C4-HSL, 3-oxo-C12-HSL and PQS, repress denitrification. As outlined in more detail below, AHLs affect the transcription of the genes encoding the denitrifying enzymes whereas PQS appears to directly impact the activity of these enzymes. Interestingly, the effect of AHLs and PQS on denitrification is predicted to result in the control of NO concentration. As discussed later, NO has important biological roles especially in the group behaviour of *P. aeruginosa.*

Under denitrifying conditions, deletion of these AHL QS system stimulate cell death (Yoon et al. 2002). This QS effect on the cell viability was attributed to the imbalance of denitrifying enzyme activity. In the *rhlR* mutant, NIR activity was greatly increased compared to NOR activity resulting in accumulation of NO. The effect of AHLs on denitrifying genes is also suggested by microarray analysis (Wagner et al. 2003). In accordance with these observations, C4-HSL and 3-oxo-C12-HSL repressed denitrifying activity via their cognate regulator, RhlR or LasR (Toyofuku et al. 2007). Regulation by the *las* quorum-sensing system was dependent on the *rhl* quorum-sensing system, indicating hierarchical regulation by the *las* system over the *rhl* system in denitrification regulation.

Unlike the AHLs, the transcription of denitrifying genes is unaffected by PQS under denitrifying conditions and PqsR is only partially involved in the PQS effect on denitrification (Toyofuku et al. 2008). It is assumed that PQS affects the activity of denitrifying enzymes post-transcriptionally. For instance, it was suggested that PQS interacts with the nitrate respiratory chain and NOR enzyme in a direct manner. During the PQS-mediated control of denitrification, NAR and NOR activity is repressed and NIR activity increased in response to PQS. This suggests that PQS would promote NO accumulation. In accordance with the PQS effects on the respiratory chain, the influence of PQS affect the growth of other bacterial species and oxygen consumption was repressed in *P. aeruginosa* (Toyofuku et al. 2010).

As explained above, the last step of PQS synthesis requires the presence of oxygen (Schertzer et al. 2010; Toyofuku et al. 2008), questioning the significance of PQS inhibition of denitrification. The PQS effect on denitrification is shown to become relevant under oxygen-limiting conditions where enough oxygen is present to synthesize PQS and denitrification is induced (Toyofuku et al. 2008). Also, the PQS effect on denitrification may be important in an environment where anoxic patches are produced by oxygen consumption of the cells, such as in biofilms.

Interestingly, while PQS did not affect *nar* gene transcription under anoxic conditions, microarray data suggested that PQS repress *nar* gene expression under oxic conditions (Rampioni et al. 2010). A similar observation was made in which PqsR had more influence on the PQS repression of denitrificaton under oxygen-limiting conditions than under denitrifying conditions (Toyofuku et al. 2008). Environmental conditions are known to interfere with the QS regulons (Wagner et al. 2003; Williams and Cámara 2009), thus the ability of PQS to differentially influence denitrification during aerobic versus anaerobic growth could be one such example on environment-specific regulation.

MV Productions Under Denitrifying Conditions

Extracellular vesicles are produced in all domains of life (Deatherage and Cookson 2012), suggesting that vesicle production is a fundamental aspect of life. In prokaryotes, vesicle formation was observed more than 45 years ago, and they were referred to as "blebs" (Knox et al. 1966), although the function of these released membrane material remained unclear. Recent studies have found that these blebs, now referred to as membrane vesicles (MVs), contain, or are associated with proteins, DNA, RNA and cell-to-cell communication signals, suggesting the potential biological functions of MVs in bacterial, host-bacterial and environmental-bacterial interactions (Tashiro et al. 2012; Mashburn-Warren and Whiteley 2006; Kuehn and Kesty 2005). MVs are produced in both Gram-positive bacteria and Gram-negative bacteria. Many Gram-negative bacteria naturally produce MVs that are mainly composed of the outer membrane. These spherical MVs are released from the outer membrane, and typically range in size from 20–500 nm in diameter. The presence of MVs-like structures in infected hosts (Irazoqui et al. 2010), as well as natural samples (Schooling and Beveridge 2006), indicate their relevance in bacterial pathogenicity and ecology. The MVs content is dependent on the environment, likely reflecting differences in cell physiology (Toyofuku et al. 2012), and recent studies have revealed that there are several stimuli and regulatory pathways that lead to altered vesiculation, implying that MV function is versatile and can be altered in response to the environment.

P. aeruginosa is one of the organisms that is extensively studied for its production of MVs, and these works have contributed significantly to our understanding of the functions and biogenesis of MVs. In *P. aeruginosa*, PQS, periplasmic stress, SOS response and environmental stresses lead to MV production under oxic conditions, presumably by distinct mechanisms (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008; Macdonald and Kuehn 2013; Tashiro et al. 2009; Maredia et al. 2012). Among the inducers of MV production, PQS-mediated MV induction is the best studied. PQS induces MV production by interacting directly with the lipopolysaccharide (LPS), which results in localized membrane curvature that will lead to MV formation (Mashburn-Warren et al. 2008; Schertzer and Whiteley 2012). PQS can also induce MVs in other bacteria, such as *Escherichia coli*, *Pseudomonas*

stuzeri, *Pseudomonas putida, Burkholderia cepacia* and even in a Gram-positive bacterium, *Bacillus subtilis* (Tashiro et al. 2010). Though PQS is involved in MV production in *P. aeruginosa* under oxic conditions, the fact that dioxygen is required for PQS synthesis, rules out the possibility of MV induction by PQS under anoxic or denitrifying conditions (Schertzer et al. 2010; Toyofuku et al. 2008).

It was demonstrated in a recent study, however, that MV are produced under denitrifying conditions by *P. aeruginosa* (Toyofuku et al. 2014). Purified MVs from denitrifying conditions attach to the neighboring cells suggesting involvement of MVs in the cell-cell interaction under such conditions. SOS response is involved in MV induction under denitrifying conditions and it is indicated that NO induced the SOS response under such conditions, as MV production decreased significantly in a *nir* deletion mutant. In another study, MV production was not observed under denitrifying conditions in *P. aeruginosa* (Schertzer et al. 2010). It was suggested that this seemingly contradictory finding is due to the difference in NO stress when different culture conditions are used, implying that environmental conditions affect MV production. Further data show that the SOS response-induced pyocin production is required for MV generation (Toyofuku et al. 2014). Pyocin production is shown to be up regulated under denitrifying conditions (Waite and Curtis 2009), explaining why this MV induction mechanism is relevant under denitrifying conditions. It is not clear whether MVs produced under denitrifying conditions contain the same complement of molecules as those produced in the presence of oxygen. The multiple pathways leading to MV production, implies that there could be environment-dependent roles of MVs.

Biofilm Formation

Biofilm Formation Under Denitrifying Conditions

One of the most significant changes that *P. aeruginosa* PAO1 expresses under denitrifying conditions is the change in cell morphology. While *P. aeruginosa* is a rod-shaped bacterium, under denitrifying conditions *P. aeruginosa* PAO1 cells elongate (Yawata et al. 2008; Yoon et al. 2011). NO is involved in the process of cell elongation where it inhibits the synthesis of Vitamin B_{12} (VB_{12}), that is a coenzyme of class II ribonucleotide reductase (RNRII) (Lee et al. 2012). RNR II is an essential enzyme in the absence of oxygen. Therefore, the DNA synthesis can be restored by the addition of VB_{12}. The presence of multiple segregating nucleoids in the elongated cells suggests that these bacteria have a defect in cell division (Yoon et al. 2011). Consistent with this idea, the expression of genes involved in Z-ring formation and peptidoglycan synthesis is reduced under denitrifying conditions. The morphological changes observed anaerobically could highly impact cell physiology and behaviours, but may provide advantages for survival in certain circumstances (Justice et al. 2008). One of the influences of cell morphology on bacterial behaviour is biofilm formation.

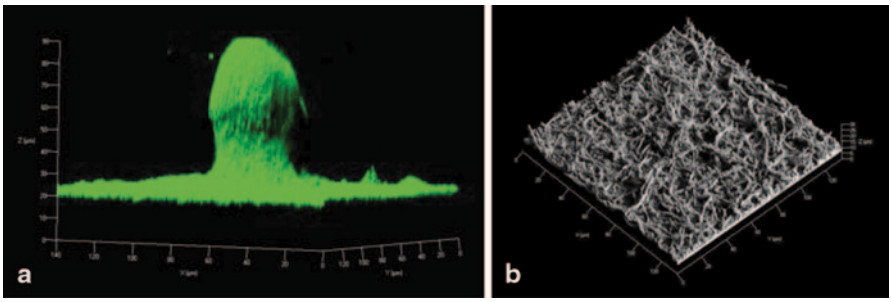

Fig. 5.2 Biofilm structures of *P. aeruginosa* PAO1 under oxic and denitrifying conditions. Under oxic conditions, mushroom-like micro-colonies are formed in biofilms (**a**), whereas flat mat-like structures with highly elongated cells are observed under denitrifying conditions (**b**)

Unlike the typical microcolony structure observed in aerobic biofilms, *P. aeruginosa* PAO1 forms a flat mat-like biofilm under denitrifying conditions wherein the cells in the interior of this community are highly elongated (Yawata et al. 2008) (Fig. 5.2).

In contrast to oxic conditions, biofilm development under denitrifying conditions has not been examined in detail. A recent study suggests that the cell elongation itself is an important factor in biofilm formation (Yoon et al. 2011). When NO accumulation is diminished, biofilm formation decreases, suggesting that cell elongation contributes to biofilm formation. This concept was also tested by elongating the cells with carbenicillin, under oxic conditions, which resulted in a more robust biofilm than the untreated cells. Furthermore, by using the 96-well biofilm assay (O'Toole and Kolter 1998), it was indicated that *lasR*, *rhlR* and *pilA* mutants have different effects on biofilm formation under oxic and anoxic conditions (Yoon et al. 2002). These results indicate that biofilm formation in oxic conditions versus denitrifying conditions may proceed via distinct mechanisms.

NO Signalling in P. aeruginosa Biofilm Dispersal

A series of studies have revealed that NO in non-toxic levels regulates dispersal of *P. aeruginosa* in biofilms. It was found that *nirS* mutants did not disperse, whereas *norCB* mutants exhibited greatly enhanced dispersal (Barraud et al. 2006). The dispersal of the cells from biofilms is linked to the motility of the cells, and consistent with this idea, NO and nitroxide compounds enhance swimming and swarming motility (Barraud et al. 2006; de la Fuente-Núñez 2013). The NO biofilm dispersal is also observed in a variety of Gram-negative and Gram-positive bacteria (Barraud et al. 2009a; McDougald et al. 2012), suggesting a conserved role of NO among bacteria. A key player in the regulation of biofilm formation is *bis*-(3'-5')-cyclic dimeric GMP (c-di-GMP). High levels of c-di-GMP in *P. aeruginosa* inhibit motility and promote biofilm formations, whereas low level of c-di-GMP support a planktonic, motile life-style (Boyd and O'Toole 2012). c-di-GMP concentration is controlled by diguanylate cyclase (DGC) and phosphodiesterase (PDE) enzymes

that synthesize or degrade c-di-GMP, respectively. The NO-induced biofilm dispersal is correlated with c-di-GMP concentration, that is, c-di-GMP levels decrease and cellular PDE activity increases (Barraud et al. 2009b). A chemotaxis transducer BdlA has been implicated in NO-induced biofilm dispersion (Barraud et al. 2009b). Deletion of the *bdlA* gene leads to accumulation of c-di-GMP even in the presence of NO, although the protein lacks DGC or PDE activity, leaving open the question on how BdlA controls c-di-GMP levels. Later studies demonstrate that BdlA is cleaved in the subpopulation of dispersal cells and becomes active (Petrova and Sauer 2012). It is proposed that BdlA affects c-di-GMP concentration by interacting with DipA, although the detailed mechanism by which these factors regulate dispersion is unknown. More recently, other proteins, including MucR and NbdA, were shown to be involved in the NO-induced biofilm dispersion (Li et al. 2013). MucR and NbdA both possess PDE activity, whereas MucR exhibits DGC activity. Among these proteins, NbdA was specific to NO, whereas MucR also was involved in the response to other environmental signals. Hence *P. aeruginosa* has several systems, that may be overlapping, to respond to NO to induce biofilm dispersal.

Phenotypic Variations Under Denitrifying Conditions

P. aeruginosa strains exhibit strain-dependent diversity (Chugani et al. 2012), that likely arises in response to selection in specific niches. Such strain variation is also observed with denitrification and related phenotypes. One of the most frequently isolated *P. aeruginosa* strains from the lungs of patients with cystic fibrosis (CF) are mucoid strains that produce excessive amount of the extracellular polysaccharide alginate (Folkesson et al. 2012). The mucoid strains have altered denitrifying activity compared to the non-mucoid strains (Yoon et al. 2006). The mucoid mutant carrying a mutant allele of the *mucA* gene possesses approximately 3.7-fold greater NAR activity compared to PAO1 or a *mucA* complemented strain. However, the mucoid strain possesses only approximately 4–20 % the NIR and NOR activity, respectively, compared to *P. aeruginosa* PAO1. Because NOR activity is low, mucoid strains are more susceptible to NO under denitrifying conditions (Yoon et al. 2006). Interestingly, another mucoid strain that has acquired secondary mutations and requires specific environmental conditions for alginate production is indicated to have high tolerance against NO. In this strain, both NO detoxifying enzymes, NOR and Fhp, are up regulated compared to the original non-mucoid strain (Firoved and Deretic 2003). These data suggest that not all mucoid strains are alike.

NO-derived reactive nitrogen species increase mutation rates and may select for variants, such as the conditional mucoid strain, that is able to survive under the low oxygen conditions of the CF lung. Small colony variants (SCV) are frequently isolated from CF patients or biofilms (Boles et al. 2004; Häussler et al. 2003), and one of the characteristics of these SCVs is up regulation in genes related to denitrification (Kirisits et al. 2005; Starkey et al. 2009). In addition, the denitrifying genes can be constitutively up regulated in clinical isolates compared to *P. aeruginosa* PAO1 (Son et al. 2007).

A study comparing several clinical isolates with *P. aeruginosa* PAO1 demonstrated that growth, cell morphology, QS activity and biofilm formation differ among these strain under denitrifying conditions (Fang et al. 2013). As already described, in PAO1, cells elongate due to NO stress and this cell elongation contributes to robust biofilm formation (Yoon et al. 2011). However, the clinical isolates remained rod-shaped under denitrifying conditions and were able to form biofilms under such conditions. These data indicate that the clinical strains used in this study may depend on mechanisms other than cell elongation to form biofilms under denitrifying conditions. In the same study, AHL production under denitrifying conditions was examined. Many clinical isolates had reduced AHL production compared to *P. aeruginosa* PAO1, whereas one of the strains had higher AHL productions than *P. aeruginosa* PAO1 under denitrifying conditions. In accordance with this result, elastase activity remained high under denitrifying conditions in the clinical isolate, whereas PAO1 and the other isolates had significantly decreased elastase activity under denitrifying conditions. These results indicate that the QS circuit may vary among strains and respond differently to changing environmental conditions. The variation in these phenotypes under denitrifying conditions would also provide a view of how *P. aeruginosa* adapts to a denitrifying environment.

Denitrification and Pathogenicity of *P. aeruginosa*

Role of Denitrifying Genes in Pathogenicity

P. aeruginosa is the most common CF airway pathogen. In the CF lung, thick mucus clogs the airway lumen producing a steep oxygen gradient. Under such conditions, *P. aeruginosa* may utilize high oxygen affinity terminal oxidase for growth (Alvarez-Ortega and Harwood 2007). Furthermore, nitrate and nitrite are detected from the CF sputum at a level that is sufficient to support anaerobic growth through denitrification (Hassett et al. 2009). Further evidence indicates that the expression of denitrifying genes in the CF lung, and sera of CF patients contain antibodies specific to nitrate reductases, NapA and NarG (Beckmann et al. 2005). In addition, a biomarker for the *P. aeruginosa*-produced anaerobic biofilm, OprF, is detected in CF mucus and antisera against OprF is produced in the CF patients (Yoon et al. 2002).

Interestingly, although the mechanism is unclear, choline, which is abundant in eukaryotic membrane and host lung surfactant, induces *nir* expression in an ANR-dependent manner in the presence of oxygen (Jackson et al. 2013). This result implies that the denitrifying genes are activated in response to the host environment and suggests the importance of denitrifying genes in host-microbe interactions. Transcriptomic analysis of *P. aeruginosa* mRNA isolated from the sputum of a chronically-infected patient revealed that denitrifying genes were significantly up regulated in the sputum compared to minimal medium supplemented with citrate (Son et al. 2007). Furthermore, a genetic screen was performed to identify the genes

associated with anaerobic growth. Most of the strains carrying a mutation in denitrification related genes had moderate or weak virulence in a lettuce assay compared to the parental *P. aeruginosa* PAO1 strain (Filiatrault et al. 2006). It is likely that denitrifying genes are involved in the pathogenicity of *P. aeruginosa* by supporting growth under microoxic or anoxic conditions in the host. Additional roles for (the) ability to grow in the absence of oxygen are discussed below.

NO Detoxification

P. aeruginosa posseses two NO detoxifying enzymes. One is the NOR enzyme that functions under anoxic conditions and is part of the denitrifying system. This enzyme reduces NO to N_2O. The other NO detoxifying enzyme is Fhp, which functions under oxic conditions and oxidizes NO to nitrate (Arai and Iiyama 2013). A recent study using the silkworm *Bombyx mori* as a host model organism, showed that a *fhp* mutant displayed slightly higher virulence than the WT, a *nor* deletion mutant exhibited a significantly decreased virulence against the host compared to the WT. NOR deficient mutant is also reported to have low survival ability in murine macrophages (Kakishima et al. 2007).

Type III Secretion

P. aeruginosa expresses the type III secretion system (T3SS), which translocates specific effector proteins directly into the host cell wherein they mediate their toxic effects. It has been shown that *nar* and *nir* mutants have low expression of T3SS components, extracellular protease and elastase and are less cytotoxic against the THP-1 human monocytic cell line compared to the WT *P. aeruginosa* PA14 (Van Alst et al. 2009). Addition of NO-generating agents restored the virulence production in the *nir* mutant suggesting that NO induces these virulence factors. Still, how NO induces T3SS is not clear. Acute virulence such as T3SS is inversely regulated with biofilm formation through the RetS/LadS/Gac/Rsm signaling network and c-di-GMP (O'Toole 2004; Moscoso et al. 2011; Goodman et al. 2004). A recent study showed that under denitrifying conditions, Anr and NarL regulate the Rsm system and induce the T3SS (O'Callaghan et al. 2011). Activated Anr induces NarL, which in turn directly represses the transcription of the *rsmY* and *rsmZ* small RNAs. This down regulation of the small RNAs allows liberation of RsmA and induction of T3SS gene expression. Anr and NarL respond to low-oxygen or nitrate, suggesting that low-oxygen is responsible for the induction of this pathway. Because NO induces low c-di-GMP, and T3SS is linked to low c-di-GMP concentration (Moscoso et al. 2011), NO may modulate T3SS expression through c-di-GMP.

In another study, the re-routing of the metabolic flux through the glyxoylate shunt in the TCA cycle was shown to affect T3SS expression. Under anoxic conditions the metabolic flux in the TCA cycle is altered compared to oxic conditions.

Although the detailed mechanism is unknown, isocitrate lyase activity was necessary for optimal T3SS expression under oxygen-limited conditions (Chung et al. 2013), an effect that may be independent of NarL.

Antibiotic Resistance

Oxygen and nitrate availability are shown to influence antibiotic tolerance. Under anoxic conditions, tolerance against ciprofloxacin was reduced while gentamicin and imipenem increased (Davey et al. 1988). In a study examining colony biofilms, the susceptibility against all tested antibiotics, carbenicillin, ceftazidime, chloramphenicol, ciprofloxacin, tetracycline and tobramycin were reduced by depletion of oxygen when the cells were exposed to 10–20 times the minimal inhibitory concentration (MIC). The susceptibility against ciprofloxacin and tobramycin was further reduced while the others did not change when nitrate was supplemented to the oxygen–depleted condition (Borriello et al. 2004). While beta-lactams, aminoglycosides, and fluoroquinolones act efficiently against rapidly dividing bacteria, the results suggest that reduced antibiotic susceptibility cannot be explained by the function of growth rate, because supplementation of nitrate under anoxic conditions promotes growth. In another study, antibiotic susceptibility was tested versus several clinical isolates and the percentage of isolates that were killed by each antibiotic was calculated under oxic and denitrifying conditions, using colistin, meropenem, tobramycin, ticarcillin-clavulate, ciprofloxacin, cefepime, ceftazidime and amikacin (Hill et al. 2005). With all the antibiotics tested, the number of isolates killed was lower under denitrifying conditions. Colistin was the most effective antibiotic for both conditions. Furthermore, colisitin combination with other antibiotics, including ciprofloxacin, contrimoxazole, ceftazidime or azithromycin were more efficient, killing 94–100 % or 75–93.8 % of the clinical isolates under oxic or anoxic conditions, respectively. Combinations of antibiotics are also shown to be effective in killing *P. aeruginsoa* in biofilms where complex microenvironments are formed and the cells exhibit heterogeneous physiology. Tetracycline or ciprofloxacin alone only kills the metabolically active subpopulation of the cells comprising the upper layer of the biofilm. On the other hand, colistin preferably targets the subpopulation of cells in the deeper layer of biofilms where it is oxygen and nutrient-limited and cells are metabolically low active. The combined treatment with either colistin and ciprofloxacin or tetracycline significantly reduced the number of viable cells in biofilms, compared to the single antibiotic treatments (Pamp et al. 2008).

Why the efficiency of antibiotics is reduced under anoxic conditions is unclear. One of the characteristics of *P. aeruginosa* under anoxic conditions is the up regulation of alginate production. This increased alginate production could be observed in a mucoid CF isolate, FRD1 (Hassett 1996), as well as a non-mucoid *P. aeruginosa* PAO1 strain (Worlitzsch et al. 2002) under denitrifying conditions. Low oxygen concentration rather than the presence of nitrate seems to contribute to this

increased alginate production (Sabra et al. 2002). Such response of extracellular matrix to low oxygen has been also observed in *Burkholderia cenocepacia* (Pessi et al. 2013). The increased alginate production may act as physical barrier against aminoglycosides, and the respiratory chain is also involved in the uptake of aminoglycoside (Bryan and Kwan 1981), which may in part explain why aminoglycoside resistance increases under anoxic conditions.

In addition, NO is reported to be involved in antibiotic tolerance in *P. aeruginosa* and other bacteria (McCollister et al. 2011; Gusarov and Nudler 2005). The mechanism of increased antibiotic resistance by NO is proposed to be the alleviation of oxidative stress and the repression of respiration and may be limited to oxic conditions. It remains unknown whether NO has similar protective roles in antibiotic resistance under denitrifying conditions.

Concluding Remarks

P. aeruginosa faces anoxic conditions in the natural environment and in the host. How the denitrifying gene expression is controlled in response to these varied environments is generally well understood. Yet, the cell physiology under such conditions is poorly understood. For instance, the altered susceptibility of antibiotic treatment under anoxic or denitrifying conditions has been recognized for a long time but the underlining mechanism(s) are not well known. Hence, better understanding of the cell physiology under such conditions is required. The absence of PQS and attenuation in AHL production is one of the examples that demonstrates the impact of anaerobiosis on *P. aeruginosa* group behaviors (Schertzer et al. 2010; Toyofuku et al. 2010; Lee et al. 2011). Because these QS systems regulate hundreds of genes, the impact of anaerobiosis on the cell physiology is likely to be large.

One of the key molecules in understanding the cell physiology under denitrifying conditions is NO. NO is studied as a signalling molecule in eukaryotic systems. In *P. aeruginosa* NO is produced as an intermediate of denitrification. At high concentrations, NO induces a stress response leading to MV production, cell elongation and biofilm formation (Toyofuku et al. 2014; Yoon et al. 2011). At low concentrations, NO acts as a biofilm dispersal signal that is regulated through modulating c-di-GMP level (Barraud et al. 2006). It is important to note that the effect of NO on biofilm dispersal is confirmed only under oxic conditions, where low amounts of nitrate or nitrite are present in the medium. We still do not know whether these mechanisms are involved in the biofilm dispersal under denitrifying conditions, where NO should be produced constitutively. The importance of NO concentrations in such group behaviour(s) is also supported by the results that suggest that QS systems affect NO concentration (Fig. 5.3).

Biofilm formation is one of the representative life-style of bacteria in natural environments. Studies under denitrifying conditions show that biofilm formation is conditional and may differ from those produced under oxic conditions. Under oxic conditions, *P. aeruginosa* forms biofilm through initial attachment to the surface,

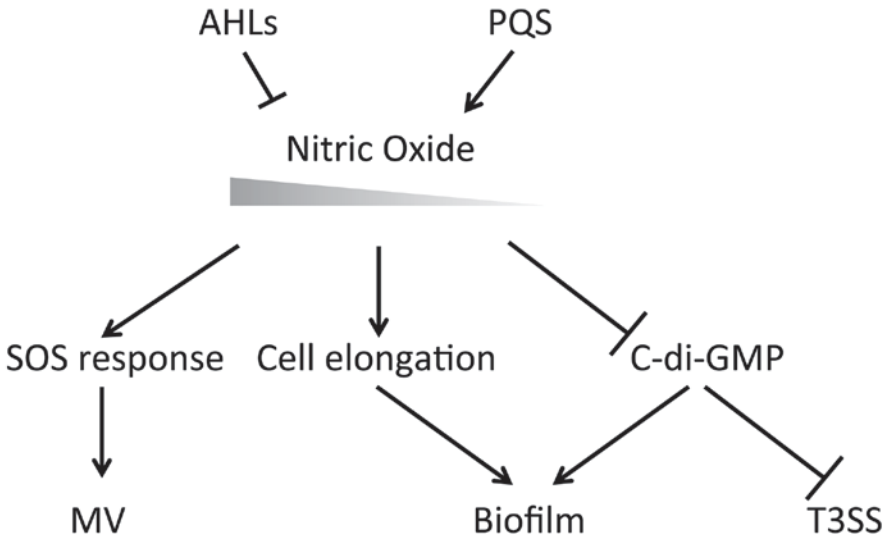

Fig. 5.3 Proposed pathways involved in the NO regulatory network. NO is linked with various phenotypes such as SOS response, cell elongation, T3SS and biofilm formation. QS systems are suggested to control the NO accumulation

microcolony formation and dispersion (Monds and O'Toole 2009). The structurally different denitrifying biofilm which does not produce microcolonies, but instead a flat mat-like structure suggests the biofilm maturation process may be different from those formed aerobically. The flat biofilm structure resembles, to some extent, the flat *lasI* deficient biofilm formed under oxic conditions (Davies et al. 1998), perhaps suggesting the flat biofilm under denitrifying conditions may be due to attenuated QS signalling.

In summary, *P. aeruginosa* has a versatile metabolism that allows the bacterium to survive under various oxygen conditions. Still, most of our knowledge is based on oxic conditions and the understanding of the broad impact of anaerobiosis on the cell physiology is limited. Denitrification is a complex system relative to aerobic respiration, and the strain variations observed in denitrifying activities implying that adaptive mutations related to denitrification may occur under particular environmental conditions. The knowledge of how *P. aeruginosa* adapts to denitrifying conditions and the application of these knowledge may benefit the dealing with bacterial infections.

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Part II
Biochemistry and Physiology

Chapter 6
Pseudomonas Strains that Exert Biocontrol of Plant Pathogens

Jesús Mercado-Blanco

Abstract An increasing number of strains of *Pseudomonas* spp. are being studied for the known benefits they provide to plants. Many of them are natural inhabitants of plant roots and the soil area influenced by them: the rhizosphere. Some are even capable to colonize the root interior and, occasionally, to spread to above-ground organs establishing themselves as beneficial endophytes. The artificial introduction of these strains into target agro-ecosystems can lead to enhanced growth and improved fitness of host plants. These positive effects are usually a consequence of the capacity of bacteria to suppress the attacks by phytopathogenic microorganisms, a phenomenon known as biological control activity. In this chapter, the main modes of action that biocontrol *Pseudomonas* strains can deploy against plant pathogens are reviewed: antibiosis, competition for nutrients and niches, induction of defense responses, etc. How these bacteria are able to colonize plant and pathogen structures, as prerequisite for effective disease suppression, will be also discussed. A concise section devoted to the complex regulatory networks controlling biological control traits is included as well. Finally, the potential that biocontrol pseudomonads pose as marketable products is briefly presented. The underlying idea is that a much better understanding of the complex, multitrophic interactions taking place among the plant, the pathogen(s), the biocontrol pseudomonads and the environment is instrumental for commercial exploitation, aiming to overcome the frequently-observed problem of biocontrol performance inconsistency. The powerful '-omics' and microscopy approaches currently available are providing fundamental information to acquire such knowledge. The use of biocontrol agents as a complementary measure within integrated disease management strategies should have a promising future in modern sustainable agriculture.

Keywords Antibiotics · Biocontrol · Endophytes · Induced resistance · Integrated disease management · Plant protection · Plant-growth promotion · Rhizosphere · Root colonization · Root hairs · Salicylic acid · Siderophores

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Biocontrol of Plant Diseases and Beneficial *Pseudomonas* spp.

Introduction

Plants and bacteria can establish three types of interactions according to the consequences for the plant: (i) negative, leading to diseases (phytopathogenic bacteria); (ii) positive, namely mutualistic symbioses, causing beneficial effects for both partners; and (iii) *a priori* neutral interactions because of the absence of evident negative or positive effects on the host plant (Mercado-Blanco and Bakker 2007). The first two types might actually represent variations on a common theme, since different studies point to a scenario where fine-tuned molecular and genetic dialogues are established between both partners—plant and bacteria, eventually leading to disease or mutualism (Baron and Zambryski 1995; Soto et al. 2006; Berendsen et al. 2012; Zamioudis and Pieterse 2012). The third type of interaction, excluding strict commensalism, actually represents a special case of a positive interaction since beneficial effects are eventually derived for the plant. Indeed, an increasing number of bacterial genera are described as able to contribute to plant growth promotion either *per se* (Glick 1995; Bashan and Holguin 1998; Lugtenberg and Kamilova 2009) or (and) by exerting biological control of phytopathogens and pests (Kerry 2000; Ramamoorthy et al. 2001; Gerhardson 2002). Moreover, some of these bacteria are able to establish themselves as endophytes causing neither deleterious effects nor evident morphological changes in colonized plant tissues (Rosenblueth and Martínez-Romero 2006; Mercado-Blanco and Bakker 2007; Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011). Overall, these intimate relationships are not considered in the literature as symbiosis *stricto sensu*, as for the well-known legume-rhizobia interaction. However, derived benefits for both the host plant (disease protection, growth promotion) and the endophyte (protected environment, constant and reliable source of nutrients) (Bacon and Hinton 2006) allow, *de facto*, to consider this type of associations as true mutualism (Mercado-Blanco and Bakker 2007).

Direct promotion of plant growth by bacteria, which can be achieved by providing microelements and bacterially-produced growth hormones and/or by mobilization of nutrients, will not be treated in this chapter. Interested readers can find excellent and comprehensive reviews on this specific topic (for instance, Lugtenberg and Kamilova 2009; Pliego et al. 2011a) as well as recent examples on how genomic approaches are providing deeper insights on the bacterial traits involved (Roca et al. 2013). Plant growth promotion can also be an indirect consequence of plant diseases suppression exerted by specific microorganisms. Protection against plant pathogens can be due to direct antagonism against the pathogens (for instance, production of antibiotic compounds), advantageous competition for nutrients and/or space, or triggering enhanced defense capacities in the host plant against pathogens attacks.

Fig. 6.1 Biocontrol exerted by *Pseudomonas fluorescens* PICF7 in nursery-produced olive (cv. Picual) plants against Verticillium wilt of olive (*Verticillium dahliae* Kleb.). The root system of the plant shown on the left was dipped in a suspension of PICF7 cells (~ 1×10^9 cfu/ml) during 15 min prior to be transferred to a pot containing *V. dahliae*-infested soil (~ 1×10^6 conidia/g). Plant on the right panel was not treated with the bacterium (control). Pictures were taken 140 days after bacterization and transplant to infested soil. For details on olive-*V. dahliae*-*P. fluorescens* PICF7 bioassays, please consult Mercado-Blanco et al. (2004) and Prieto et al. (2009). Photopraphs were kindly provided by M. Mercedes Maldonado-González

Biological Control of Plant Diseases

Biological control, that is, "*the use of natural or modified organisms, genes or gene products to reduce the effects of pests and disease*" (Cook 1988) is an environmentally-friendly approach that can be combined with other control measures (chemical, physical, agronomical, etc.) within integrated disease/pest management (IPM) frameworks. Biological control agents (BCAs) can be used either as preventive (before-planting) or palliative (post-planting) tools within the target agro-ecosystem. The first ones are obviously the most plausible from an economical perspective. For instance, application of antagonistic bacteria during nursery propagation of pathogen-free certified olive (*Olea europaea* L.) plants to protect them from eventual infections by the soil-borne fungal pathogen *Verticillium dahliae* has been proposed as a plausible approach to manage Verticillium wilt of olive (VWO; Mercado-Blanco et al. 2004; López-Escudero and Mercado-Blanco 2011) (Fig. 6.1).

It must be emphasized that the successful use of BCAs, either alone or in combination with other control tools, should be sustained on a solid knowledge of the targeted system where they will be deployed. When implementing a biocontrol

strategy it must be kept in mind that the pathogen, the host plant, the introduced BCA and the environment (including biotic and abiotic components) establish a complex array of interactions. The outcome of this tightly-regulated multitrophic interplay will eventually determine the effectiveness of the applied biological control measure (Weller 1988). Moreover, beneficial effects exerted by a given BCA relies on the successful colonization and persistence of adequate population levels of the former on/in the target niche (rhizosphere, phyllosphere, inner plant tissue, etc.), which in turn can also be conditioned by the above-mentioned factors (Haas and Défago 2005). For instance, efficient colonization of plant roots by a BCA depends on the soil (type, water content, temperature, pH, etc.), the host plant (genotype, composition of root exudates, etc.), the plant-associated microbiome (rhizoplane, endophytic), and specific BCA traits (motility, exoenzymes production, etc.) which are all dynamically interacting to set-up the so-called phenomenon '*rhizospheric competence*' (O'Sullivan and O'Gara 1992; Lugtenberg et al. 2001; Berg et al. 2006; Mercado-Blanco and Bakker 2007; Raaijmakers et al. 2009).

Beneficial Pseudomonas spp.

The focus of this chapter will be on *Pseudomonas* spp. (Proteobacteria γ subclass) strains able to suppress plant diseases, thereby interesting to be exploited in agriculture as biocontrol tools to increase crop productivity (O'Sullivan and O'Gara 1992; Mercado-Blanco and Bakker 2007; Stockwell and Stack 2007; Weller 2007; Höfte and Altier 2010). This genus, comprising more than a hundred species (Mulet et al. 2010; Loper et al. 2012), is characterized by the broad colonization ability (*environmental ubiquity*) of many of its representatives, a consequence of their remarkable capability to produce ample repertories of secondary metabolites and to utilize a diversity of organic molecules as source of energy (*metabolic versatility*) (Bender et al. 1999; Gross and Loper 2009; Wu et al. 2010). Many pseudomonads are natural inhabitants of plant surfaces, water and/or soil. Some strains are able to establish as plants commensals; others are beneficial to their hosts by protecting them from pathogens infections and/or directly promoting their growth and fitness (Weller 1988; Haas and Défago 2005; Mercado-Blanco and Bakker 2007; Lugtenberg and Kamilova 2009; Pliego et al. 2011a). Some species are pathogenic to plants causing leaf spots, blights, and wilts; for instance, *Pseudomonas syringae* which is subdivided in numerous pathovars according to host specificity (Young 2010; Baltrus et al. 2011). Finally, others can behave as opportunistic pathogens for humans (i.e. *Pseudomonas aeruginosa* in immuno-compromised patients) (Stover et al. 2000).

Literature available on the identification and use of plant-associated, beneficial *Pseudomonas* spp. strains that stimulate plant growth and/or suppress phytopathogens is continuously growing (Table 6.1). Moreover, novel and powerful '-omics' technologies are helping to uncover the amazing diversity of these bacterial group, to redefine its taxonomy, and to better understand mechanisms underlying biocontrol activity exerted by them. Thus, the genomes of several beneficial, plant-associated

Table 6.1 Examples of biocontrol *Pseudomonas* spp. strains mentioned in the text. This is not an exhaustive list; the literature is full of examples of effective biocontrol *Pseudomonas* spp. strains.

Strain	Target Pathosystem(s)[a]	Relevant traits for/related to biocontrol	Reference(s)[b]	Genome sequence availability
P. aeruginosa				
7NSK2	Tomato, bean-*Botrytis cinerea*; tomato-*Pythium* spp.; rice-*Magnaporthe grisea*	ISR[c]; iron-regulated metabolites (SA[d], siderophores), antibiotics	Buysens et al. (1996); De Meyer and Höfte (1997); Audenaert et al. (2002); De Vleesschauwer et al. (2006)	
P. brassicacearum				
Q8r1-96	Wheat-G*aeumannomyces graminis* var. *tritici*;	Antibiotics; secretion systems; root colonization studies	Raaijmakers and Weller (1998); Mavrodi et al., (2006b, 2011)	Loper et al. (2012)
P. chlororaphis				
PCL 1391	Tomato- *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL)	Antibiotics production; efficient root colonizer.	Chin-A-Woeng et al. (1998)	
O6	Cucumber-*Corynespora cassiicola*; tobacco- *Erwinia carotovora*; tobacco-TMV; tomato- root-knot nematode	ISR; 2R,3R-butanediol; antiviral peptides; unknown nematicidal compound	Kim et al. (2004); Han et al., (2006a, 2006b); Lee et al. (2011); Park et al. (2012)	Loper et al. (2012)
30-84	Wheat-G. *graminis* var. *tritici*	Antibiotics production	Pierson et al. (1994); Thomashow et al. (1990)	Loper et al. (2012)
P. fluorescens				
CHA0	Diverse plant pathogenic fungi and nematodes; tobacco-TNV; *A. thaliana-Peronospora parasitica*	Production of wide range of antibiotics and lytic exoenzymes; siderophores; ISR; model for biocontrol regulatory processes	Maurhofer et al. (1994); Voisard et al. (1994); Iavicoli et al. (2003); Siddiqui and Shoukat (2003); Humair et al. (2009)	
F113	Potato-cyst nematode *Globodera rostochiensis*; potato- *Erwinia carotovora* subsp. *atroseptica*	Antibiotic production; model strain to study root colonization	Cronin et al. (1997a, 1997b); Sánchez-Contreras et al. (2002); Barahona et al. (2011)	Redondo-Nieto et al. (2012)

Table 6.1 (continued)

Strain	Target Pathosystem(s)[a]	Relevant traits for/related to biocontrol	Reference(s)[b]	Genome sequence availability
PCL1751	Tomato-FORL	Competition for nutrients and niches	Kamilova et al. (2005)	
Pf29Arp	Wheat-G. *graminis* var. *tritici*	Activation of host defense responses	Daval et al. (2011)	
PICF7	Olive-*Verticillium dahliae*	Root endophyte; activation of host defense responses	Mercado-Blanco et al. (2004); Prieto et al. (2009); Schilirò et al. (2012)	
Q2-87	Wheat-*G. graminis* var. *tritici*; *Arabidopsis thaliana-Pseudomonas syringae* pv. tomato (Pst)	Antibiotics production; ISR	Thomashow and Weller (1990); Weller et al. (2012)	Loper et al. (2012)
SBW25	Pea (and other hosts)-*Pythium ultimum*	Growth promotion; High rhizosphere colonization ability	Naseby et al. (2001); Preston et al. (2003)	Silby et al. (2009)
WCS365	Tomato-FORL	Outstanding root colonizing ability; model strain	Geels and Schippers (1983); Lugtenberg et al. (2001); Pliego et al. (2011a)	
WCS374	Radish- *Fusarium oxysporum* f.sp. *raphani* (FOR)	Competition for Fe^{3+}; production of diverse siderophores; Lipopolysaccharides; ISR	Geels and Schippers (1983); Leeman et al. (1995, 1996a)	
WCS417	Radish- *F. oxysporum* f.sp. *raphani*; carnation- *F. oxysporum* f. sp. *dianthi* (FOD); *A. thaliana*-Pst	ISR; Lipopolysaccharides, iron-regulated metabolites	Van Peer and Schippers (1992); Leeman et al., (1995, 1996); Van Wees et al. (1997)	
P. pseudoalcaligenes				
AVO110	Avocado-*Rosellinia necatrix*	Competition for nutrients and niches	Pliego et al., (2007;2008)	
P. protegens				
Pf-5	Diverse soilborne fungal, oomycete and bacterial pathogens	Wide range of antibiotics	Howell and Stipanovic (1979); Rodriguez and Pfender (1997); Ramette et al. (2011)	Paulsen et al. (2005)

Table 6.1 (continued)

Strain	Target Pathosystem(s)[a]	Relevant traits for/related to biocontrol	Reference(s)[b]	Genome sequence availability
P. putida				
BTP1	Bean-*B. cinerea*; cucumber-*Pythium aphanidermatum*	Iron-regulated metabolite (*N*-alkylated benzylamine derivative); ISR	Ongena et al., (1999, 2005)	
KT2440	*A. thaliana*-Pst	Model strain for rhizosphere colonization studies; ISR	Ramos-González et al. (2005); Matilla et al., (2007, 2010); Fernández et al. (2013)	Nelson et al. (2002)
WCS358	*A. thaliana*-Pst; Eucalyptus-*Ralstonia solanacerum*; carnation-FOD; radish-FOR; Tomato, bean-*B. cinerea*; bean-*Colletotrichum lindemuthianum*	Enhanced rhizosphere competence; Iron competition; ISR; flagella; siderophores; Lipopolysaccharides	Geels and Schippers (1983); Duijff et al., (1994a); Leeman et al., (1996a); van Wees et al. (1997); Meziane et al. (2005); Ran et al. (2005a)	

[a]More target pathosystems can be found in the literature for each particular strain
[b]Included in this chapter. The reader can find additional literature dealing with different aspects of each strain included in this list
[c]Induced Systemic Resistance
[d]Salicylic acid

and/or biocontrol *Pseudomonas* spp. strains are currently available (Paulsen et al. 2005; Silby et al. 2009; Kimbrel et al. 2010; Ortet et al. 2011; Loper et al. 2012; Redondo-Nieto et al. 2012; Shen et al. 2012), and the number will undoubtedly increase in the near future. Genome comparisons studies of *Pseudomonas* spp. strains have stressed the wide diversity of this group and the fact that only about 53 % of the genome (core genome) is present in all strains examined (Paulsen et al. 2005; Silby et al. 2009; Kimbrel et al. 2010; Ramette et al. 2011; Loper et al. 2012). Recently, the study performed by Loper and co-workers (2012) has extended our knowledge of the *P. fluorescens* group by analyzing a number of strains originating from distant geographical areas, diverse habitats, and displaying biological control against different phytopathogens. Besides relevant information about the phylogeny of this genus and its ecological and physiological heterogeneity, these authors performed a genomic survey of known biocontrol traits that will be reviewed in this chapter. According to them, this type of studies will definitively provide valuable information to unravel how these traits may have evolved, to shed light on their mechanisms of inheritance, and to explain the strain-specificity frequently observed for biological control. Moreover, genome-wide analyses may serve to predict and discover novel potential biocontrol traits.

Pseudomonas spp. strains displaying effective biological control may share traits such as: (i) good colonizing capacity of plant surfaces, inner plant tissues (endophytes), and/or phytopathogen (fungi and oomycetes) structures (Lugtenberg et al. 2001; Rosenblueth and Martínez-Romero 2006; Mercado-Blanco and Bakker 2007; Pliego et al. 2011a); (ii) ability to synthesize diverse classes of antibiotics enabling them to advantageously compete in targeted niches against indigenous microbiota and to suppress plant pathogens (Mavrodi et al. 2006a; Weller et al. 2007; Raaijmakers et al. 2010; Haas and Défago 2005); (iii) capability of advantageously use specific nutrients present, for instance, in root or seed exudates enabling them to outcompete indigenous microbiota, including pathogens (Nelson 2004; Kamilova et al. 2005; Kidarsa et al. 2013); and/or (iv) capacity to induce (systemic) resistance responses in the host plant (Van Loon et al. 1998; Bakker et al. 2007). These biocontrol traits will be reviewed in the following sections, with emphasis in the most recent findings. *Pseudomonas* spp. strains can also directly increase plant biomass *per se* by producing phytohormones and/or altering plant hormones levels or by mobilizing mineral nutrients (Arshad and Frankenberger 1998; Lugtenberg and Kamilova 2009). These aspects will not be treated in this chapter unless they serve to illustrate specific issues.

Finally, pseudomonads are one of the essential biotic components of the rhizosphere, a term defined for the first time by L. Hiltner as "*the soil compartment influenced by the roots*" (Hiltner 1904; Smalla et al. 2006; Pinton et al. 2007). This complex ecological niche is the primary place where many relevant signaling events within plant-microbe and microbe-microbe interplays take place. The consequences of these events occurring below ground level are of extraordinary relevance for the whole plant, eventually determining its fitness, development and productivity (Mercado-Blanco and Bakker 2007; Perry et al. 2007; Uren 2007; Raaijmakers et al. 2009; Pliego et al. 2011a; Berendsen et al. 2012). Therefore, the rhizosphere will be the scenario here considered to review biocontrol *Pseudomonas* spp. Nevertheless, specific pseudomonads strains have also shown to be effective in the biocontrol of postharvest and fruit trees disease (see, for instance, Nunes et al. 2007; Bonaterra et al. 2012) although they will not be treated in this chapter.

Colonization Abilities of Biocontrol *Pseudomonas*: Plant Root and Pathogen Surfaces

Technologies for Direct Monitoring of Multitrophic Interactions in the Rhizosphere

Since effective biocontrol is a consequence of multiple interactions established among participating (micro)organisms and the environment, detailed observations of bipartite/tripartite interactions (involving BCA, pathogen, plant) are essential to better understand mechanisms of biocontrol. The development of diverse bioreporter technologies is enhancing our knowledge of the molecular basis underlying multitrophic interactions occurring, for instance, in the rhizosphere (Pliego et al.

2011a). Functional genomics techniques such as *In Vivo Expression Technology* (IVET), a promoter-trap strategy enabling the identification of up-regulated genes under specific environment conditions (Rainey and Preston 2000; Ramos-González et al. 2005; Fernández et al. 2013), *Signature Tagged Mutagenesis* (STM), assisting in the identification of mutants in mixed populations with modified adaptation to specific environment and facilitating the identification of fitness or competition mutants otherwise difficult to obtain by standard mutagenesis approaches (Holden and Hensel 1998), and DNA microarrays (Wang et al. 2005; Mendes et al. 2011) are powerful tools providing holistic insights helping to understand multilevel trophic scenarios hardly envisaged a few years ago. In addition, they also enable the identification of microbial gene clusters potentially involved in biocontrol.

Some bio-reporters are very appropriate tools to monitor the fitness and activity of tagged microorganisms in a given environment. This is the case of the *lux* reporter gene that, for instance, can be used to assess bioluminescence emission over time by metabolically active *Pseudomonas* cells when artificially inoculated in bulk and rhizosphere soils (Porteous et al. 2000). On the other hand, autofluorescent proteins (AFP) enable the direct visualization of individual AFP-tagged microorganisms when used in combination with fluorescence and/or confocal laser-scanning microscopy (CLSM). The green fluorescent protein (GFP) (Tsien 1998) is the most commonly used AFP, although derivatives and variants (i.e. Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP) [Yang et al. 1998; Ellenberg et al. 1999; Shaner et al. 2004]) are available and increasingly utilized. The combined use of different AFPs enables the tagging of several species and/or strains differing in fluorescence emission, thus facilitating their simultaneous imaging *in situ* (Lagendijk et al. 2010). This approach provides crucial information not only about where microorganisms are located but also on the type of interactions they may establish with the plant and/or target phytopathogens, even at the single cell level (Lagopodi et al. 2002; Prieto et al. 2009; 2011). Moreover, competition for target sites in a given pathosystem can also be important to explain the biocontrol ability of a BCA. These methodological approaches are providing relevant information as well. Thus, different studies serve to illustrate that the early colonization by the BCA of the preferential penetration spots used by the phytopathogen to invade host tissues is important to explain biocontrol activity of strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 against *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) in tomato (*Solanum lycopersicum* Mill.) (Bolwerk et al. 2003), *Pseudomonas pseudoalcaligenes* AVO110 against *Rosellinia necatrix* in avocado (*Persea americana* Mill.) (Pliego et al. 2008), or *P. fluorescens* PICF7 against *V. dahliae* in olive (Prieto et al. 2009).

Biocontrol Pseudomonas spp.-Phytopathogens Interactions

Microscopy studies demonstrate that biocontrol *Pseudomonas* spp. operating in the rhizosphere may exert their beneficial effects by directly interacting with fungal phytopathogens. For instance, combination of biotechnological (AFP-tagging)

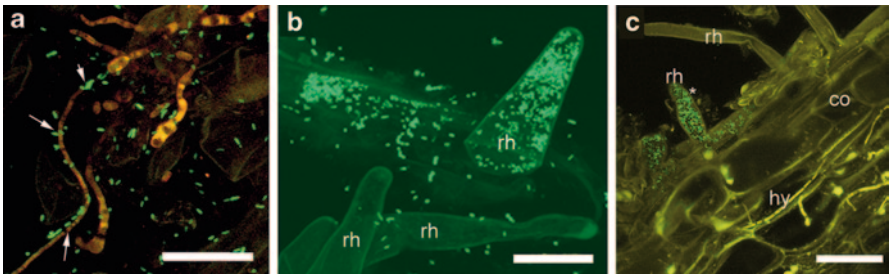

Fig. 6.2 Confocal laser scanning microscopy (CLSM) showing different colonization events of olive cv. Arbequina root tissues by *Pseudomonas fluorescens* PICF7 (enhanced green fluorescent protein [EGFP]-tagged) and *Verticillium dahliae* VDAT-36I (enhanced yellow florescent protein [EYFP]-tagged transformant of a defoliating isolate). **a** Colonization of VDAT-36I hyphae by PICF7 cells (arrowed) on the differentiation zone of the olive root surface five days after inoculation (DAI); **b** Detection of EGFP-tagged PICF7 cells inside a root hair cell at three DAI. Non-colonized root hairs are also shown; **c** Simultaneous detection of PICF7 cells, within root hairs, and of VDAT-36I EYFP-tagged hyphae inside the cortical tissue of an olive root. This image was taken from a vibratome longitudinal section of a representative olive root from a plant sampled 10 DAI. Images were projections of 10 (**a**), 20 (**c**) and 30 adjacent confocal optical sections (**b**). The focal step size between confocal optical sections was 1 mm. Asterisk in **c** indicates a root hair colonized by PICF7 cells. Bars, 15 μm in panel **a**, and 20 mm in panels **b** and **c**. rh, root hair; co, cortex; hy, hypha. These CLSM microphotographs are reproduced from Prieto et al. (2009) Microbial Biotechnol 2: 499–511; doi:10.1111/j.1751-7915.2009.00105.x, with permission of the Publisher (Wiley and Sons)

and microscopy tools have undoubtedly revealed direct contact between beneficial *Pseudomonas* spp. and phytopathogenic soil-borne fungi, what can be important to explain biocontrol activity. It has been demonstrated that *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 do not only colonize roots but also the hyphae of FORL (Lagopodi et al. 2002; Bolwerk et al. 2003), a situation also observed between the biocontrol strains *P. pseudoalcaligenes* AVO110 and the avocado white root rot fungus *R. necatrix* (Pliego et al. 2008) and *P. fluorescens* PICF7 and the VWO causal agent *V. dahliae* (Prieto et al. 2009) (Fig. 6.2a). Similarly to what happens in root-bacteria interactions (see Sect 2.3), some antagonistic *Pseudomonas* strains are chemotactically attracted by fungal exudates, facilitating the attachment of bacterial cells to fungal structures (De Weert et al. 2004). Approach and anchorage to fungal surfaces as well as subsequent passive or active feeding from fungal biomass can be achieved thanks to production of lytic enzymes (Nielsen and Sorensen 1999) or biosurfactants (Braun et al. 2001) by bacteria. It has been suggested that improved biocontrol activity can be explained by BCA feeding on deleterious fungi thereby inhibiting their growth (Leveau and Preston 2008). Moreover, both the BCA and the fungal pathogen can establish a molecular dialogue enhancing biocontrol activity of the former. For instance, the biocontrol strain *P. fluorescens* Pf29Arp can perceive signals emitted by *Gauemannomyces graminis* var. *tritici*, the causal agent of take-all in wheat (*Triticum* spp.), provoking important changes at the transcriptome level, including genes for bacterial motility and stress responses (Barret et al. 2009). These authors have demonstrated that a number of bacterial

genes were induced before and after direct contact is established between the BCA and the fungus when both were jointly cultured on plates. Furthermore, the presence of the fungus even enhanced the growth of strain Pf29Arp. Similarly, stimulation of *P. fluorescens* growth when associated with *Pythium debaryanum* was earlier reported, and utilization of the sugar trehalose seemed to play an important role in both bacterial growth and inhibition of the oomycete (Gaballa et al. 1997). Finally, IVET has been also employed to uncover induction of diverse genes in the biocontrol strain *Pseudomonas putida* 06909 when interacting with the oomycete *Phytophthora* sp. (Lee and Cooksey 2000; Ahn et al. 2007).

Pseudomonas spp. Traits Involved in Root Colonization

An essential prerequisite for a BCA to succeed in biocontrol is the efficient colonization of the target niche. Thus, soil inhabiting *Pseudomonas* strains should efficiently colonize the rhizosphere prior to be able to protect plant roots from pathogen's attack. In fact, root colonization and the bacterial traits involved in this complex process are key elements in biocontrol efficacy, as demonstrated by insertional mutagenesis studies for *P. chlororaphis* PCL1391. Mutants of this strain impaired in root colonization have lost biocontrol activity against tomato foot and root rot (Chin-A-Woeng et al. 2000). Identification of *Pseudomonas* traits involved in rhizosphere and/or root colonization has been a thoroughly-pursued aim (Lugtenberg et al. 2001) and appropriate experimental tools and study systems have been developed to achieve it (Simons et al. 1996). It is worth mentioning that successful rhizosphere colonization is a consequence of a delicate equilibrium between biotic (the host plant, the introduced BCA, resident rhizosphere and endophytic microbiota) and abiotic (soil type and structure, water and nutrients availability, pH, temperature, composition of root exudates, etc.) factors (Mercado-Blanco and Bakker 2007, and references therein). Inconsistent performance observed upon introduction of a BCA in a given agro-ecosystem could be explained either by an insufficient understanding of these factors and their dynamic interactions (Thomashow 1996) or the absence of adequate screening procedures to select suitable BCAs for a determined soil and pathosystem (Pliego et al. 2011b).

Plant roots and the rhizosphere are very attractive niches for soil-borne microbes compared to the nutrient-limiting and hostile bulk soil environment since they are important sources of nutrients (Degenhardt et al. 2003; Bacon and Hinton 2006). Mutant analysis enabled to identify that motility and chemotaxis are essential for root colonization by *P. fluorescens* strains (Lugtenberg and Bloemberg 2004). While flagella seem not to be enough to explain competitive colonization in some cases (De Weert et al. 20022002), its involvement in attachment to a number of diverse (a) biotic surfaces has been clearly elucidated for both pathogenic and non-pathogenic *Pseudomonas* strains (Duque et al. 2013). Root colonization process by biocontrol *Pseudomonas* spp. begins with the movement of free living bacteria in response to chemoattractants present in root exudates (Bais et al. 2004; Uren 2007) by means of flagellar motility (De Weger et al. 1987; Turnbull et al. 2001a; 2001b; De Weert

et al. 2002). While motility seems to be essential for root colonization in some cases (Capdevila et al. 2004; Martínez-Granero et al. 2006), other studies point to only a minor role in colonization for flagella (Howie et al. 1987; Scher et al. 1988). One of the best-studied strains is *P. fluorescens* WCS365 which has served as a model to understand mechanisms involved in the root colonization process by beneficial pseudomonads (Lugtenberg and Dekkers 1999; Lugtenberg et al. 2001; Lugtenberg and Kamilova 2009). Strain WCS365 is a good colonizer of potato (*Solanum tuberosum* L.) and tomato roots and able to control the soil-borne fungus FORL in tomato (Geels and Schippers 1983; Brand et al. 1991; Simons et al. 1996; Dekkers et al. 2000). WCS365 is chemically attracted by carboxylic acids (mainly malic and citric acids) and amino acids present in tomato root exudates (Simons et al. 1997; Kravchenko et al. 2003). It is known that, besides flagellar motility, the lipopolysaccharide (LPS) O-antigen, synthesis of vitamin B1, production of NADH dehydrogenase, and high bacterial growth rate are bacterial traits contributing to the outstanding root colonization ability of *P. fluorescens* WCS365 (Simons et al. 1996; Dekkers et al. 1998b; Camacho-Carvajal et al. 2002). Carbon sources present in root exudates also influence colonization of tomato roots by strain WCS365. While synthesis of specific amino acids is essential for effective colonization (Simons et al. 1997) the presence of the polyamine putrescine negatively affects WCS365 root colonization competitiveness (Kuiper et al. 2001). On the other hand, the capacity to use specific sugars seems not to be decisive for such ability (Lugtenberg et al. 1999).

Strain WCS365 also carries a *sss*/ *xerC* homologue encoding a site-specific recombinase (regulatory role in process of DNA rearrangements causing phase/phenotypic variation [Höfte et al. 1994]) shown to be important in competitive colonization of the rhizosphere of several plants (Dekkers et al. 1998a). Phenotypic variation can affect bacterial traits involved in root colonization and biocontrol (Sánchez-Contreras et al. 2002; Van den Broek et al. 2003, 2005a). Influence on colonization traits by site-specific recombinases has been demonstrated in biocontrol strains *P. fluorescens* F113 (Martínez-Granero et al. 2005) and Q8r1-96 (Mavrodi et al. 2006b) as well. Interestingly enough, the colonization ability of poor root colonizing *Pseudomonas* strains can be improved by the transfer of site-specific recombinase genes (Dekkers et al. 2000). Similarly, a triple mutant strain (KSW) of *P. fluorescens* F113 affected in *kinB*, *sadB* and *wspR* showed increased motility and rhizosphere colonization ability than that of the wild-type strain (Barahona et al. 2011). In addition, KSW strain showed improved biocontrol activity against the fungal pathogen FORL in tomato and the oomycete *Phytophthora cactorum* in wild strawberry (*Fragaria vesca* L.). This finding further supports the notion that good (or even increased) colonization ability of a BCA leads to enhanced biocontrol capacity. More importantly, this can be achieved by the *ad hoc* design of improved BCAs what opens promising perspectives for the implementation of novel biological control strategies (Barahona et al. 2011).

The implementation of some of the aforementioned methodologies is providing abundant information on genes involved in the rhizosphere colonization process. Moreover, they allow the assessment of in vivo expression of large arrays of genes simultaneously. Thus, IVET has been utilized to identify genes of *P. fluorescens*

SBW25 expressed in the sugar beet rhizosphere (Gal et al. 2003) and of *P. putida* KT2440 while colonizing the rhizosphere of different plants (Ramos-González et al. 2005; Fernández et al. 2013). By using IVET it was also suggested that the type III secretion system (T3SS) (see below) might have a functional significance for a beneficial rhizosphere *P. fluorescens* strain (Preston et al. 2001). However the true involvement of T3SS was elusive since a mutation in the *hrcC* gene (encoding a component of the T3SS) had no effect on root colonization capacity (Preston et al. 2001). Later, De Weert and co-workers (2007) suggested that T3SS of strain SBW25 is involved in both injection of proteins into plant cells and absorption of nutrients from them. Similarly, IVET allowed the identification in strain SBW25 of one gene (*wssE*) of the *wss* operon involved in acetylated cellulose polymers biosynthesis and shown to contribute to ecological fitness in the rhizosphere (Rainey and Preston 2000; Gal et al. 2003). Fernández and associates (2013) using IVET to analyze gene expression of strain KT2440 in the rhizosphere of diverse plants (pine, [*Pinus halepensis* Mill.], evergreen oak [*Quercus ilex* L.], cypress [*Cupressus sempervirens* L.], and rosemary [*Rosemarinus officinalis* L], have recently suggested that this strain senses plant-specific signals leading to gene expression involved in its successful rhizosphere establishment. Gene expression of strain KT2440 during colonization of maize (*Zea mays* L.) roots was also analyzed by Matilla and co-workers (2007) using microarrays. They found that bacterial genes involved in amino acid uptake, metabolism of aromatic compounds, bacterial efflux pumps, enzymes related with glutathione metabolism, and flagellar and chemotaxis proteins were induced in the maize rhizosphere. Interestingly, a gene (*rup4959*) encoding a protein containing GGDEF/EAL response regulator was identified as preferentially induced. These protein domains are involved in the metabolism of the secondary messenger cyclic diguanylate (c-di-GMP) that regulates a broad diversity of physiological processes in bacteria (Römling 2012). The promoter of *rup4959* gene has been demonstrated to be induced by maize root exudates and microaerobiosis. Manipulation of the expression levels of this gene led to negative effects on biofilm formation (upon inactivation) or to total inhibition of swarming motility, decreased swimming motility, and lower root tip colonization efficiency (upon overexpression) (Matilla et al. 2011).

Endophytic Beneficial *Pseudomonas* spp.

Some plant-associated *Pseudomonas* spp. can establish themselves as endophytes. The interested reader can find excellent reviews on bacterial endophytes, traits involved in endophytism, and potential to utilize them in plant-growth promotion and biological control among other applications (Hallmann et al. 1997; Sturz et al. 2000; Lodewyckx et al. 2002; Rosenblueth and Martínez-Romero 2006; Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011). Criteria for proper identification of true endophytic bacteria were proposed by Reinhold-Hurek and Hurek (1998) and should not be just based on the simple isolation from surface-disinfected plant

tissues, but must be confirmed by microscopic evidences and the capability of the endophyte to re-infect disinfected seedlings (Rosenblueth and Martínez-Romero 2006). It is assumed that, once endophytic, bacteria take advantage of being within a protected niche, less exposed to (a)biotic stresses and with a constant source of nutrients that diminish competition with other microorganisms (Bacon and Hinton 2006). Our current knowledge on endophytic bacteria has been enlarged by implementing culturing-independent identification techniques as well as metagenomics approaches. These are uncovering a much wider diversity and abundance of endophytic communities than those obtained by traditional culturing-dependent methods (see, for instance, Garbeva et al. 2001; Sessitsch et al. 2002; Manter et al. 2010; Lucero et al. 2011; Sessitsch et al. 2012). Beneficial endophytic *Pseudomonas* spp. have been identified, alone or as part of complex bacterial consortia, in many plant species from diverse geographical origins and from different plant organs and tissues (Mercado-Blanco and Bakker 2007, and references therein).

Mechanisms involved in the endophytic colonization of bacteria, including beneficial *Pseudomonas* spp., are thus far mostly unknown. Bacterial traits potentially involved in endophytic colonization of plant roots have been reviewed (Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011). It is suggested that diverse cell wall-degrading enzymes are involved in the penetration process (Hallmann et al. 1997; Compant et al. 2005). Duijff and co-workers (1997) also reported that the O-antigen of LPS is involved in endophytic colonization of tomato roots by the biocontrol strain *P. fluorescens* WCS417. Similarly, definitive proofs of preferred sites used by endophytic bacteria to gain entrance into root tissues are scant, although root cracks, injuries (caused by arthropods, nematodes, etc.), emergence sites of lateral roots, zones of elongation and differentiation, and/or intercellular spaces in the epidermis are generally admitted as 'hot spots' for bacterial penetration (Rosenblueth and Martínez-Romero 2006; Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011).

Recent studies have reported that root hairs can also play an important role during the endophytic colonization of plant roots by certain pseudomonads strains. Prieto and associates (2011) have demonstrated using AFP-tagged bacteria and CLSM that strains of *Pseudomonas* spp. (*P. fluorescens* PICF7 and *P. putida* PICP2) effective against VWO (Mercado-Blanco et al. 2004; López-Escudero and Mercado-Blanco 2011), can internally and simultaneously colonize root hairs of this woody host (Fig. 6.2b). Moreover, they are able to colonize these epidermal structures and establish in the intercellular spaces of the root cortex under different (gnotobiotic and non-gnotobiotic) experimental conditions (Prieto and Mercado-Blanco 2008; Prieto et al. 2009; 2011). However, the well-known events taken place during the infection process of legume root hairs by symbiotic members of the Rhizobiaceae family (Kijne 1992) were not observed. Therefore, it remains to be elucidated whether these bacteria enter root hairs *via* active or passive processes (Mercado-Blanco and Prieto 2012). From the initial sites of penetration endophytic pseudomonads may eventually spread to distant parts of the plant. For instance, vascular transport from the roots to aerial tissues may explain the extensive inner colonization of several plant species observed for *Pseudomonas aureofaciens* strain L11 (Lamb et al. 1996). On the contrary, *P. fluorescens* PICF7 was never observed to colonize the

xylem vessels, bacteria being confined to the root cortex without no evidence of translocation to above-ground tissues in olive (Prieto and Mercado-Blanco 2008; Maldonado-González et al. 2013).

Presence of endophytic *Pseudomonas* spp. has proven as beneficial for the host plant in several studies, and plant growth promotion exerted by consortia of bacteria including *Pseudomonas* spp. has been reported (Chanway et al. 2000; Nejad and Johnson 2000; Adhikari et al. 2001; Kuklinsky-Sobral et al. 2004). Nevertheless, the contribution to plant growth of each partner in the consortia is not well established. Promotion of plant growth by endophytic *Pseudomonas* spp. can also be due to control of phytopathogens. Despite the fact that numerous studies have proved in vitro antagonism between a cultured endophyte and a target pathogen, effective *in-planta* biocontrol activity of the endophyte is difficult to prove, although examples of biocontrol activity exerted by endophytic pseudomonads are available (Adhikari et al. 2001; Chen et al. 1995; Brooks et al. 1994; Grosch et al. 2005; Prieto et al. 2009). However, the biocontrol mechanism(s) involved remain to be elucidated in most of the cases. It is suggested that beneficial effects deployed by bacterial endophytes seem to operate by means of similar mechanisms described for rhizosphere-associated bacteria, including induction of resistance (Kloepper and Ryu 2006). Duijff and co-workers (1997) early suggested that induction of resistance exerted by *P. fluorescens* WCS417 was related to the internal colonization of tomato root tissues by this strain. It has been proposed that biocontrol of *V. dahliae* in olive by *P. fluorescens* PICF7 requires of the early colonization of olive roots, including endophyte establishment, before infection by the pathogen (Prieto et al. 2009) (Fig. 6.2c). Moreover, functional genomics analysis has revealed that root colonization by strain PICF7 induces a wide array of defense responses to different stresses in olive root tissues, including up-regulation of genes involved in induced systemic resistance (ISR) and systemic acquired resistance (SAR) (see below) responses (Schilirò et al. 2012). However, PICF7 was not effective to control olive knot disease caused by *P. savastanoi* pv. savastanoi when the BCA was applied to the roots and the pathogen inoculated into the stems (Maldonado-González et al. 2013).

Overall, very little information is available about the molecular/genetics changes that endophytic *Pseudomonas* produce in the host plant once internal colonization has been established. The same accounts about the effects that an introduced bacterial endophyte can provoke on the indigenous endophytic microbiome or *vice versa*. Accurate knowledge about these effects is essential for the successful application of biocontrol endophytes. Microarray analysis performed by Wang and associates (2005) have revealed that root colonization by strain *P. fluorescens* FPT9601-T5 (endophytic in the model plant *Arabidopsis thaliana* and in tomato roots) of Arabidopsis roots produces the up-regulation of a number of genes involved in metabolism, signal transduction, stress response, and interestingly, putative auxin-regulated genes and nodulin-like genes. Results also shown that 105 genes were down-regulated, including some ethylene responsive genes. This study suggests that similar genetic responses are triggered/repressed when rhizobacteria, endophytic PGPR, and to some extent rhizobia, interact with their host plants.

Similarly, computational analysis of some 445 olive ESTs induced during the colonization of olive roots by the endophytic strain *P. fluorescens* PICF7 revealed that nearly 45 % of the up-regulated genes were related to plant defense and response to different stresses. Validation by quantitative real-time PCR (qRT-PCR) confirmed the induction of lipoxygenase, phenylpropanoid, terpenoids and plant hormones biosynthesis transcripts, and diverse transcription factors such as bHLH, WRKYs and GRAS1 (Schilirò et al. 2012). These type of approaches, as well as IVET, will be instrumental to enhance our understanding of plant-endophyte relations, similarly as they have been implemented for gene expression studies in the rhizosphere.

Finally, the composition, abundance, distribution and functionality of a given endophytic microbiome, including *Pseudomonas* spp., can be influenced by different factors: the host plant genotype (Siciliano and Germida 1999; Germida and Siciliano 2001; Van Overbeek and Van Elsas 2008), developmental and/or growth stages (Pirttilä et al. 2005; Van Overbeek and Van Elsas 2008), temperature (Pillay and Nowak 1997), presence of plant pathogens and pests (Hallmann et al. 1998; Reiter et al. 2002; 2003; Lian et al. 2008), seasonal variations (Mocali et al. 2003; Pirttilä et al. 2005), plant organ (Mocali et al. 2003), introduction/recruitment of endophytes (Andreote et al. 2009; Ardanov et al. 2012), or certain crop management strategies (Kuklinsky-Sobral et al. 2005).

Pseudomonas spp. Traits Involved in Biological Control

Introduction

After being able to externally and/or internally colonize host plant tissues and persist on/within them, biocontrol *Pseudomonas* strains may deploy diverse mechanisms contributing to plant fitness. The ultimate consequence is enhancement of plant growth that can be achieved either by growth promotion *per se* or by protection against phytopathogens. It is worth mentioning that effectiveness of many biological control traits depends on the population levels reached by the BCA on/in the specific target niche, and that some of these traits (i.e. antibiotics production) are subjected to a cell-density dependent regulation (see below) (Pierson et al. 1994; Raaijmakers et al. 1999). For instance, *P. fluorescens* WCS374 fails to elicit induced systemic resistance in *Arabidopsis* plants when applied at high inoculum densities ($>10^7$ cfu/g of soil) (Van Wees et al. 1997; Djavaheri et al. 2012). However, when strain WCS374 is applied at low cell densities (i.e. 10^3 cfu/g of soil), the BCA is able to trigger systemic resistance against *P. syringae* pv. tomato DC3000 (Djavaheri et al. 2012), suggesting that active growth of WCS374 is needed for effective biocontrol.

Disease suppression by pseudomonads can be based on direct antagonism of the pathogen (i.e. production of antibiotics) or indirect mechanisms such as competition for (micro)nutrients (predominantly Fe^{3+} through biosynthesis of siderophores) and

niches or induction of systemic resistance responses. Direct and indirect mechanisms are not mutually exclusive and can operate simultaneously or being triggered at different stages (spatially and temporarily) along the plant-BCA-pathogen interaction. Moreover, competition for nutrients, microelements, and antibiosis can be exerted without interacting with the plant. However, disease suppression mediated by induction of systemic resistance is a consequence of a direct (or even intimate) interaction between the BCA and the host plant. Remarkably, signal transduction pathways leading to enhanced resistance in plants upon interaction with beneficial *Pseudomonas* spp. are better known than bacterial traits responsible to trigger such defense responses. The current knowledge on *Pseudomonas* traits involved in biocontrol of plant disease will be reviewed in the following sections.

Competition for Nutrients and Niches

This biocontrol mechanism was suggested by Kamilova and co-workers (2005) to explain the enhanced root tip colonization ability that strain *P. fluorescens* PCL1751 showed compared to other highly-efficient root colonizers (i.e. *P. fluorescens* WCS365). By using an enrichment procedure enabling the selection of fast root-tip colonizing strains, these authors were able to identify strain PCL1751 that reduced tomato foot and root rot disease caused by FORL. A non-motile spontaneous mutant of strain PCL1751 was as competitive as its parent when both were grown in culturing medium and in tomato root exudates. However, in competitive tomato root tip colonization assays under gnotobiotic experimental conditions, the mutant was outcompeted by the wild type strain in different parts of the tomato root. Biocontrol assays under greenhouse conditions showed no significant biocontrol activity against FORL by the mutant, compared to the wild type. Moreover, enhanced colonizers grew much better on root exudates than randomly isolated strains. All these evidences led authors to conclude that competitive colonization, or competition for niches and nutrients, was required for the biocontrol activity exerted by *P. fluorescens* PCL1751 (Kamilova et al. 2005). By using a similar enrichment approach, Pliego and co-workers (2007) concluded that despite the fact that two strains (*P. pseudoalcaligenes* AVO110 and *P. alcaligenes* AVO73) were good colonizers of avocado root tips, only strain AVO110 showed significant protection against avocado white root rot caused by *R. necatrix*. The key difference was that strain AVO110 colonized the preferential infection sites of *R. necatrix* whereas AVO73 was predominantly found in regions not colonized by the pathogen (Pliego et al. 2008). Therefore, it appears that biocontrol pseudomonads acting through the competition for nutrients and niches mechanism must efficiently and/or previously colonize the same plant tissue areas preferred by the pathogen (Pliego et al. 2011a).

Finally, it is worth mentioning that the plant spermosphere usually represents the first interaction point between some pathogens and BCAs. Considering that germinating seeds are an important source of exudates, nutrients present in them are a clear target of competition, and can affect expression of bacteria biocontrol traits on the seed surface (Nelson 2004; Kidarsa et al. 2013).

Competition for Iron: Siderophores of Beneficial Pseudomonas spp.

In spite of its abundance, iron is basically unavailable for soil-inhabitant microorganisms which in turn have developed a strategy for its assimilation. This strategy involves the biosynthesis of low-molecular-weight, iron-chelating molecules called siderophores. These metabolites are classified according to their iron chelating groups and show an ample structural diversity (Höfte 1993). These compounds show a high affinity for ferric iron (Fe^{3+}) and its production, and that of protein receptors that recognize Fe^{3+}-siderophore complexes, is induced during iron-limiting growth conditions. Competition for Fe^{3+} is likely the best documented mechanism of competition exerted by BCA (Leong 1986), particularly for *Pseudomonas* spp. which produce a large variety of siderophores to cope with iron-limiting conditions (Bultreys 2007). Among them, pyoverdines (=pseudobactins) are the prevalent class, showing a complex and variable structure. They carry both catechol and hydroxamate groups responsible of iron chelation (Leong 1986). Molecular structure of pyoverdines as well as the genetics and regulation of their biosynthesis, release and uptake, which involve a large number of genes, have been comprehensively studied and reviewed (Meyer 2000; Ravel and Cornelis 2003; Visca et al. 2007; Cornelis et al. 2009). Pyoverdines differ in the number and composition of the amino acids present in the peptide chain. This has application in bacterial taxonomy since a given *Pseudomonas* species or strain synthesizes a specific type of pyoverdine (Fuchs et al. 2001; Meyer 2007; Djavaheri et al. 2012).

Production of *Pseudomonas* siderophores, including pyoverdines, can be influenced by diverse environmental factors, or by the composition of root exudates, thereby affecting the potential beneficial effects they exert on the plant (Duffy and Défago 1999; Audenaert et al. 2002; Ran et al. 2005b; Djavaheri et al. 2012). On the other hand, certain *Pseudomonas* strains are able to use heterologous siderophores, conferring them selective advantages in iron-limiting environments and potentially enhancing their ability to control plant pathogens (Bakker et al. 1988; Mirleau et al. 2000; Mercado-Blanco and Bakker 2007). For instance, the strain *P. putida* WCS358 shows an enhanced rhizosphere competence because of its ability to use not only its own siderophore pseudobactin 358 (Psb-358) but also others produced by different bacteria due to the presence of additional receptors. Moreover, Psb-358 can only be taken up by few pseudomonads further enhancing the competence of strain WCS358 over other bacteria present in the soil (Koster et al. 1993; Raaijmakers et al. 1994). Enhanced rhizosphere competence can thus be engineered by introducing either iron-regulated siderophore promoters or siderophore receptor genes in heterologous genetic backgrounds (Raaijmakers et al. 1995b; Loper and Henkels 1999).

Many plant beneficial *Pseudomonas* spp. strains produce additional, secondary siderophores, or at least harbour the gene clusters needed for their biosynthesis and uptake. Some of these are pyochelin (Buysens et al. 1996), enantio-pyochelin (Youard et al. 2007), pseudomonine (Mercado-Blanco et al. 2001; Loper et al. 2012), or achromobactin (Loper et al. 2012). Moreover, the genomes of strains *P.*

protegens Pf-5, *Pseudomonas* sp. BG33R, and *P. fluorescens* SBW25 and SS101 carry the entire genes set for the biosynthesis and efflux of a protein (hemophore) (Wandersman and Delepelaire 2004) with high affinity to chelate the heme group (Loper et al. 2012).

Salicylic acid (SA; 2-hydroxybenzoic acid) is another compound earlier suggested to act as an endogenous siderophore under iron limiting conditions in different bacteria, including *Pseudomonas* spp. (Akenbauer and Cox 1988; Meyer et al. 1992; Visca et al. 1993; Anthoni et al. 1995). However, the role as a siderophore was questioned since SA is not able to bind Fe^{3+} at pH values above 6 (Chipperfiled and Ratledge 2000). Salicylate biosynthesis genes in bacteria were first identified in *P. aeruginosa* PAO1 by Serino and co-workers (1995), demonstrating the SA proceeds via the chorismate/isochorismate pathway. SA biosynthesis genes (*pmsB* and *pmsC*) were also identified in the PGPR, biocontrol strain *P. fluorescens* WCS374, as part of the operon *pmsCEAB* involved in the synthesis of the siderophore pseudomonine (Mercado-Blanco et al. 2001). The ecological and physiological relevance of *Pseudomonas*-produced SA may lie as an essential building block in the synthesis of siderophores like pyochelin, dihydroaeruginoic acid or pseudomonine (Cox et al. 1981; Anthoni et al. 1995; Serino et al. 1995; 1997; Mercado-Blanco et al. 2001).

The involvement of *Pseudomonas*-produced siderophores in biological control of diseases is variable. Siderophores may act through competition by sequestering Fe^{3+} thereby limiting its availability for pathogens (Bakker et al. 1986; Loper and Buyer 1991). Thus, the ability of strain *P. putida* WCS358 to suppress Fusarium wilt of carnation was attributed to production of the siderophore Psb-358 (Lemanceau et al. 1992). Disease suppression mediated by *Pseudomonas* siderophores has also been shown against Fusarium wilt of radish (*Raphanus sativus* L.) (Raaijmakers et al. 1995a), *Pythium* damping-off (Buysens et al. 1996) and *Botrytis cinerea* (Audenaert et al. 2002) in tomato. In contrast, only a minor role (or no involvement at all) was reported in suppression of Pythium damping-off of cucumber (*Cucumis sativus* L.) (Kraus and Loper 1992), *Pythium aphanidermatum* root rot of cucumber (Ongena et al. 1999), Take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller 1990; Hamdan et al. 1991), or *Pseudomonas syringae* pv. tomato-Arabidopsis bacterial speck (Djavaheri et al. 2012).

The involvement of siderophores (and that of SA itself or as an essential moiety of some siderophores) synthesized by *Pseudomonas* strains in suppression of diseases *via* induction of systemic resistance responses is also controversial. On the one hand, SA produced by *P. aeruginosa* and *P. fluorescens* strains has been demonstrated to play a role in inducing systemic resistance against plants pathogens (Leeman et al. 1996b; De Meyer and Höfte 1997; Maurhofer et al. 1998; De Meyer et al. 1999; Audenaert et al. 2002). On the other hand, siderophores produced by strain *P. fluorescens* WCS374, including SA and the SA-based siderophore pseudomonine, have been shown as not needed for eliciting this defensive response against *Pseudomonas syringae* pv. tomato in Arabidopsis (Ran et al. 2005b; Djavaheri et al. 2012). Therefore, the role of SA, and that of siderophores in general in systemic resistance, appears to be highly dependent on the plant-BCA interaction under study (Höfte and Bakker 2007; Mercado-Blanco and Bakker 2007).

Siderophores produced by beneficial *Pseudomonads* spp. can also have a variable influence (negative, neutral or positive) in iron plant nutrition (Barness et al. 1991). For instance, iron uptake in maize and pea was inhibited upon addition of pseudobactin under gnotobiotic conditions (Becker et al. 1985), but nearly no influence on iron acquisition was observed when roots of oat (*Avena sativa* L.) plants were inoculated with *Pseudomonas* strains producing high amounts of siderophores (Alexander and Zuberer 1993). In contrast, barley (*Hordeum vulgare* L.) seedlings grown under hydroponics conditions used Psb-358 as iron source and the synthesis of chlorophyll was enhanced in these plants (Duijff et al. 1994b). Enhanced chlorophyll levels, less chlorosis, and increase in total and physiological available iron were also observed in mung bean plants (*Vigna radiata* L. Wilzeck) upon treatment with a siderophore-producing *Pseudomonas* sp. strain (Sharma et al. 2003). Similarly, amelioration of lime-induced chlorosis on peanut was achieved by amending Fe^{3+} -pyoverdines (Jurkevitch et al. 1986). Finally, plant phytosiderophores can also influence siderophore production of rhizosphere pseudomonads (Marschner and Crowley 1998).

Antibiosis: Antibiotics, Bacteriocins and Insect Toxins Produced by Biocontrol Pseudomonas spp.

Antibiotics

Production of antibiotics and/or additional toxic compounds is one of the best studied mechanisms of biocontrol of plant diseases exerted by beneficial *Pseudomonas* spp., a topic reviewed in detail elsewhere (for instance, Raaijmakers et al. 2002; Haas and Keel 2003; Mavrodi et al. 2006a; Pierson and Pierson 2010). Many strains produce a wide repertoire of secondary toxic compounds which are effective against diverse phytopathogenic fungi, oomycetes, and bacteria (Haas and Keel 2003; Weller et al. 2007). Antibiosis is therefore an attractive and powerful mode of action to be exploited in biocontrol of plant diseases (Mercado-Blanco and Bakker 2007). However, from a practical perspective, several issues should be considered. First, occurrence of antibiotic resistance in the target pathogen, leading to loss of biocontrol effectiveness, is a possibility that should not be excluded to occur under natural conditions (Mazzola et al. 1995). Second, biosynthesis of antimicrobial metabolites is largely influenced by diverse environmental and biotic factors that can rapidly change in dynamic scenarios such as the rhizosphere (Notz and Défago 1999; Duffy and Défago 1999; Lugtenberg and Bloemberg 2004). Finally, potential utilization of genetically-modified *Pseudomonas* spp. with enhanced antibiosis ability (for instance, by introducing antibiotic biosynthesis genes form heterologous strains) under field conditions raises public concern about undesirable effects on non-target organisms and ecosystems (Mercado-Blanco and Bakker 2007). However, diverse studies carried out so far have indicated that introduction of engineered *Pseudomonas* spp. strains can lead to changes, but these are minor and transient compared to

common agricultural practices such as, for example, crop rotation (Glandorf et al. 2001; Viebahn et al. 2005; Blouin-Bankhead et al. 2004; Timms-Wilson et al. 2004).

The list of antimicrobial metabolites synthesized by biocontrol *Pseudomonas* strains is continuously growing and new compounds with antimicrobial activity can now be predicted by genomics studies (Loper et al. 2012). Besides, metagenomics approaches reveal that a wide array of effective antibiotic molecules can be discovered in soil ecosystems (Handelsman 2004). Antibiotics compounds that have been demonstrated to play a role in biocontrol include phenazines (Phz) (Mavrodi et al. 2013), phloroglucinols (Phl) (Keel et al. 1992), pyoluteorin (Thompson et al. 1999), pyrrolnitrin (Dikin et al. 2007), hydrogen cyanide (HCN) (Voisard et al. 1989; Laville et al. 1998), cyclic lipopeptides (Raaijmakers et al. 2006), 2-hydroxymethyl-chroman-4-one (Kang et al. 2004), and 2-hexyl–5-propyl resorcinol (HPR) (Nowak-Thompson et al. 2003; Cazorla et al. 2006; Loper et al. 2012). It is worth mentioning that some of the best up-to-date characterized strains (for instance, *P. protegens* Pf-5 or *P. fluorescens* CHA0) can produce a broad range of different antibiotic compounds (Howell and Stipanovic 1980; Nowak-Thompson et al. 1994; Haas and Keel 2003; Loper et al. 2008). Moreover, recent comparative genomics analyses of diverse plant-associated *Pseudomonas* spp. have demonstrated the presence in their genomes of gene clusters involved in the synthesis of antibiotics such as Phz, DAPG, rhizoxin and pyoluteorin, as well as HCN and pyrrolnitrin (Gross and Loper 2009; Loper et al. 2012).

While in vitro antibiosis can be easily demonstrated by performing antagonistic assays confronting the BCA and the target pathogen(s) on plates and monitoring the growth inhibition effect on the latter (Lugtenberg and Bloemberg 2004), the true contribution of antibiotics in biological control *in situ* is not always easy to assess. Nevertheless, several studies have clearly shown the involvement of antibiotics such as pyoluterin, phenazine-1-carboxamide (PCA), DAPG, pyrrolnitrin, pyocyanine, HCN, and viscosanamide in biocontrol (reviewed by Haas and Défago 2005). A classical example of inhibition of a pathogen through the production of antibiotics is provided by the suppressive effect observed in the so-called *disease suppressive soils* (Schroth and Hancock 1982). Pathogens potentially present in these soils do not cause disease to susceptible plants because of the presence of beneficial microorganisms, mainly *Pseudomonas* spp. and *Bacillus* spp. On the contrary, *conducive soils* are those ones favoring disease incidence and severity because of the absence of this antagonist microbiota. A natural, spontaneous decrease in the incidence and severity of take-all of wheat caused by the phytopathogenic fungus *G. graminis* var. *tritici* has been documented after severe outbreaks of the disease in fields devoted to wheat monoculture for many years. This phenomenon is known as take-all decline (TAD), and it endures over extended time periods (Weller et al. 2002). The involvement of the antibiotic PCA produced by *P. fluorescens* in suppression of take-all of wheat was early reported by Thomashow and co-workers using PCA mutants analysis. They demonstrated that the wild-type strain as well as a complemented PCA mutant derivative effectively suppressed the disease (Thomashow and Weller 1988), and that PCA was produced in the rhizosphere of plants generated from seeds treated with pseudomonads producing this antibiotic

(Thomashow et al. 1990). Furthermore, several evidences suggest that TAD is explained by the presence of populations of DAPG-producing *Pseudomonas* spp.: (i) TAD *suppressive soils* worldwide contain higher populations of DAPG-producing pseudomonads compared with *conducive soils* (Raaijmakers and Weller 1998); (ii) Effective amounts of DAPG could be detected on wheat roots from TAD soils by HPLC mass spectrometry (Raaijmakers et al. 1999); and (iii) The suppressive effect can be transferred to disease *conducive soils* by amending them with TAD soil and that effect correlated with DAPG-producing pseudomonads reaching the threshold population level needed for disease suppression (Weller et al. 2002).

Cyclic lipopeptides (CLPs) are produced by many plant-associated and biocontrol *Pseudomonas* spp., and genes coding for production of CLPs such as orfamide A, massetolide A and viscosin have been found in the genomes of different strains (De Bruijn et al. 2007, 2008; Gross et al. 2007; Loper et al. 2012). Synthesis of CLPs proceeds *via* non-ribosomal peptide synthases. Their core chemical structure consists of a lipid tail (variable in length and composition) bound to a cyclic oligopeptide (diverse in type, number and configuration) which enable them to insert into membranes thereby disturbing their function and integrity. CLPs have surfactant, antibacterial, antifungal, anti-predation and cytotoxic properties (Haas and Défago 2005; Raaijmakers et al. 2010; Mazzola et al. 2009).

Bacteriocins

Some plant-associated, beneficial *Pseudomonas* produced bacteriocins and insect toxins. The involvement that these metabolites may have in the biocontrol of plant pathogens and pests, respectively, has not been sufficiently investigated yet. Bacteriocins (proteinaceous toxins inhibiting or killing strains closely related to the bacteriocin-producing ones) produced by beneficial *Pseudomonas* spp. may play a dual role. On the one hand, they can contribute to biocontrol of bacterial phytopathogens, as reported for *Xanthomonas* spp. (Hert et al. 2009). On the other hand, they may provide environmental advantages to bacteriocin producers in those ecological niches where resources are limited, outcompeting closely-related bacterial neighbors. Loper and co-workers (2012) reported the presence in the genomes of several plant-associated *Pseudomonas* spp. of genes encoding for different bacteriocins such as diverse pyocins, colicin M-like and lectin-like Llp, previously reported to be produced by pseudomonads (Sano et al. 1993; Michel-Briand and Baysse 2002; Parret and De Mot 2002; Parret et al. 2005; Barreteau et al. 2009). Furthermore, microcin B17, a bacteriocin produced by Enterobacteria (Duquesne et al. 2007), was predicted for the first time in *Pseudomonas* spp. to be encoded by the genome of *P. fluorescens* A506. The comparative genomics analysis performed also enabled to identify putative novel bacteriocins (namely N1, N2 and N3) in the predicted proteomes of the studied *P. fluorescens* group strains (Loper et al. 2012). The functions of these bacteriocins remain mostly elusive, setting aside a few exceptions (Parret et al. 2005; Barreteau et al. 2009). Nevertheless, it can be hypothesized that due to the prevalence and diversity they show, bacteriocins could be important for the ecological competitiveness within and among *Pseudomonas* spp. Whether biocontrol

Pseudomonas spp. strains produce bacteriocins effective against phytopathogenic relatives is an interesting research field yet to be explored.

Insect Toxins

Likewise, a novel study area deserving attention is the toxic effects that specific *Pseudomonas* strains display against some insects. The issue of insect toxicity goes beyond the scope of this chapter. However, it is worth mentioning that exploiting the enthomopathogenic behavior of some pseudomonads as a biocontrol tool against some pests is a promising research field that has been seldom investigated (Ruffner et al. 2013). Insect toxicity by *P. fluorescens* group's strains is related, in some cases, to the presence of the Mcf (Makes Caterpillars Floppy) toxin or Tc (Toxin complexes) gene clusters (Péchy-Tarr et al. 2008; Olcott et al. 2010; Liu et al. 2010). *P. protegens* Pf-5 and *P. chlororaphis* strains O6 and 30-84 harbor the *fitABCDEFGH* locus (Loper et al. 2012), which includes genes for regulation and efflux of the *fitD* (Fluorescens Insect Toxin, FIT) gene product, closely related to *mcf* and linked with Pf-5's lethality against the tobacco hornworm *Manduca sexta* (Péchy-Tarr et al. 2008). The elegant comparative genomics study performed by Loper and co-workers (2012) showed that while genes distantly related to *fitD* are present in *P. brassicacearum* Q8r1-96 and *P. fluorescens* Q2-87 and Pf0-1 genomes, other genes of the *fit* locus are absent in these strains. These authors concluded that genomes analyzed have collectively six distinct types of Tc clusters, which can be distinguished by their location in the genomes as well as the number and organization of their constituent genes. Despite the fact that Tc clusters seem to be widely distributed, the ecological relevance they pose for toxin-producing strains is unclear yet (Loper et al. 2012). Recently, Péchy-Tarr and co-workers (2013) have shown that induction of FIT production by the biocontrol (pest and disease) strain *P. fluorescens* CHA0 responded to the host environment and that two regulators (namely FitH and FitG) play a key role in such activity. Similarly, the global regulator GacA (see below) was demonstrated to be necessary for the full insecticidal activity (via oral infection) displayed by strains *P. fluorescens* CHA0 and *P. chlororaphis* PCL1391 (Ruffner et al. 2013).

Induced Systemic Resistance (ISR) and Involved Pseudomonas spp. determinants

Plants can build up an enhanced defensive state called induced systemic resistance (ISR) when properly stimulated (Van Loon et al. 1998; Pieterse et al. 2003; Bakker et al. 2007). Disease suppression mediated by ISR was discovered as a disease suppression mechanism in PGPR *Pseudomonas* spp. by two independent research groups (Van Peer et al. 1991; Wei et al. 1991). To undoubtedly demonstrate that ISR is the operative mechanism, the BCA and the pathogen must be spatially separated on the plant during *ad hoc* bioassays. This experimental strategy will exclude

that direct interaction between microorganisms under evaluation can take place, confirming that disease suppression is truly mediated by the plant. ISR is phenotypically similar to systemic acquired resistance (SAR), a response in which SA plays a major role (Sticher et al. 1997; Gaffney et al. 1993). On the contrary, ISR operates through jasmonic acid (JA) and ethylene (ET) signaling pathways in most cases (Pieterse et al. 1998), although exceptions have been reported (Audenaert et al. 2002). ISR and SAR responses, as well as possibilities to be implemented as a disease control tool, have been extensively reviewed elsewhere (Ramamoorthy et al. 2001; Vallad and Goodman 2004; Bakker et al. 2007). It is interesting to emphasize that SA, JA and ET pathways cross-communicate, and that ISR and SAR might have fuzzy boundaries at some points (Koornneef and Pieterse 2008; Van Wees et al. 2008; Van Verk et al. 2009; Zamioudis and Pieterse 2012), a situation exemplified by responses triggered in host plants by beneficial endophytic bacteria (Conn et al. 2008; Schilirò et al. 2012). Moreover, simultaneous triggering of the SA and the ET/JA signaling pathways in *A. thaliana* led to enhanced disease suppression pointing to an scenario where combined bacterial traits able to trigger both signaling pathways can improve implementation of biological control (Van Wees et al. 2000). However, knowledge on the systemic defensive responses in plants is much better than that for the bacterial determinants involved in such responses, as it was discussed above for the controversial role of *Pseudomonas* spp. iron-regulated metabolites. Indeed, siderophores produced by beneficial *Pseudomonads* spp. were early thought to act in disease suppression mainly through competition for iron with the pathogen (see above), but diverse studies also suggested that these molecules can be bacterial signals triggering ISR (Leeman et al. 1996b; Meziane et al. 2005, Ran et al. 2005a). In some case, however, siderophore mutants were equally effective in ISR induction as the wild-type strains (Leeman et al. 1996b; Meziane et al. 2005; Djavaheri et al. 2012).

Since SA is well known to trigger SAR when applied exogenously (Sticher et al. 1997), SA produced by beneficial *Pseudomonas* spp. was suggested to be involved in induced resistance (Leeman et al. 1996b; De Meyer and Höfte 1997; Maurhofer et al. 1998). However, many studies have concluded that bacterially-produced SA is not the signal leading to ISR (Press et al. 1997; Ran et al. 2005b; Djavaheri et al. 2012). Similarly, SA-based siderophores (i.e. pyochelin and pseudomonine), and even pyoverdine, were shown not to be implicated in induced resistance either (Audenaert et al. 2002; Djavaheri et al. 2012).

Besides the major role that antibiotics have in direct inhibition of pathogens, they have been implicated in ISR as well. For instance, pyocyanin produced by *P. aeruginosa* 7NSK2 has been involved in ISR against *B. cinerea* in tomato (Audenaert et al. 2002). Interestingly, the effects on diseases development in rice by 7NSK2-produced pyocyanin varied depending on the pathogen. Thus, while pyocyanin elicited ISR against *Magnaporte grisea*, it enhanced susceptibility to *Rhizoctonia solani* (De Vleesschauwer et al. 2006). The antibiotic DPAG was also demonstrated to trigger ISR in *A. thaliana* against *Peronospora parasitica* (Iavicoli et al. 2003) and *P. syringae* pv. tomato (Weller et al. 2012), as well as against the root-knot nematode *Meloidogyne javanica* in tomato (Siddiqui and Shoukat 2003).

Additional *Pseudomonas* traits which have been proposed to be involved or influence ISR are: N-alkylated benzylamine derivative (Ongena et al. 2005), the O-antigen of LPS (Van Peer and Schippers 1992; Leeman et al. 1995, 1996; Van Wees et al. 1997; Meziane et al. 2005), flagella (Meziane et al. 2005), *N*-acyl-homoserine lactone (Schuhegger et al. 2006), 2,3-butanediol (Han et al. 2006b), and others (Han et al. 2006a). Apparently, multiple determinants present in a given *Pseudomonas* strain can trigger ISR. It is expected that more will be uncovered in the future. Some studies are revealing a redundancy of ISR-triggering traits what becomes evident when one trait is knocked out but others still lead to effective ISR (Bakker et al. 2003; Djavaheri et al. 2012). This redundancy can obviously difficult the studies on bacterial ISR determinants but, on the other hand, it is clearly indicating that ISR is a robust disease suppression mechanism (Bakker et al. 2007).

Additional Traits Potentially Involved in Biocontrol by *Pseudomonas* spp. strains

Among *Pseudomonas* spp. traits involved in the interaction with plants some are not related to biocontrol *stricto sensu* or, so far, have not been directly linked to plant protection against biotic constraints. However, they could be key elements to promote plant growth and/or alter their metabolism and physiology. Moreover, production of specific secondary metabolites and proteins may influence biocontrol activity. It is therefore interesting to briefly mention them, particularly because genome sequencing projects are providing comprehensive information on the genetics of these traits, hence enhancing our current knowledge on *Pseudomonas*-plant interactions.

Plant Hormones Metabolism

Some strains produce plant hormones and/or are able to degrade them (Lugtenberg and Kamilova 2009). For instance, *P. chlororaphis* strains 30-84 and O6 carry the genes encoding for tryptophan-2-monooxygenase and indole-3-acetamide hydrolase which are involved in the indole-3-acetamide pathway leading to indole-3-acetic acid (IAA), the main auxin hormone in plants implicated in many physiological processes. While genetic information is present in both genomes, production of IAA in culture has been only confirmed for strain O6 (Dimkpa et al. 2012; Loper et al. 2012). A putative IAA degradation gene cluster is present in *Pseudomonas* spp. (*P. synxantha*) BG33R (Loper et al. 2012), a strain originated from the rhizosphere of peach and suppressive of the plant-parasitic nematode *Mesocriconema* (*Criconemella*) *xenoplax* (Kluepfel et al. 1993). The comparative genomics analysis recently conducted by Loper and co-workers (2012) also revealed the presence of: (i) genes involved in the catabolism of phenylacetic acid (PAA), an auxin (Wightman

and Lighty 1982) with antimicrobial properties (Kim et al. 2004), in the genomes of strains 30-84 and O6, as well as in *P. protegens* Pf-5; (ii) gene *acdS* encoding for aminocyclopropane-1-carboxylic acid (ACC) deaminase in *P. brassicacearum* Q8r1-96, an enzyme that reduces the levels of the plant hormone ethylene (converting ACC into ammonia and a-ketobutyrate) thus leading to improved plant tolerance to diverse (a)biotic stresses and stimulation of root growth (Glick 1995), as the beneficial effects demonstrated for the ACC deaminase-producing strain *P. putida* UW4 (Hao et al. 2007; Toklikishvili et al. 2010; Nascimento et al. 2013); and (iii) a putative acetoin reductase gene in some of the analyzed strains, including *P. chlororaphis* O6 that was previously demonstrated to produce the volatile 2,3-butanediol implicated in systemic resistance (Han et al. 2006b).

Exoenzymes

Biocontrol *Pseudomonas* spp. strains can also secrete enzymes playing different roles. For instance, strain *P. fluorescens* SBW25, originated from the phyllosphere of sugar beet, shows pectolytic activity in potato what correlated to the presence of a pectate lyase gene in its genome (Nikaidou et al. 1993; Silby et al. 2009). However, this enzymatic activity was not found in other biocontrol strains (Loper et al. 2012). On the other hand, exoprotease AprA is produced by a number of plant-associated pseudomonads (Loper et al. 2012), although the role that this exoenzyme plays in biocontrol of fire blight disease of pear and apple (caused by *Erwinia amylovora*) has yet to be clarified (Anderson et al. 2004; Stockwell et al. 2011). Finally, chitinase-coding genes and in vitro chitinolytic activity, what can contribute to biocontrol of fungal phytopathogens (Nielsen et al. 1998), have been identified in diverse plant-associated pseudomonads as well (Loper et al. 2012). An interesting aspect not yet investigated is whether endophytic *Pseudomonas* spp. may produce enzymes important for the endophytic colonization process of their plant hosts (see above), as shown/suggested for different plant-endophytic bacteria associations (Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011).

Secretion Systems in Beneficial Pseudomonas spp.: Do They Play a Role in Biocontrol?

Specific bacterial secretion systems are clearly related to pathogenesis (virulence factors). For instance, *P. syringae* group bacteria are able to cause disease by injecting type III effector proteins (T3Es) into plant cells using type III secretion systems (T3SSs) (Cornelis 2010), thereby suppressing plant defense responses and altering eukaryotic cell physiology (Chang et al. 2004; Lindeberg et al. 2008). Thus, T3SS-mediated protein secretion and delivery of effector proteins leading to activation of effector-triggered immunity upon recognition by the plant are important strategies to suppress host defense responses not only in gram-negative pathogens but also in

rhizobial symbionts (Jones and Dangl 2006; Reinhold-Hurek and Hurek 2011). On the other hand, T3SSs have been involved in plant root colonization, rhizosphere competence, defense against protozoa predation, environmental competition, or oomycete suppression observed for some *Pseudomonas* strains (Rezzonico et al. 2005; Matz et al. 2008; Mavrodi et al. 2011). Therefore, bacterial secretion systems could also participate in plant-beneficial bacteria interactions, and may well concern the proper development of endophytic lifestyles. However, the role that secretion systems can play in the interaction of beneficial bacterial endophytes, including *Pseudomonas* spp., has been hardly investigated.

Unlike general and commonly-found systems such as T1SSs and T2SSs, the latter involved in the transport of many extracellular enzymes and with multiple gene clusters in several strains (Loper et al. 2012), secretion systems like T3SS and T4SS were thought to be rarer among studied beneficial and/or endophytic strains. On the contrary, T5SSs and T6SSs seem to be more widespread (Reinhold-Hurek and Hurek 2011) and in some cases with a multiplicity of gene clusters (Pukatzki et al. 2009; Loper et al. 2012). For instance, and regarding beneficial pseudomonads, *Pseudomonas stutzeri* A1501 (Yan et al. 2008) carries a T5SS gene cluster, and *P. putida* W619 harbors several T5SSs and T6SSs (Yan et al. 2008; Taghavi et al. 2009; Reinhold-Hurek and Hurek 2011). Nevertheless, the overall picture regarding secretion systems is rapidly changing as more genomic data are available. For instance, T3SSs have been identified in plant-growth promoting *P. fluorescens* strains SBW25 (Preston et al. 2001), BBc6R8 (Cusano et al. 2011) and *P. brassicacearum* (*P. fluorescens*) Q8r1-96 (Mavrodi et al. 2011). Strain Q8r1-96, as well as *P. fluorescens* A506, Q2-87, SS101, SBW25 and *Pseudomonas* sp. BG33R, harbour gene clusters encoding for *rsp/rsc* (rhizosphere-expressed secretion protein and rsp-conserved) T3SS resembling *hrc/hrp* T3SS of plant pathogenic *P. syringae* (Loper et al. 2012). Similarly, these authors have identified several putative T3Es in the genomes of examined strains, although it remains to be proven whether these proteins constitute novel T3Es. Finally, the original thought that T6SSs systems could exclusively be implicated in the delivery of virulence effectors to eukaryotic hosts has changed towards a key role in the interaction among bacteria, according to novel findings showing their prevalence in environmental bacteria and plant-associated *Pseudomonas* spp. (Records 2011; Russell et al. 2011; Loper et al. 2012). Regarding to this, one to three gene clusters coding for T6SS are present in the genomes of *P. fluorescens* group strains analyzed by Loper and co-workers (2012).

Regulation of Biocontrol Traits in *Pseudomonas* spp.

Many biocontrol traits of *Pseudomonas* spp. are hierarchically controlled by a complex regulatory network comprising the GacS/GacA two-component system, QS systems, small regulatory RNAs, RNA-binding proteins, and diverse sigma factors (Heeb and Haas 2001; Haas et al. 2002; Haas and Keel 2003; Humair et al. 2009; Kidarsa et al. 2013). Overall, expression of biocontrol factors depends on the Gac/

Rsm signal transduction pathway (Lapouge et al. 2008). The sequence of events can be summarized as follows: three small RNAs (sRNAs), namely RsmX, RsmY and RsmZ, are transcriptionally activated by the GacS/GacA two-component regulatory system. The triggering factor for such activation seems to be high bacterial cell population densities, although sRNAs expression takes place at different moments: RmsX and RsmY are expressed along the growth curve, whereas RsmZ is preferentially expressed in the stationary phase (Heeb et al. 2002; Valverde et al. 2003; Kay et al. 2005; 2006). sRNAs subsequently bind to the sRNA-binding proteins RsmA and RsmE, belonging to the RsmA/CsrA family, easing the translational repression they exert and making target mRNAs available to ribosomes (Reimmann et al. 2005; Lapouge et al. 2008).

The activity of the Gac/Rsm pathway is regulated by unknown autoinducing signal molecules apparently through activate phosphorylation of the GacS sensor (Heeb et al. 2002; Dubuis et al. 2007). Subsequently, phosphorylated GacS activates the response regulator GacA, as shown in the human pathogen *Pseudomonas aeruginosa* (Goodman et al. 2009). Moreover, the Gac/Rsm pathway can be influenced by environmental factors like temperature thereby affecting the expression of biocontrol factors such as antibiotics and HCN production in strain *P. fluorescens* CHA0 (Humair et al. 2009). These authors have provided evidences showing that the Gac/Rsm pathway is modulated by the interaction between GacS and RetS (a sensor kinase antagonizing the GacS/GacA system) and between GacS and LadS (a sensor kinase activating the GacS/GacA system), and that temperature affects the RetS-GacS interaction.

Examples of biocontrol traits regulated by the Gac/Rsm signal transduction pathway are antibiotics production and HCN synthesis in *P. protegens* Pf-5 and *P. fluorescens* CHA0 (Blumer et al. 1999; Kay et al. 2005; Lapouge et al. 2008), although the stationary-phase sigma factor RpoS (σ^S) is also involved (Sarniguet et al. 1995; Heeb et al. 2005). Mutations affecting the GacS/GacA system or sRNAs in strain CHA0 impaired effective biocontrol of several phytopathogens, whereas mutation in RpoS of strain Pf-5 induced better biocontrol of *Pythium ultimun* damping-off in cucumber but lower inhibition of *Pyrenophora tritici-repentis* in wheat straw (Pfender et al. 1993; Sarniguet et al. 1995; Valverde et al. 2003; Kay et al. 2005). Recently, Kidarsa and co-workers (2013) have demonstrated that GacA and RpoS influenced the transcription of a large number of genes in strain Pf-5 when colonizing the surface of pea seeds. Many of these genes (i.e. motility, antibiotics production, exoenzymes, secretion systems, etc.) are involved in either biocontrol or colonization by pseudomonads, as discussed in previous sections. Finally, the GacS/GacA system also regulates the expression of AHL QS systems (see below) in *P. aureofaciens* 30-84 (Chancey et al. 1999) and *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 2000). On the contrary, the GacS/GacA system has been shown to control the expression of pseudomonads biocontrol traits but in which AHL signals have not been detected (Whistler et al. 1998; Bull et al. 2001).

Quorum-sensing (QS) is a gene regulatory mechanism identified in a large number of bacterial species, including *Pseudomonas* spp. This topic has been comprehensively reviewed elsewhere (see, for instance, Venturi 2006; Hartmann and

Schikora 2012, and references therein). The most common QS molecular signals in Gram-negative bacteria are *N*-acyl-homoserine lactones (AHLs) (Cámara et al. 1998), although other signal molecules as the so-called *Pseudomonas* quinolone signal (PQS) has been reported (Pesci et al. 1999). QS systems are known to be based in the activities of two proteins belonging to the LuxI (biosynthesis of the intercellular AHLs signals) and LuxR (transcriptional regulators) families (Fuqua et al. 2001; Miller and Bassler 2001). Biocontrol and plant growth-promoting *Pseudomonas* spp. strains also carry QS systems which, in addition, display an intimate cross-talk with additional global regulators (Bertani and Venturi 2004). Elasri and coworkers (2001) have shown that production of autoinducer signals is more usually present among plant-associated *Pseudomonas* isolates than in free-living ones, suggesting an important role of AHL QS systems in plant-*Pseudomonas* interactions. Moreover, plants and animals may influence bacterial processes mediated by QS systems and important for such interactions. Indeed, it is known that higher plants can produce and secrete AHL-interfering compounds (Teplitski et al. 2000; Gao et al. 2003; Bauer and Mathesius 2004), and that AHL molecules can also be implicated in the sensing of bacteria by *Caenorhabditis elegans* (Beale et al. 2006). Considering that beneficial endophytes may reach high populations densities in specific spots (Chi et al. 2005), the possible production of QS signals inside plant tissues and how they may operate in endophytic-mediated processes is an interesting area of study. Some examples of QS systems operating in biocontrol *Pseudomonas* spp. are: (i) two AHL QS systems reported for strain *P. aureofaciens* 30-84: the PhzI-PhzR QS system, involved in the regulation of the operon *phzFABCD* involved in Phz biosynthesis (Pierson et al. 1994; Wood et al. 1997), a key antibiotic in the control of wheat take-all disease and the CsaI-CsaR QS system involved in rhizosphere competitiveness and regulation of cell-surface components biosynthesis (Zhang and Pierson 2001); (ii) production of the antifungal metabolite phenazine-1-carboxamide in strain *P. chlororaphis* PCL1391is also regulated by the PhzI-PhzR QS system (Chin-A-Woeng et al. 2001); or (iii) the QS system PcoI-PcoR involved in biofilm formation, colonization of wheat rhizosphere and biocontrol of take-all disease by biocontrol strain *P. fluorescens* 2P24 (Wei and Zhang 2006).

Besides Gac/Rsm and QS systems, different sigma factors have been shown to be involved in the regulation of diverse pseudomonads biocontrol traits. Thus, the housekeeping sigma factor RpoD (σ^{70}) in *P. fluorescens* CHA0 regulates pyoluteorin and DAPG production, improving protection against the oomycete *P. ultimun* in cucumber (Schnider et al. 1995). Similarly, Péchy-Tarr and co-workers (2005) have shown that the environmental sigma factor RpoN (σ^{54}) is a major regulator of several important biocontrol traits in the same strain. Finally, the above-mentioned sigma factor RpoS influences antibiotics production, biocontrol activity, and survival of strain *P. protegens* Pf-5 on cucumber seedlings surface (Sarniguet et al. 1995).

An example of the high complexity and cross-talks established among these regulatory elements is that of *Pseudomonas* sp. PCL1171. A functional GacA/GacS system, together with RpoS and MutS (involved in mutation repair) are involved in phase variation in this strain. It has been previously mentioned that phase variation

influences plant root colonization and biocontrol traits (i.e. motility, siderophore production, etc.). Moreover, spontaneous mutation affecting the GacA/GacS system is considered one of the mechanisms of phenotypic variation which in turn affect the expression of genes involved in secondary metabolism like antimicrobial compounds (Van den Broek et al. 2005a, b).

Use of Biocontrol *Pseudomonas* spp. Strains within Integrated Disease Management Strategies: From Basic Knowledge to Field Application

The use of BCAs is an environmentally-friendly approach fitting the criteria of modern sustainable agriculture (Hamblin 1995). Moreover, it is a promising control measure which can be implemented in combination with others within IPM frameworks (López-Escudero and Mercado-Blanco 2011). IPM strategies include the use of chemical and biological control tools, among other measures such as plant breeding, plant certification schemes, adequate agricultural practices, etc. Despite the fact that BCAs cannot currently substitute chemicals in many agronomical scenarios, biocontrol measures should aim to a gradual decrease in the use of chemicals thereby overcoming public concerns on the risk that chemical biocides pose for the environment and human and animal health. To achieve that, in-depth studies are still needed not only to unravel mechanisms underlying biocontrol, which have been reviewed in previous sections, but also to understand how these BCAs can be commercialized and applied with both efficacy and safety. Furthermore, they should be able to complement and be compatible with culturing practices such as irrigation, fertilization and other crop protection measures used in targeted agro-ecosystems.

As it was already mentioned, the positive affect observed for a given biocontrol *Pseudomonas* strain (or any other BCA) under laboratory and/or controlled-growth conditions does not necessarily mean it can perform well under natural environments (*biocontrol inconsistency*) (Lindow 1988; Kraus and Loper 1992). Thus, the development of formulations harboring BCA(s) which can be used as effective commercial products usually poses a long and arduous process. Current status on the identification and practical use of BCAs as well as production, registration and commercialization of microbial pesticides have been reviewed elsewhere (for instance, Montesinos 2003; Fravel 2005; Alabouvette et al. 2006; Höfte and Altier 2010; Pliego et al. 2011a; Bonaterra et al. 2012, and references therein).

It must be emphasized that an adequate screening and selection strategy of BCAs is the first key step for the subsequent successful development of effective formulations (Fravel 2005; Höfte and Altier 2010; Pliego et al. 2011b). Moreover, taking advantage of the basic knowledge on biocontrol traits gathered from studies predominantly carried out with isolates from culture collection, the search, isolation and identification of new strains adapted to field conditions is encouraged. This approach will serve to overcome failures on biocontrol performance sometimes predicted and usually observed for culture collection strains (Campbell 1989). Höfte and Altier (2010) have recently summarized two case studies exemplifying

approaches aimed to isolate and screen novel, indigenous and effective biocontrol *Pseudomonas* spp. strains in South America and Africa.

The interest that biocontrol companies may have in developing novel microbial products (*decision making*) will mainly depend, among other issues, in how important is the target crop (*market size*) and what is the incidence and severity of the phytopathological problem(s) affecting it (*level of economical losses to overcome = financial profit*). If evaluation of opportunities turns out to be positive, companies should then be in the position to evaluate at the field level what academic research is most of the times only able to examine at laboratory or (semi)controlled conditions (*know-how transfer*). Finally, important issues to be considered are the proper evaluation of the environmental safety of novel bioproducts (*risk/safety assessment*), development of procedures for the production, long-term storage and delivery of stable and effective products (*bio-formulation technology*), and assessment of their performance against different pathogens (or their pathogenic varieties, i.e. strains, isolates, pathovars, etc.) and/or crops, as well as under diverse agronomical, geographical and environmental conditions (*effective range of the bioproduct*).

BCAs safety undergoes a severe scrutiny and is submitted to strict regulations issued by food authorities prior to be approved as marketable (Pliego et al. 2011a). Indeed, concerns are sustained by studies alerting on the risk that potential BCA may pose for human health (Berg et al. 2005; Kumar et al. 2013). Therefore, proper identification of strains that could eventually behave as opportunistic pathogens under specific circumstances (i.e. immuno-depressed patients) is compulsory. Available molecular procedures and/or novel promising approaches such as the assay with the model nematode *Caenorhabditis elegans* to assess the pathogenic potential of a BCA (Zachow et al. 2009) are potent tools to accomplish with that aim. The same accounts for BCAs whose biocontrol activity is based on the synthesis and release of new antibiotics and/or toxic metabolites. The possibility that these compounds, or the BCAs themselves, may pose undesirable effects on non-target microbiota (soil microorganisms, particularly beneficial) and/or flora and fauna of the target application site requires of thorough analyses to determine potential toxicological and environmental impacts (for instance, Scherwinski et al. 2008; Adesina et al. 2009). A good example on the concern raised by a BCA is the evaluation report on *Pseudomonas chlororaphis* MA342 performed by the scientific committee on plants of the Health and Consumer Protection Directorate-General of the EU (http://ec.europa.eu/food/fs/sc/scp/out120_ppp_en.pdf). This strain has been formulated and used for the control of seed-borne *Tilletia caries, Fusarium* sp*., Microdochium nivale* and *Septoria nodorum* in cereals (Hökeberg 2006).

Effective bioformulations should meet requisites such as good preservation and long-storage properties of the active component (the BCA), easiness to be delivered to their targets, and proven activity (Burges 1998). On the other hand, production line of the bioformulation must be cost effective as well, able to overcome cases of low productivity of specific BCAs, and not be detrimental to any biocontrol trait(s) aimed to be exploited (Fravel et al. 2005; Pliego et al. 2011a). Unlike spore-forming biocontrol *Bacillus* spp. strains, *Pseudomonas* spp. strains have the disadvantage that must be formulated as vegetative cells. The former can endure for longer periods of time when produced as powder or granular formulations. On the contrary,

they need time to revert from the quiescent stage to metabolically-active forms. For biocontrol *Pseudomonas* spp. strains, seed-coating, freeze-dried and liquid formulations can be good options, although the latter one may pose problems of viability loss along time. It is worth mentioning that the choice of a bioformulation may also depend on the target crop, cropping practices and irrigation system available. Development of improved *Pseudomonas* spp.-based bioformulations still need of further technical inputs to overcome problems of stability and storage, although methodological approaches such as freeze-drying or bacteria fixation on solid supports such as clay look promising (Muñoz-Rojas et al. 2006; Jiang et al. 2007).

Concluding Remarks

Many strains of *Pseudomonas* spp. can reduce diseases of plants significantly by means of diverse mechanisms which are not mutually exclusive. Some strains can even establish a mutualistic type of interaction with their plant hosts, developing an endophytic lifestyle. Basic knowledge on the molecular, physiological and ecological mechanisms underlining biocontrol *Pseudomonas*-plant interactions have increased at steady and rapid rate during the last decades. This is particularly true for mechanisms such as antibiosis, although others like induction of defensive responses such as ISR or SAR still need of better understanding. Enhancement of the colonization ability of the targeted niches and/or phytopathogens by biocontrol pseudomonads are interesting areas to further investigate. Regarding to the rhizosphere and to *Pseudomonas*-root interactions, a better knowledge on the composition and function of organic volatiles and root exudates is clearly needed. This should be accomplished by implementing multidisciplinary approaches and taking advantage of the currently-available and powerful technologies to explore plant-bacteria-soil interactions. New areas of research like insecticidal activity and endophytism pose promising perspectives. The use of endophytic *Pseudomonas* spp. strains offers the advantage of dealing with bacteria ecologically adapted to the target niche (inner plant tissues) where they may deploy their beneficial effects. Nevertheless, insights on the endophytic colonization process as well as on the biocontrol mechanisms operating once endophytic bacteria are well established inside plant tissues are still needed. The same accounts to comprehend how application of endophytic *Pseudomonas* spp. may overcome the plant's defensive barriers to successfully colonize them. Likewise, more studies are needed to understand whether the biocontrol potential of introduced biocontrol pseudomonads (either endophytic or not) can be affected by the innate microbiome of the host plant; and *vice versa*, how the indigenous plant microbiota can be influenced by the external applications of BCAs and whether this effect may alter host plant development and fitness. More efforts are also needed to improve the effectiveness, stability and storage of *Pseudomonas*-based bioformulations, as well as to improve the current burdensome registration process of biocontrol products. Research and information efforts should also aim to overcome the negative perception that consumers and politicians may have about the use of these bioproducts, which should be viewed as more

environmental-friendly tools compared with chemical biocides, provided that all precautions mentioned above have been taken into account. The use of biocontrol and plant growth promotion microorganisms, including beneficial *Pseudomonas* spp. strains, should play a more relevant role in agriculture. Biocontrol measures must be viewed as excellent tools to use in combination with other disease control measures within integrated disease management frameworks.

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Chapter 7
Resistance and Response to Anti-Pseudomonas Agents and Biocides

Yuji Morita, Junko Tomida and Yoshiaki Kawamura

Abstract *Pseudomonas aeruginosa* possesses an innate resistance to various antimicrobial agents, owing much to its low membrane permeability and multidrug efflux pumps. Although anti-pseudomonas agents are effective against this organism, their repeated use has been linked to the development of drug resistance with the emergence of multidrug resistant *Pseudomonas aeruginosa* strains in clinical settings of special concern. Inappropriate use of disinfectants can be also causes of *P. aeruginosa* infection.

Multidrug resistance in *P. aeruginosa* is complex, and attributable to both chromosomal mutations and the acquisition of extraneous resistance genes. Recent genome-wide analyses of *P. aeruginosa* have uncovered novel intrinsic resistance mechanisms and global responses to antimicrobials. Among these strategies, two in particular deserve special mention: two-component systems, and intracellular proteases.

Introduction

Pseudomonas aeruginosa possesses an intrinsic resistance to many antimicrobials, owing to the low permeability of its outer membrane barrier and the presence of multidrug efflux transporters (Poole 2011). Although broad-spectrum β-lactams (e.g., imipenem), fluoroquinolones (e.g., ciprofloxacin), and anti-pseudomonas aminoglycosides (e.g., amikacin) are often available for treatment, *P. aeruginosa* readily acquires resistance to these specific agents via chromosomal mutations and lateral gene transfer. As only a limited number of new antimicrobial agents are in development, the emergence and spread of multidrug resistant *P. aeruginosa* infections, against which very few agents are currently effective, is of great concern. Here we discuss how *P. aeruginosa* responds to anti-pseudomonas agents to survive as a pathogen.

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Carbapenems and Other Anti-Pseudomonas β-Lactams

β-lactams bind to cell wall transpeptidases (penicillin binding proteins (PBPs)) blocking an important step in peptideglycan biosynthesis (Poole 2004). Among anti-pseudomonas β-lactams, carbapenems (e.g., imipenem and meropenem) are an important class of anti-pseudomonal β-lactams owing to their stability when exposed to most β-lactamases. Under clinical conditions, the acquisition of extraneous carbapenases, especially metallo-β-lactamases (MBLs) is of greatest concern, as it is the major cause of high-level resistance to almost all β-lactams, including carbapenems. This resistance is then frequently co-transferred with genes encoding aminoglycoside-modifying enzymes (or rarely 16S rRNA methylases) (Poole 2011). The most common mechanism through which carbapenem resistance is acquired is loss or alteration of the outer membrane OprD porin protein (e.g., Shu et al. 2012; Khuntayaporn et al. 2013; Fournier et al. 2013), the major portal for entry for carbapenems (Trias and Nikaido 1990). Other additional mechanisms such as resistance, nodulation, and cell division (RND)-type multidrug efflux pumps (such as MexAB/MexXY-OprM and/or AmpC β-lactamase) further strengthen their resistance (e.g., Riera et al. 2011).

Causes responsible for the loss of porin OprD in *P. aeruginosa* are a lack or alteration of OprD production through disruption of *oprD*'s coding or upstream regions and downregulated OprD production through mutations of the two-component ParRS system, the two-component CzcRS system, and the probable oxidoreductase MexS with inverse coregulation of active RND-type efflux systems (Fournier et al. 2013). Notably, some amino acid substitutions of the ParRS system cause not only downregulation of *oprD*, but also upregulation of the RND-efflux *mexXY* pump gene and lipopolysaccharide modification *arnBCADTEF-ugd* operon, interconnecting resistance to polymyxins, aminoglycosides, fluoroquinolones, and some β-lactams (e.g., carbapenems and cefepime) (Muller et al. 2011). Additionally, some amino acid substitutions of the MexS causes downregulation of *oprD* and upregulation of RND-efflux *mexEF-oprN* pump genes, both of which depend on the activator gene *mexT,* yielding increased resistance to carbapenems and fluoroquinolones (Sobel et al. 2005). Sub-inhibitory concentrations of imipenem were shown to strongly induce *ampC* gene expression both in planktonic cells and biofilm, although imipenem is not itself a substrate of the AmpC enzyme (Bagge et al. 2004). In addition, *P. aeruginosa* biofilms exposed to imipenem induced genes coding for alginate biosynthesis, causing thicker and more robust biofilms (Bagge et al. 2004).

In addition to carbapenems, penicillins (e.g., ticarcillin, piperacillin), cephalosporin (e.g., ceftazidime, cefepime) and monobactams (e.g., aztreonam) are commonly used to treat pseudomonal infection. Mutational de-repression of *ampC* is the most common mechanism of β-lactam resistance (Poole 2011). High-level clinical β-lactams (except to carbapenems) resistance was shown to be driven by inactivation of *dacB*-encoded nonessential PBP4, triggering overproduction of the chromosomal β-lactamase AmpC and the specific activation of CreBC (BlrAB) two-component regulator (Moya et al. 2009). Among the RND multidrug efflux pumps,

MexAB-OprM accommodates the broadest range of β-lactams (e.g., ticarcillin, piperacillin, ceftazidime, and aztreonam) and is most frequently linked to β-lactam resistance, although MexXY-OprM has been linked to some β-lactams (e.g., cefepime) as well (Hocquet et al. 2006; Poole 2011). Ceftazidime, a PBP3 inhibitor, which does not induce *ampC* gene expression, but is rather a substrate of AmpC, impacts the transcription of a large number of genes in *P. aeruginosa,* including those of SOS response repressor LexA-like proteins (Blázquez et al. 2006). Noticeably, this antimicrobial agent induces the error-prone DNA polymerase DinB, causing induced mutagenesis and decreasing ciprofloxacin toxicity (Blázquez et al. 2006). The acquisition of extraneous β-lactamases including ESBL frequently co-transferring with genes encoding aminoglycoside-modifying enzymes also deserves attention (Poole 2011).

The mutants' profiles of susceptibility for three β-lactams, namely ceftazidime, imipenem, and meropenem, suggested that a wide array of elements, but distinct mechanisms of action and resistance contributes to β-lactam resistance despite these compounds belonging to the same structural family (Alvarez-Ortega et al. 2010). Only three mutants demonstrated reduced (PA0908) or increased (*glnK* and *ftsK*) susceptibility to all three β-lactams (Alvarez-Ortega et al. 2010). The mechanisms involved vary, as *glnK* encodes a nitrogen regulatory protein PII-2 and *ftsK* encodes a protein involved in cell division and stress response (Alvarez-Ortega et al. 2010).

Fluoroquinolones

Fluoroquinolones, particularly ciprofloxacin, are commonly used in the treatment of *P. aeruginosa* infections (Poole 2011). This class of agents interacts with complexes composed of DNA and either of the two target enzymes, DNA gyrase or topoisomerase IV (Hooper 1998). Resistance to these agents, particularly high-level resistance, is predominantly mediated by mutations in these flouroquinolones-targeted enzymes, though efflux is a significant contributing factor, often in combination with target site mutations (Poole 2011). The four RND-type multidrug efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) are well recognized as significant determinants of fluoroquinolone resistance (Poole 2011) although fluoroquinolone-resistant mutants which overproduce other RND multidrug efflux pumps might exist. Some, such as MexGH-OpmD and MexVW can be isolated from mutants lacking the four major RND multidrug efflux pumps (Sekiya et al. 2003; Li et al. 2003) and other efflux pump-bolstering fluoroquinolone resistance may be coded for in the genome (e.g., NorM ortholog of *P. aeruginosa* (Morita et al. 1998)).

The *mexAB-oprM* operon is constitutively expressed in wild-type cells under usual laboratory conditions, where it contributes to *P. aeruginosa's* intrinsic resistance to quinolones and other many antimicrobial agents (e.g., carbenicillin, aztreonam, chloramphenicol, and tetracycline) (Poole 2013; Morita et al. 2001). Highly

expressed MexAB-OprM quinolone-resistant mutants are classified as *nalB*, *nalC*, or *nalD*. *NalB* mutants carry lesions in the *mexR* gene encoding a local repressor of the *mexAB-oprM* operon (Srikumar et al. 2000). *NalC* mutants carry a mutation in a local *nalC* repressor gene of an adjacent two-gene operon, PA3720-PA3721 (Cao et al. 2004). PA3721 was named *armR* after its function as an anti-repressor of MexR, where AmrR binds to MexR and negatively impacts *mexAB-oprM* expression (Daigle et al. 2007). While MexR binds to the first (efflux-operon distant) promoter (Morita et al. 2006a), yet a third gene, *nalD* also encodes a repressor, but instead binds to the second (efflux-operon proximal) promoter upstream of *mexAB-oprM*. The *mexCD-oprJ* operon and the *mexEF-oprN* operon are almost unnoticeable in wild type cells and are overexpressed in the quinolone-resistant mutants known as *nfxB* and *nfxC,* respectively (Poole 2013; Kohler et al. 1997). *NfxB* mutants, which carry lesions in their local repressor gene, *nfxB,* of the *mexCD-oprJ* operon, showed increased resistance to antimicrobial agents including fluoroquinolone and zwitterionic cephems, and decreased resistance to both various β-lactams (ticarcillin, cefepime, ceftazidime, and aztreonam) and aminoglycosides (Poole 2013 Jeannot et al. 2008). Notably, MexCD-OprJ-overproducing mutants are rarely seen under clinical conditions (Jeannot et al. 2008). *NfxC* mutants which carry lesions in the local activator *mexT* gene of the *mexEF-oprN* operon, or the probable oxidoreductase *mexS* gene showed increased resistance to antimicrobial agents including fluoroquinolone and carbapenems, and decreased resistance to various β-lactams (ticarcillin, cefepime, ceftazidime, and aztreonam) and aminoglycosides (Sobel et al. 2005; Llanes et al. 2011). The molecular mechanisms of *mexEF-oprN* overexpression in *nfxC* mutants are described above. Although the expression of *mexXY* in wild type cells is repressed by *mexZ,* a cognate regulator gene, overexpression of *mexXY* increased the level of fluoroquinolones resistance in *P. aeruginosa* cells (Aires et al. 1999; Morita et al. 2001). The incidence of MexXY-OprM overproducers among clinical isolates has been linked to the use of various antibiotics, including ciprofloxacin, cefepime and amikacin, but not imipenem (Hocquet et al. 2008).

Transcriptional responses of *P. aeruginosa* to sub-inhibitory and inhibitory ciprofloxacin exposure demonstrated the expression of hundreds of promoted or repressed genes (Brazas and Hancock 2005; Cirz et al. 2006; Brazas et al. 2007). Surprisingly, genes for bacteriophage-like pyocins were upregulated and mediated fluoroquinolone susceptibility (Brazas and Hancock 2005). At least one-third of upregulated genes occur in regulons that are likely controlled by LexA-like SOS response repressor proteins in response to inhibitory concentrations of ciprofloxacin, while downregulated genes appear to involve virtually every facet of cellular metabolism (Cirz et al. 2006; Brazas et al. 2007). The overall pattern of expression in the DNA replication enzymes suggests a shift from canonical DNA replication enzymes to inducible polymerases in response to inhibitory ciprofloxacin concentrations (Cirz et al. 2006). These inhibitory concentrations of ciprofloxacin create selection pressure in favor of mutants with increased *ampC* expression (Wolter et al. 2007), while sub-inhibitory levels of ciprofloxacin or ofloxacin enhance the mutation frequency for carbapenem (esp., meropenem)-selected carbapenem resistance (Tanimoto et al. 2008).

Several mutants with increased or decreased ciprofloxacin susceptibilities were identified through random transposon insertion libraries, highlighting complex ciprofloxacin resistomes (Brazas et al. 2007; Breidenstein et al. 2008). Among the ciprofloxacin mutants with increased susceptibility (revealing genes involved in intrinsic resistance) were an exceptionally high number of mutants with mutations involving DNA replication and repair (Brazas et al. 2007; Breidenstein et al. 2008). Among the most unusual were mutants with alterations in *ftsK* encoding DNA segregation ATPase (Breidenstein et al. 2008) and *lon* encoding ATP-dependent intracellular protease, respectively (Brazas et al. 2007; Breidenstein et al. 2008). The *ftsK* mutants also showed increased susceptibility to the β-lactams described above (Alvarez-Ortega et al. 2010). Lon modulates SOS response and consequently ciprofloxacin susceptibility (Breidenstein et al. 2012).

Aminoglycosides

Aminoglycosides bind to the 30S ribosomal subunit and interfere with protein synthesis, causing mistranslation and ultimately cell death without lysis (Davis 1987). Anti-pseudomonas aminoglycosides (e.g., amikacin, gentamicin, and tobramycin) can therefore be used in the treatment of *Pseudomonas aeruginosa* infections (Poole 2005). Aminoglycoside uptake and subsequent action within bacterial cells is a complex process that involves lipopolysaccharide binding and outer membrane permeation, cytoplasmic membrane (CM) traversal driven by membrane potential, and ribosome disruption, leading to the production of membrane-damaging mistranslated polypeptides (Davis 1987; Krahn et al. 2012). The antagonism of aminoglycosides by divalent cations Mg^{2+} and Ca^{2+} is well documented in *P. aeruginosa*, which requires a MexXY multidrug efflux system (Mao et al. 2001; Morita et al. 2012b).

Aminoglycoside resistance typically results from the acquisition of aminoglycosides-modifying enzymes (AMEs) the genes for which are typically found on MBL-containing integrons in multidrug resistant *P. aeruginosa* (Poole 2011). Upregulation of the MexXY multidrug efflux system, in addition to AME acquisition, leads to *P. aeruginosa* developing high levels of aminoglycoside resistance (Morita et al. 2012b). Extremely high resistance to all anti-pseudomonas aminoglycosides is also promoted by 16S rRNA methylases (RMTs), recently discovered in *P. aeruginosa* but rarely observed (Poole 2011). This is not, however, the case for cystic fibrosis (CF) patients of isolates, where the mechanism are almost unknown but the most commonly observed aminoglycoside adaptive resistance phenotype of *P. aeruginosa* is impermeability phenotype and up-regulation of the MexXY multidrug efflux pump is predominant (Poole 2011). In wild type *P. aeruginosa* cells, the MexXY efflux system is inducible with sub-inhibitory concentrations of aminoglycoside- and ribosome-targeting antimicrobials (e.g., chloramphenicol and tetracycline), which are involved in the gene product PA5471 (recently dubbed ArmZ for anti-repressor MexZ (Hay et al. 2013)) (Morita et al. 2006b). The PA5471 system is also inducible

through interference, with translation of the leader peptide PA5471.1 (Morita et al. 2009). Overall the MexXY system has been increasingly recognized as the favored determinant of aminoglycoside resistance (Morita et al. 2012a).

P. aeruginosa possesses and exploits a great number of genes (at least 135) the inactivation of which contributes to low-level aminoglycoside resistances through genome-wide analysis (Schurek et al. 2008). These genes were involved predominantly in energy metabolism, which is reasonable when considering that aminoglycoside uptake by bacteria is energy-dependent (Schurek et al. 2008, p. 5). In previous studies, most of these mutant strains did not exhibit growth defects under the conditions tested, although some exhibited a small-colony phenotype and/or growth defects under anaerobic conditions (Schurek et al. 2008), Several additional genes involved in LPS biosynthesis which contribute to outer membrane permeability were also identified (Schurek et al. 2008). Schurek's results are consistent with previous reports on both impermeability and adaptive aminoglycoside resistance (Poole 2005). Furthermore, the deletion of such genes compromised the high level pan-aminoglycoside resistance of clinical isolates, emphasizing their importance to acquired resistance (Krahn et al. 2002).

Genome-scale identification of intrinsic aminoglycoside resistance in *P. aeruginosa* has also been performed (Lee et al. 2009; Struble and Gill 2009; Gallagher et al. 2011; Krahn et al. 2002). Although there are many newly identified intrinsic aminoglycoside determinants, the most noteworthy include an AmgRS two-component system, potassium, phosphate transporters (Trk and Pts systems), lipid biosynthesis or metabolism (LptA and FaoA), and membrane protein folding (PpiD) (Lee et al. 2009; Struble and Gill 2009; Gallagher et al. 2011; Krahn et al. 2012). The AmgRS two-component system was shown to control an adaptive response to membrane stress, which can be caused by aminoglycoside-induced translational misreading, in order to enhance aminoglycoside susceptibility in planktonic cells and biofilm (Lee et al. 2009; Krahn et al. 2012). Interestingly, mutational activation of AmgRS was recently found in pan-aminoglycoside resistant *P. aeruginosa* clinical isolates (Lau et al. 2013). AmgRS strongly regulated the expression of several genes involved in proteolysis (*htpX, nlpD,* and *yccA*), membrane transport (*yegH, ygiT,* and *sugE*) and other functions (*yebE*, PA5528, *yceJ*), but not the MexXY multidrug efflux pump (Lee et al. 2009). The combined effects of three genes (*yccA, htpX,* and PA5528) largely accounts for AmgRS-controlled resistance (Hinz et al. 2011). The first, *yccA*, encodes a protein whose homologue modulates the activity of membrane protease FtsH; the second, *htpX*, encodes a membrane protease itself; and the third, PA5528, encodes a membrane protein of unknown function, indicating that proteolysis plays a central part in aminoglycoside resistance (Hinz et al. 2011). The membrane protease FtsH was shown to be particularly important to *P. aeruginosa*'s aminoglycoside resistance (Hinz et al. 2011). The network of proteases provides robust protection from aminoglycosides and other substances through the elimination of membrane-disruptive mistranslation products (Hinz et al. 2011).

Transcriptomic analyses confirm that aminoglycosides impact the expression of a myriad of genes (Kindrachuk et al. 2011). While prolonged exposure to subinhibitory concentrations of tobramycin caused increased levels of expression pre-

dominantly of the *mexXY* efflux pump genes, the greatest increases in gene expression levels in response to lethal concentrations of tobramycin involved a number of *P. aeruginosa*'s heat shock genes (e.g., *htpG*, *ibpA*, *groES,* and *asrA*) (Kindrachuk et al. 2011). Under these conditions, the likely intracellular ATP-dependent AsrA protease is noteworthy because of a modest positive impact on aminoglycoside resistance (Kindrachuk et al. 2011). The Lon protease is also inducible by aminoglycoside (Marr et al. 2007).

Sub-inhibitory concentrations of aminoglycosides, especially tobramycin, induce biofilm formation in *P. aeruginosa* (Hoffman et al. 2005). Aminoglycoside response regulator (Arr) is predicted to encode an inner membrane phosphodiesterase whose substrate is cyclic di-guanosine monophosphate (c-di-GMP). A bacterial second messenger that regulates cell surface adhesiveness is essential for this induction and contributes to biofilm-specific aminoglycoside resistance (Hoffman et al. 2005). *ndvB* and PA1874-1877 genes involved in biofilm-specific resistance to aminogylycosides and other compounds (e.g., fluoroquinolone) have also been reported (Mah et al. 2003; Zhang and Mah 2008). The *ndvB* gene is involved in the production of highly glycerol-phosphorylated β-(1→3)-glucans, which bind to aminoglycosides (Sadovskaya et al. 2009) and affects the expression of multiple genes in biofilms. Ethanol oxidation genes are also linked to biofilm-specific antibiotic resistance (Beaudoin et al. 2012)

Polymyxins

Owing to the increased prevalence of multidrug-resistant *P. aeruginosa,* polymyxin B and colistin (also called polymyxin E), belonging to a family of antimicrobial cyclic oligopeptides, have returned to favor as a last resort treatment option, although these agents have strong side effects (e.g., nephrotoxicity) with high incidence (Poole 2011). The mechanism of polymyxins involves an initial stage of interaction with the lipid A of lipopolysaccharides (LPS), leading to self-promoted uptake across the membrane, followed by cell death (Zhang et al. 2000; Fernandez et al. 2013). The most common resistance mechanism to polymyxin has been shown to arise from substitution of LPS lipid A with 4-amino-L-arabinose *in vitro* and in cystic fibrosis isolates, while other unknown mechanisms remain under investigation (Poole 2011; Miller et al. 2011; Moskowitz et al. 2012). This modification is carried out by the products of the *arnBCADTEF-ugd* operon, otherwise known as *pmrHFIJKLM-ugd* (McPhee et al. 2003; Yan et al. 2007). Expression of the *arn* operon is activated by a series of two-component systems (involving at least PhoPQ, PmrAB, ParRS, and CprRS) after recognizing environmental signals including low Mg^{2+} (PhoPQ and PmrAB) or cationic peptides (ParRS and CprRS) (Macfarlane et al. 2000; McPhee et al. 2003; Fernandez et al. 2010, 2012), or when mutations in *phoQ*, *pmrB*, and *parR* promote low to moderate polymyxin resistance (Miller et al. 2011; Moskowitz et al. 2012; Muller et al. 2011). The set of genes directly or indirectly responding to mutated ParRS included the *pmrAB* genes (Muller et al. 2011),

consistent with previous data that polymixin induced *pmrAB* gene expression to a weaker extent than did other antimicrobial catioinic peptides or low Mg^{2+} (McPhee et al. 2003). Polymixin B resistance in *P. aeruginosa* is fairly limited and includes LPS-related genes (*galU*, *lptC*, *wapR,* and *ssg*) (Fernandez et al. 2013). Recently, polymyxin resistance in *P. aeruginosa phoQ* mutants was shown to be dependent on additional two-component systems: ColRS and CprRS. The addition of 4-amino-L-arabinose to lipid A is not the only mechanism to acquire this resistance, (Gutu et al. 2013), although it results in a less virulent but more inflammatory phenotype (Gellatly et al. 2012).

Approximately 0.5% of genes showed significantly altered expression in the sub-inhibitory concentration of colistin (Cummins et al. 2009), which is no less dramatic than the other anti-pseudomonas agents (e.g., ceftazidime, ciprofloxacin, and tobramycin) described above. The most noticeable alternation was upregulation of Pseudomonas quinolone signal (PQS) biosynthetic genes such as the *pqsABCDE* operon, the phenazine biosynthetic operon, or the *arn* operon (Cummins et al. 2009).

Development of colistin tolerance was observed in spatially distinct sub-populations of metabolically active *P. aeruginosa* cells on a biofilm, unlike those exhibiting low metabolic activity and dependence on the *arn* and efflux pump operons (*mexAB-oprM*, *mexCD-oprJ*, and *muxABC-opmB*) (Pamp et al. 2008; Chiang et al. 2012). MuxABC-OpmB, originally characterized as a multidrug efflux pump only in mutants lacking the major RND multidrug efflux pumps (Mima et al. 2009), was shown to contribute to intrinsic carbenicillin resistance and twitching motility of *P. aeruginosa* cells (Yang et al. 2011).

Macrolides

Macrolides such as erythromycin and azithromycin are widely used antibiotics which block translation by binding to the 50S ribosomal subunit. While *P. aeruginosa* cells are intrinsically very resistant to macrolides in standard broths such as Mueller-Hinton, low-dosage macrolides such as azithromycin are effective treatments in patients with chronic lung infections (Kudoh et al. 1998; Jaffe et al. 1998). Macrolides (e.g., azithromycin, erythromycin, clarithromycin) even far below their MICs (e.g., 2 μg/ml of azithromycin) exhibit exposure-dependent bactericidal effects to inhibit protein and autoinducer synthesis, leading to a reduction in virulence factor production (Tateda et al. 1996, 2001). Genome-wide approaches uncover the quorum-sensing antagonistic activities of azithromycin (i.e., inhibition of quorum sensing, reduction of virulence factor production and strong induction of type III secretion systems) (Nalca et al. 2006). This modulation causes downregulation of MexAB-OprM efflux pump expression in *P. aeruginosa* (Sugimura et al. 2008). Sub-MIC levels of azithromycin does not affect mRNA expression of quorum-sensing-related genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *vft*, and *rsaL*), but lowers expression of most N-acylhomoserine lactone (AHL) synthesis enzymes, which act upstream

of *lasI* or *rhlI* (Kai et al. 2009). Azithromycin additionally inhibits expression of the small RNAs RsmY and RsmZ, dependent on the GacA/Rsm signal transduction pathway which positively controls *P. aeruginosa* quorum sensing (Perez-Martinez and Haas 2011).

Both effects of azithromycin on quorum-sensing dependent virulence factor production and cell death requires azithromycin to interact with ribosomes (Kohler et al. 2007). The stationary-phase killing of azithromycin is further enhanced by the production of rhamnolipids, which likely facilitate macrolide uptake (Kohler et al. 2007). The mode of action of azithromycin *in vivo* is also described through mutations in 23S rRNA conferring azithromycin resistance to cystic fibrosis patients chronically infected with *P. aeruginosa* (Marvig et al. 2012). The clinical efficacy of macrolides in treating pseudomonal infections can be partially explained by the increased susceptibility of *P. aeruginosa* to these compounds in eukaryotic cell culture media and biological fluids, due to decreased *oprM* expression of and increased outer-membrane permeability (Buyck et al. 2012).

Biocides

P. aeruginosa has also been reported to contaminate disinfectants (e.g., chlorhexidine, benzalkonium, and triclosan) in hospital or other such environments, thereby compromising their ability to reduce or eliminate bacterial contamination. Chlorhexidine and benzalkonium are a cationic biguanide and a nitrogen-based quaternary ammonium compound, respectively. They function by affecting the cell membrane, resulting in lysis and the loss of cytoplasmic material (Morita et al. 2014). The RND-type MexCD-OprJ multidrug efflux pump is inducible at sub-inhibitory concentrations of disinfectants such as benzalkonium chloride or chlorhexidine (Morita et al. 2003), dependent upon the AlgU stress response factor (Fraud et al. 2008). Global transcriptome response to chlorhexidine included upregulation of the *mexCD-oprD* and *oprH-phoPQ* operons and downregulation of membrane transport, oxidative phosphorylation electron transport and DNA repairs (Nde et al. 2009). The variant highly adapted to benzalkonium showed increased resistance to fluoroquinolones, owing to mutations in the quinolone resistance-determining region of *gyrA* and repressor genes (*mexR* and *nfxB*) of *mexAB-oprM* and *mexCD-oprJ*, respectively (Mc Cay et al. 2010). Development of chlorhexidine-tolerant sub-populations on the *P. aeruginosa* biofilm was also dependent on the *mexCD-oprJ* genes (Chiang et al. 2012).

Triclosan specifically inhibits fatty acid synthesis through inhibition of bacterial enoyl-acyl carrier protein reductase, although *P. aeruginosa* is intrinsically highly resistant to triclosan due to FabV (triclosan resistant enoyl-acyl carrier protein reductase) and active efflux (Zhu et al. 2010). This innate resistance stems from at least five RND efflux pumps including MexAB-OprM (Zhu et al. 2010; Mima et al. 2007). In *P. aeruginosa* mutant cells lacking the *mexAB-oprM* genes, sub-inhibitory concentrations of triclosan led to alterations in almost half the genome, with 28%

of genes being significantly upregulated and 16% being significantly downregulated (Chuanchuen and Schweizer 2012). Quorum sensing-regulating genes were among the most strongly downregulated, and surprisingly, iron homeostasis was completely blocked in triclosan-exposed cells, thus mimicking conditions with excess iron (Chuanchuen and Schweizer 2012).

Further Prospects

Our findings regarding mode of action, resistance mechanisms, and global responses to antimicrobial agents paves the ways to conquer *P. aeuginosa* infections. We are aware of potential molecular targets for novel genomic anti-pseudomonas agents, including essential gene products (Morita et al. 2010). However, we first need to address the problems of low membrane permeability and multidrug efflux pumps in *P. aeruginsoa* to develop these novel agents. While many labs, including our own, are currently screening, so far there are no efflux pump inhibitors available for clinical settings and screening novel antibacterial agents, including an efflux inhibitor, is currently in progress in many labs including ours.

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Chapter 8
Lessons from the QSCR Structure for Quorum Sensing

Christina L. Wysoczynski and Mair E.A. Churchill

Abstract *Pseudomonas aeruginosa*, along with various other gram-negative bacterial species, utilizes the process of quorum sensing to sense the surrounding environment and respond accordingly, which ultimately promotes its survival and virulence in human hosts. The small, lipid-diffusible signaling molecules known as acyl-homoserine lactones (AHLs) play an integral role in regulating the quorum sensing system in *P. aeruginosa*. 3OC12-HSL is recognized by the AHL receptors LasR and QscR. The crystallographic structure of full-length QscR revealed a unique, symmetrical criss-crossed homodimer that is poised to bind DNA. Thus, QscR is the only full-length quorum sensing receptor bound to its endogenous signaling ligand (3OC12-HSL) that has been captured in the activated state. This chapter discusses the lessons learned from the structural studies of QscR, which have provided valuable insights into the varied mechanisms that QscR and other AHL receptors, including *P. aeruginosa* LasR, may use to respond to AHLs.

Keywords Quorum sensing · *Pseudomonas aeruginosa* · Transcription factor · QscR

Quorum Sensing Introduction

Many bacterial species possess the intrinsic capability to intercept and interpret information from their surroundings, either from other living organisms or nonliving environmental factors, and respond accordingly to the proximate cues. Bacteria use the signaling process of quorum sensing to elicit and coordinate a group re-

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sponse, typically at high bacterial cell densities; the specific responses promote a synergistic behavior that benefits the community of cells that cannot be achieved if bacteria were acting as individuals. To date, more than one hundred gram-negative bacterial species have been identified to use the quorum sensing process to coordinate their behaviors (Fuqua and Winans 1994; Lerat and Moran 2004; Smith and Iglewski 2003), including symbiosis and colonization, as well as pathogenesis (Churchill and Chen 2011; Davies et al. 1998; Fuqua et al. 1996; Manefield and Turner 2002; Nickel et al. 1985; Rutherford and Bassler 2012; Stevens et al. 2012). As many human pathogenic bacteria use quorum sensing to regulate their virulence, understanding the molecular mechanisms underlying quorum sensing and how quorum sensing regulates bacterial virulence will yield new therapeutic options for combating infectious diseases in humans (Davies et al. 1998; Galloway et al. 2012; Gambello et al. 1993; Geske et al. 2005; Jakobsen et al. 2013; Kalia 2013; Passador et al. 1993; Rumbaugh et al. 1999; Smith et al. 2003; Stevens et al. 2011; Tang et al. 1996).

The study of the behaviors of gram-negative Proteobacteria group has led to a better understanding of the quorum sensing process. Specifically, understanding the origin of the fluorescent properties of *Vibrio harveyi* and *Vibrio fisheri*, a marine symbiont of the Hawaiian bobtail squid *Euprymna scolopes* (Joo and Otto 2012; Ruby 1996; Ruby and McFall-Ngai 1992), revealed fundamental properties of the "quorum sensing" mechanism of inter-cellular communication. When the bacteria exist as dense cell populations in the light organ of the squid, they emit light in response to quorum sensing (Boettcher and Ruby 1995; Fuqua et al. 1996; Rumbaugh et al. 1999; Tang et al. 1996; Whiteley et al. 2001). Small lipid-diffusible signaling molecules known as autoinducers, or acyl-homoserine lactones (AHLs) were later discovered to serve as the mediators of this quorum sensing process (Eberhard et al. 1981; O'Toole and Kolter 1998; Ruby 1996; Ruby and McFall-Ngai 1992). Although there are other signaling molecules used for quorum sensing in a wide variety of bacteria (reviewed in (Galloway et al. 2011; Rutherford and Bassler 2012), this chapter focuses on the AHLs because of their important role in *P. aeruginosa.*

AHL-mediated quorum sensing not only results in light emission as a result of bacterial communication, but it is used in a variety of ways by pathogens and symbionts of many eukaryotic organisms, such as plants and humans (Dunny et al. 1995; Keller and Surette 2006; Latifi et al. 1995; Miyashiro and Ruby 2012; Pirhonen et al. 1993; Stevens et al. 2012). Pathogens promote group-mediated responses related to infections in agriculture and patients that can be detrimental in crop production and infectious diseases of humans (Fuqua and Winans 1994; Ham et al. 2011; Jimenez et al. 2012; Kalia 2013; Manefield and Turner 2002; Smith and Iglewski 2003). In particular, the human opportunistic pathogen *Pseudomonas aeruginosa* utilizes the process of quorum sensing to regulate the formation of biofilms that encapsulate sessile bacterial cells at high cell densities (Fig. 8.1). These biofilms, which are made of exo-polysaccharide matrices, further complicate the *P. aeruginosa* infections of cystic fibrosis patients and immune-compromised patients due

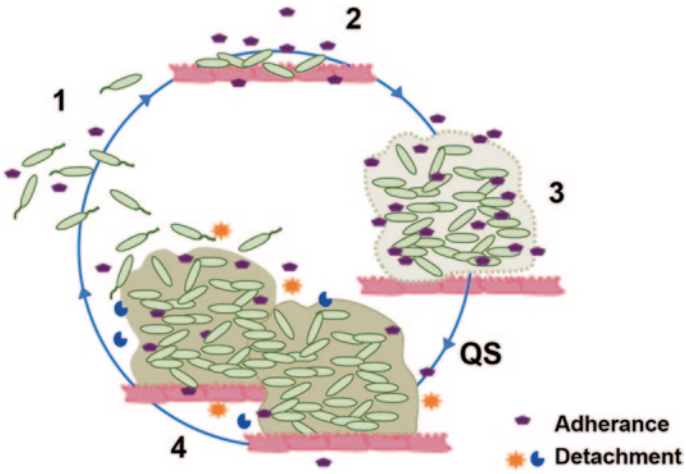

Fig. 8.1 Biofilm formation in *P. aeruginosa*. [*#1*] Motile planktonic bacteria are inhaled or ingested. [*#2*] Attachment of bacterial cells to a biotic surface through protein-protein interactions or to an abiotic surface through hydrophobic interactions. [*#3*] Bacterial cell maturation and initial biofilm formation and secretion of adherence factors. [*#4*] Mature biofilm communities form, and detachment of bacterial cells occurs to allow for recolonization of new biofilm colonies. Detachment factors such as proteases are secreted to release motile bacterial cells into the environment

to the increased drug resistance they exhibit and host tissue damage they promote (Churchill and Chen 2011; Cooley et al. 2008; Davies et al. 1998; Fuqua et al. 1996; Nickel et al. 1985; Rumbaugh et al. 1999; Tang et al. 1996).

Upon initial contact of *P. aeruginosa* with a suitable adherent surface, such as the mucosal tracts in the trachea and lung cavities of cystic fibrosis patients, the bacterial cells suppress expression of genes involved in virulence that trigger host immune responses (Davies et al. 1998; Drescher et al. 2013; Gambello et al. 1993; Passador et al. 1993; Ruby 1996; Ruby and McFall-Ngai 1992; Sauer et al. 2002). This initial phase of biofilm formation is denoted as (#1 in Fig. 8.1). Down regulation of virulence factors at the early stages of infection allows for cell growth and proliferation of *P. aeruginosa* without being detected by immune surveillance systems. After attachment and adaptation to the surrounding environment through protein-protein interactions and hydrophobic interactions on abiotic surfaces, the cells divide and establish a sessile community, losing their flagella (#2 in Fig. 8.1; Boettcher and Ruby 1995; Fuqua et al. 1996; Joo and Otto 2012; Rumbaugh et al. 1999; Tang et al. 1996). Subsequently, when the bacterial cells have reached a certain cell density, or number of cells that have the potential to overwhelm the host immune system, *P. aeruginosa* adopts a virulent phenotype that subsequently forms biofilms, damages surrounding host tissues, and promotes persistent infections in the host (#3 in Fig. 8.1; Cooley et al. 2008; Eberhard et al. 1981; Ruby 1996; Ruby

and McFall-Ngai 1992; Whiteley et al. 2001). The process of gene regulation that transforms *P. aeruginosa* as cell densities accumulate is initiated by the quorum sensing process. As the many cells adapt to their new conditions, the biofilm communities become quiescent and adopt a more dormant lifestyle until new colonization is needed and cells with flagella are released back into the surrounding environment (#4 in Fig. 8.1; Daniels et al. 2004; Dunny et al. 1995; Latifi et al. 1995; O'Toole and Kolter 1998; Pirhonen et al. 1993). This process comes full circle as the bacterial cells can be released as planktonic cells into the surrounding environment (Kostakioti et al. 2013).

AHL Signaling Molecules

AHLs are responsible for promoting quorum sensing and allowing cells to perceive cues from the surrounding environment in many gram negative bacteria (Cao and Meighen 1989; Eberhard et al. 1981; Engebrecht et al. 1983; Kaplan and Greenberg 1985). The chemical composition of an acyl homoserine lactone, and structures of AHLs described in this chapter, are depicted in Fig. 8.2a. A common feature of all AHLs is the characteristic homoserine lactone ring, which is conjugated to a fatty acid chain of differing carbon lengths, denoted as the second R group (R2) through an amide linkage. The lactone ring of an AHL contains a chiral carbon. Further examination of the isomeric functions of AHLs revealed that the L-enantiomer is biologically active in quorum sensing systems (Ikeda et al. 2002; Pomini and Marsaioli 2008). However, the racemic mixture of L- and D- stereoisomers of AHLs can exist upon chemical synthesis (Ikeda et al. 2002). The third carbon of the fatty acyl chain, considered as the first R group (R1), can be substituted with a hydrogen, oxo, or hydroxyl moiety. Thin-layer chromatography techniques coupled with mass spectrometry have advanced the sensitivity and accuracy of AHL detection (Chandler et al. 2009; Duerkop et al. 2008; Gould et al. 2006; Khan et al. 2007; Kirwan et al. 2006; Shaw et al. 1997; Zan et al. 2012), and have not only have greatly increased the number of bacterial species known to produce AHLs, but have revealed the exquisite specificity of some species in AHL production. Additionally, it has recently been discovered that the carbon chain of an AHL (R2 group) can be substituted with moieties such as phenyl rings or branched-chain alkanes (Lindemann et al. 2011; Schaefer et al. 2008; Thiel et al. 2009).

Upon diffusion into neighboring cells (Pearson et al. 1999), the AHL signaling molecules bind to their cognate quorum sensing receptors. In some systems, the AHLs can be detected by cell-surface receptors (Swem et al. 2008), but in *P. aeruginosa* they are detected by the intracellular DNA binding receptors (Fukushima et al. 1994; You et al. 1996). In turn, these cognate receptors bind to promoters that regulate sets of genes that generate positive feedback loops to activate the transcription of synthase proteins and further enhance the quorum sensing process (Swartzman et al. 1992).

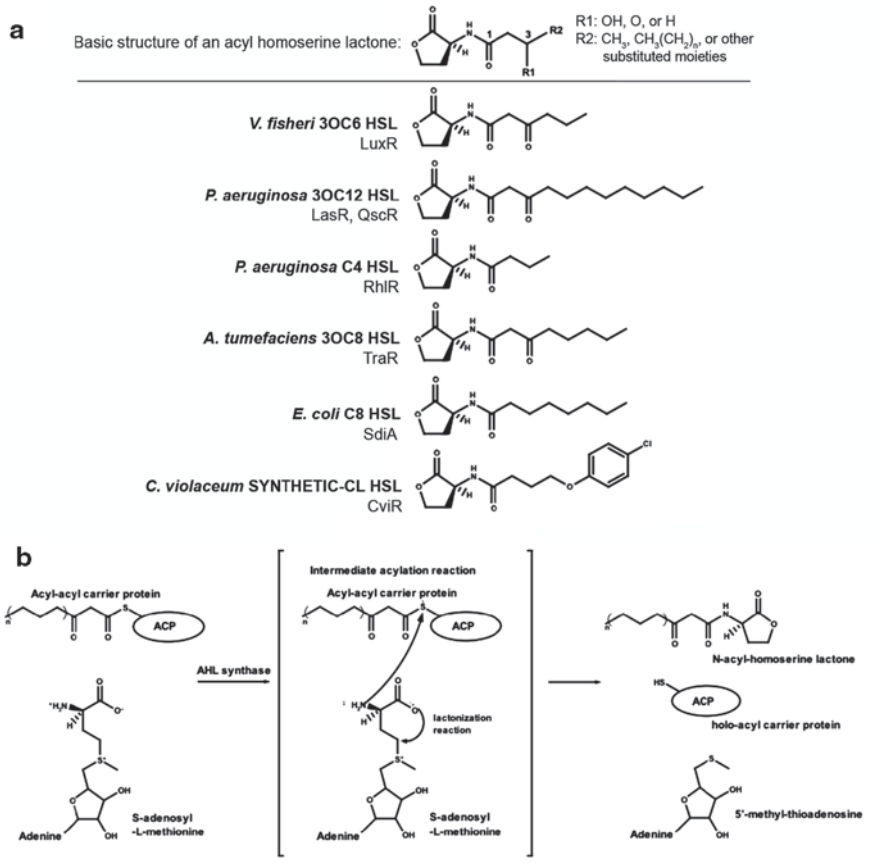

Fig. 8.2 Acyl-homoserine lactones. **a** Depiction of AHLs found in different QS systems discussed in this chapter. The first position of the acyl chain contains a carbonyl group, and the carbon at the third position can be either unsubstituted or have an oxo or hydroxyl group. The fatty acyl chain also can contain different moieties, such as branching and unsaturation, which are found in the different AHLs mentioned in this review. **b** The AHL synthesis process. AHL synthases facilitate the addition of the acyl chain of an acyl-acyl carrier protein to S-adenosyl methionine (SAM) and subsequent lactonization of the SAM to form the AHL. The byproducts of the reaction are holo-acyl carrier proteins and 5′-methyl-thioadenosine

AHL Synthases

Pioneering studies of the *V. fisheri* quorum sensing system, known as the *lux* system, have laid the groundwork for the general understanding and current nomenclature of the components of quorum sensing systems. In the *lux* system, the enzyme that synthesizes the autoinducer AHL N-(ß -ketocaproyl)-L-homoserine lactone, or

3OC6-HSL (Fig. 8.2b), is known as LuxI (Schaefer et al. 1996b). The receptor that recognizes 3OC6-HSL, known as LuxR, is an AHL-sensitive transcription factor that binds to the promoter region of the *luxICDABEG* operon to activate genes that are pertinent for the quorum sensing system in *V. fisheri* (Engebrecht et al. 1983). Originating from the *lux* system, the term "I" proteins and "R" proteins correspond to the inducers and receptors of the quorum sensing system. The LuxI/LuxR nomenclature has been used to designate other synthase and receptor proteins used in quorum sensing systems in more than 150 species of Proteobacteria (Lerat and Moran 2004).

Each quorum system typically has distinct genes for the AHL synthases. Some species have more than one quorum sensing system, and thus make more than one type of AHL, which further fine tunes and regulates the response of the bacteria to these cell density-dependent communication systems. Generally speaking though, these quorum sensing systems include cognate pairs of AHL synthases and AHL receptor proteins (Pearson et al. 1994).

The LuxI-type enzymes in quorum sensing systems make use of the substrates S-adenosyl-L-methionine (SAM) and an acyl carrier protein that is conjugated to a fatty acyl chain of variable carbon lengths (acyl-ACP) to synthesize AHL molecules (Moré et al. 1996; Parsek et al. 1999). SAM contains a nucleophilic amino group that attacks the C1 carbonyl carbon of the acyl chain that is conjugated to the acyl carrier protein carrying the phosphopantetheine prosthetic moiety through a thioester linkage (Watson et al. 2002). Figure 8.2a displays the general pathway for an AHL synthesis reaction used by the LuxI-type synthases. The AHL synthesis process has also been confirmed using an *in vivo* system based on the TraI-TraR system with purified substrates in *A. tumefaciens*, and *in vitro* studies using RhlI in *P. aeruginosa* (Parsek et al. 1999; Pearson et al. 1994, 1995).

The crystallographic structures of EsaI and LasI have helped to confirm the enzymatic process of AHL synthesis through analysis of the active site binding pockets that recognize SAM and acyl-ACP (Gould et al. 2004a, b; Moré et al. 1996; Parsek et al. 1999; Watson et al. 2002). Interestingly, the structures of the AHL synthases EsaI and LasI also revealed an uncanny resemblance to the histone acetyltransferase family of enzymes (Churchill 2006; Gould et al. 2004b; Watson et al. 2002), which is consistent with a proposed common mechanism for acylation reaction (Churchill 2006; Parsek et al. 1999; Watson et al. 2002). In the AHL synthase binding pocket, SAM and the acyl-ACP react to produce the particular AHL signaling molecules, and in some cases the AHL is highly specific to each AHL synthase (Watson et al. 2002). For example, TofI, a quorum sensing synthase from the *Burkholderia glumae,* is responsible for the production of C8-HSL (Chung et al. 2011). The recent structure of TofI binding to a synthetic modulator, the synthetic antagonist known as C8-J8, reveals a mechanism for blocking the binding of the acylated acyl-carrier protein to impede AHL synthesis and ultimately halt the quorum sensing process.

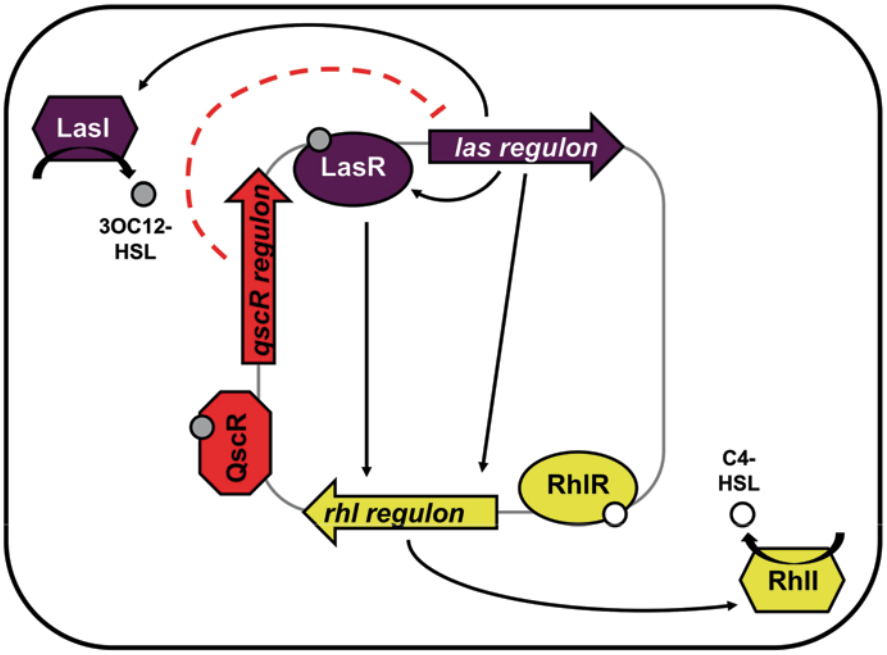

Fig. 8.3 Simplified model of the AHL dependent QS circuitry in *P. aeruginosa*. LasI and RhlI synthesize the signaling molecules 3OC12-HSL and C4-HSL, respectively. LasR subsequently binds to DNA after 3OC12-HSL recognition and regulates sets of genes that control the *rhl* regulon, the sets of genes controlled by RhlR. LasR directly regulates some of the *rhl* genes as well. RhlR recognizes C4-HSL and subsequently binds DNA. The and *las* and *rhl* regulons include the genes for the AHL synthases LasI and RhlI, which in turn make more signaling molecules. QscR does not have a cognate synthase, but it recognizes 3OC12-HSL and can bind to DNA to regulate the genes of the *qscR* regulon that exerts some control over the *las* regulon

Quorum Sensing Machinery in *Pseudomonas Aeruginosa*

The quorum sensing machinery in *P. aeruginosa* is more complex than those found in many other Proteobacteria (Fig. 8.3). *P. aeruginosa* bacteria use two main lipid-diffusible AHLs known as N- (3-oxododecanoyl)- L-homoserine lactone (3OC12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL) to communicate and sense neighboring bacterial cells (Fig. 8.2a; Pearson et al. 1994, 1995). Specifically, the AHL synthase enzymes LasI and RhlI produce 3OC12-HSL and C4-HSL, respectively (Parsek et al. 1999; Pearson et al. 1994, 1995). AHL binding to the AHL receptors LasR and RhlR, promotes the interactions with their particular DNA binding sites, putative promoters and operators, to regulate genes involved in production of the virulence factors and biofilm formation (Gilbert et al. 2009; Whiteley and Greenberg 2001). 3OC12-HSL is recognized by the receptor LasR, which in turn binds to DNA and serves to activate many genes of the *las* system of genes known

as the *las* regulon, which is involved in the virulence of *P. aeruginosa* (Gilbert et al. 2009; Schuster et al. 2004). C4-HSL is recognized by the receptor RhlR, which in turn binds to DNA and regulates the *rhl* system of genes known as the *rhl* regulon, also involved in the virulence of *P. aeruginosa* (Latifi et al. 1995; Schuster and Greenberg 2007). The *las* system exerts dominance over the *rhl* system, and as such the *las* system is considered to lie at the top of the hierarchical system of quorum sensing in *P. aeruginosa* (Dekimpe and Déziel 2009; Latifi et al. 1996; Pesci et al. 1997; Seed et al. 1995). These two signals regulate two distinct but interdependent signaling networks that contribute to different quorum sensing circuitry systems (Gilbert et al. 2009; Schuster and Greenberg 2007; Schuster et al. 2003). In addition, these systems employ the production of virulence factors which also serve to damage host tissues (Ochsner and Reiser 1995; Pearson et al. 1997).

In addition to the *las* and *rhl* systems, which have cognate AHL synthases and receptors, a third AHL receptor was discovered in the *P. aeruginosa* quorum sensing circuitry that opposes the function of LasR and RhlR (Chugani et al. 2001; Fuqua 2006). The quorum sensing control repressor, or QscR does not have a cognate inducer protein and is thus considered an 'orphan receptor' (Chugani et al. 2001; Patankar and González 2009). QscR binds and responds to 3OC12-HSL, as well as other AHLs of longer chain lengths that *P. aeruginosa* does not even synthesize (Lee et al. 2006). Liganded QscR recognizes a specific *lux*-box DNA promoter sequence similar to the sequence recognized by LasR, and when bound to 3OC12-HSL the receptor facilitates the expression of unknown genes that collectively are responsible for the suppression of LasI and RhlI activity (Lequette et al. 2006; Oinuma and Greenberg 2011). Upon deletion of the QscR gene *in vivo*, *P. aeruginosa* becomes hypervirulent in a *Drosophila melanogaster* feeding and virulence model (Chugani et al. 2001). Many genes in the *qscR* regulon overlap with the *las* regulon (Lequette et al. 2006), and although QscR and LasR have opposing functions, these receptors have a similar mechanism of action to regulate gene expression through AHL and subsequent DNA binding.

Structure Function Analysis of AHL-Receptors

LuxR

LuxR from *V. fisheri* was the first AHL-dependent transcription factor to be identified. Initial structural analyses of LuxR revealed that the receptor has two characteristic domains: the N-terminal ligand binding domain (LBD) recognizes 3OC6-HSL and the C-terminal DNA binding domain (DBD) binds to the *lux* promoter sequence of DNA (Hanzelka and Greenberg 1995). The binding site is known as the *lux*-box, the term used to describe the DNA binding sites in the *V. fisheri* quorum sensing system (Devine et al. 1989). In *V. fisheri*, the *lux* box contains a 20 base-pair near palindromic region of DNA that is a *cis*-acting promoter upstream of the *lux* op-

eron (Engebrecht and Silverman 1987; Fuqua and Winans 1996). The near dyad symmetry suggests that the C-terminal DNA binding domain of LuxR recognizes DNA as a dimer (Antunes et al. 2008). Overexpression of the N-terminal AHL binding domain resulted in inactive multimers containing the full length and truncated receptors in the presence of 3OC6-HSL, suggesting that the full-length receptor dimerizes in the N-terminal region in the presence of an activating ligand (Hanzelka and Greenberg 1995). Signal recognition (3OC6-HSL) is reversible, giving *V. fisheri* a possible detection mechanism of cell density (Urbanowski et al. 2004). As cell numbers in the environment decrease, signal concentrations decrease as well, and LuxR will then become inactivated and DNA binding is abolished (Urbanowski et al. 2004). Interestingly, when the DNA binding domain of LuxR is expressed in cells, transcription can occur with the truncated protein (Choi and Greenberg 1991). This suggests that the DNA binding region can make sufficient contacts with the RNA polymerase to form a functional transcription complex to activate genes (Choi and Greenberg 1991; Egland and Greenberg 2000; Trott and Stevens 2001). The full-length structure of LuxR is not known, but the data gathered from the analyses of the receptor have given insight into the structures and functions of *P. aeruginosa* QscR, LasR, and *Chromobacterium violaceum* CviR and how each receptor is subsequently activated upon AHL recognition.

LuxR-Like AHL Receptors

In addition to the studies of LuxR that have served to characterize this class of receptors, there are studies of several other AHL-receptors that have shaped the current understanding of their structure and function. *Agrobacterium tumefaciens* TraR (Luo and Farrand 1999; Qin et al. 2000), *C. violaceum* CviR (Stauff and Bassler 2011), together with *P. aeruginosa* LasR (Kiratisin et al. 2002) and QscR (Lee et al. 2006) are examples of cytosolic transcription factors that are generally activated upon AHL binding. Although these AHL receptors share only around 30% amino acid identity, they all function by recognizing AHLs resulting in subsequent DNA binding and regulation of their cognate genes. Based on findings with TraR (Qin et al. 2000; Zhu and Winans 2001), other AHL receptors are generally thought to multimerize and bind to a *lux*-box-like sequence of DNA. This can either positively regulate genes to promote quorum sensing or serve to influence genes that inhibit quorum sensing.

The LuxR-like receptors have a characteristic LBD and DBD, as well as a flexible linker region connecting the LBD to the DNA binding domain (DBD) (Hanzelka and Greenberg 1995). Interestingly, each linker and C-terminal sequence varies in amino acid length with each type receptor, and it is hypothesized that these linkers and C-terminus play important roles in the structural conformations and functions of the receptors (Lintz et al. 2011; Vannini et al. 2004). Generally, LuxR-like receptors activate transcription in the presence of AHL, but it is important to note that there exist other AHL receptors that act in an opposite fashion when bound

to an AHL, such as EsaR, and that can act as repressors under certain circumstances (Bodman et al. 1998; Minogue et al. 2002; Schu et al. 2011; Tsai and Winans 2010).

QscR

P. aeruginosa QscR is the most recent AHL receptor to provide new and interesting insights into the function of the quorum sensing receptors. The activity of QscR was dissected using a variety of footprinting, gel-shift assays, and *in vitro* ligand binding studies to investigate its biochemical properties (Lee et al. 2006; Oinuma and Greenberg 2011). At the genetic level, studies of QscR using transcriptional profiling revealed that a distinct regulon under the control of QscR exists (Lequette et al. 2006). QscR regulates a gene set that has the ability to control quorum sensing at different levels of cell densities through either repression or activation of these genes (Lequette et al. 2006). QscR also is responsible for activating *P. aeruginosa* PAO1 PA1897, a gene of unknown function (Lee et al. 2006). Further analysis of the DNA region that is bound by QscR revealed very similar characteristics to the LasB promoter sequence that is recognized by *P. aeruginosa* LasR, except for a two base-pair difference in the DNA sequences (Lee et al. 2006). Gel shift assays confirmed that QscR distinctly recognizes the PA1897 promoter sequence and the difference of two base-pairs of DNA deter its binding to the LasB promoter site (Lee et al. 2006).

QscR has the ability to bind to certain promoter regions that are not recognized by LasR or RhlR, indicating that QscR exerts a specific control over the quorum sensing system; when QscR is overexpressed, higher levels of transcription initiates from the promoters that are recognized by QscR are evident, showing the role of being a limiting factor in the quorum sensing system of *P. aeruginosa*. Some genes controlled by QscR overlap with the *las* regulon, indicating the integral role of QscR to bind AHLs and influence extra genes to further enhance the regulation of quorum sensing in *P. aeruginosa* (Lequette et al. 2006).

QscR, like many quorum sensing receptors, cannot be purified in an exogenous system without the addition of an AHL. However, addition of 3OC12-HSL to an overexpression system in *E. coli* produced soluble protein amenable for *in vitro* studies of the QscR (Lee et al. 2006). One feature of QscR and other AHL receptors that tends to increase the stability and the solubility of the protein is homodimerization. At high concentrations in gel filtration analysis QscR dimerizes in the presence of 3OC12-HSL (Oinuma and Greenberg 2011). This is similar to LuxR that also dimerizes when activated, which facilitates subsequent DNA binding and activation of transcription (Kolibachuk and Greenberg 1993; Oinuma and Greenberg 2011). Interestingly, when QscR is truncated to the DBD alone, it can still bind DNA, but unlike LuxR, is does not activate transcription (Park et al. 2013).

QscR is a relatively promiscuous AHL receptor. Using a *ß-galactosidase* reporter assay set up in *E. coli*, QscR was found to recognize a range of AHLs other than 3OC12-HSL (Lee et al. 2006). QscR is activated by other long-chain AHLs such

as C12-HSL and C10-HSL, revealing that this receptor is capable of intercepting and responding to signals that are not native to *P. aeruginosa* (Lee et al. 2006). The affinity of QscR for the PA1897 promoter sequence in the presence of these different ligands, shows that AHLs with longer fatty acyl chains tend to have a tighter interaction with QscR (Oinuma and Greenberg 2011). Since 3OC6-HSL is a weaker binding ligand for QscR, the affinities of different acyl-chain HSLs were measured showing that QscR binds DNA in the presence of relatively low concentrations of 3OC12-HSL and C12-HSL (Oinuma and Greenberg 2011). The relative affinity of QscR for 3OC12-HSL is weaker than LasR, but QscR has gained the ability to recognize both 3-oxo and unsubstituted long-acyl chain AHLs and potentially serve a different role in the cell. These results suggest that QscR has a promiscuous mode of AHL recognition, and could serve as an interspecies communicator with other bacterial organisms (Ha et al. 2012).

Structural Insights from Analyis of QSCR

Overall Structural Conformation of the QS Receptors

It has proved difficult to purify and study AHL receptors, and therefore only a few structures for these receptors have been determined. The full-length crystal structures of *A. tumefaciens* TraR bound to DNA (Vannini et al. 2002; Zhang et al. 2002) and *C. violaceum* CviR (Chen et al. 2011) are known, and recently the *P. aeruginosa* QscR structure have been described (Fig. 8.4) (Lintz et al. 2011). It is important

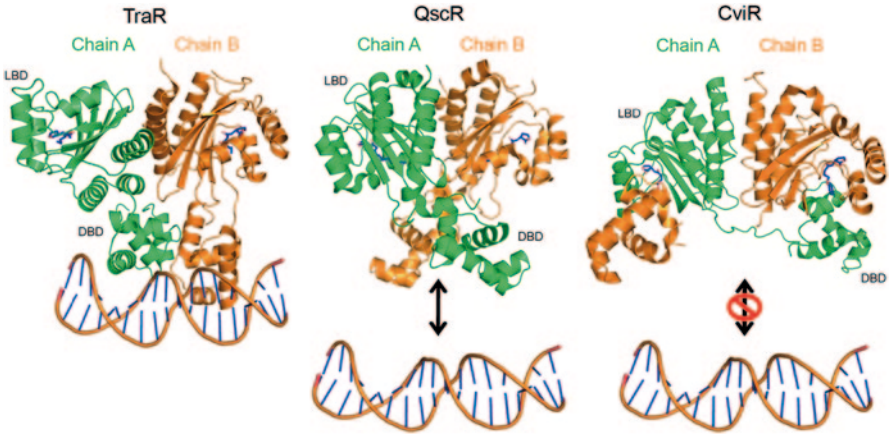

Fig. 8.4 Conformations of QS receptors from x-ray crystallography studies. The full-length receptor conformations of QscR (PDB ID: 3SZT), TraR (PDB ID: 1L3 L, 1HOM), and CviR (PDB ID: 3QP5) are shown with one subunit in *orange* and the other in *green*. The DBD regions of QscR and TraR are poised to bind DNA, whereas CviR adopts a conformation, when bound to an antagonist, which does not allow for DNA binding

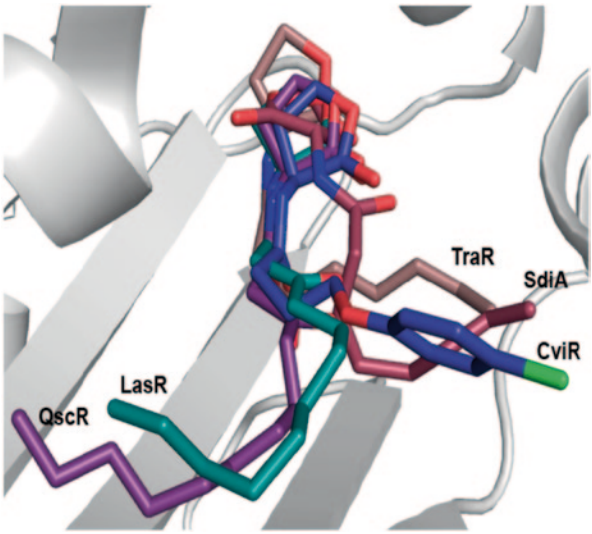

Fig. 8.5 Recognition of AHL molecules by different QS receptors. An overlay of the QS receptor binding pockets of TraR (PDB ID: 1L3 L, 1HOM) (Vannini et al. 2002; Zhang et al. 2002), SdiA (PDB ID: 2AVX) (Yao et al. 2006), CviR (PDB ID: 3QP5) (Chen et al. 2011), LasR (PDB ID: 2UVO) (Bottomley et al. 2007), and QscR (PDB ID: 3SZT) (Lintz et al. 2011) reveals different modes of AHL recognition between the classes of QS receptor classes. TraR, SdiA, and CviR recognize shorter chain AHLs that protrude from the binding pocket towards the aqueous environment. LasR and QscR recognize longer chain AHLs that are more hydrophobic and are sequestered into the binding pocket of the receptor serving to further stabilize the receptor

to note that the full-length structure of CviR is bound to a synthetic antagonist. Although the general form of the LBD and the DBDs are the same, these structures show an interesting range of domain configurations, dimerization interfaces, and degrees of interaction between the LBDs and DBDs.

The overall conformations attained from the crystallographic structures vary with each full-length receptor, with differences largely attributed to the placement of the LBD and DBD regions within the proteins. The overall conformations of TraR, QscR, and CviR are shown in Fig. 8.4. The structure of TraR is a non-symmetrical homodimer bound to DNA (Vannini et al. 2002; Zhang et al. 2002). The crystallographic structure of CviR represents a symmetrical, criss-crossed receptor that is inactive due to the binding of the antagonist N-4-chlorophenyoxybutanyl homoserine lactone (CL), which renders the receptor more stable but unable to bind DNA (Chen et al. 2011). The full-length structure of QscR bound to 3OC12-HSL reveals a symmetrical, criss-crossed homodimer, where each monomeric LBD is connected to the DBD through a short linker of ten amino acids (Lintz et al. 2011). The interface between the monomeric subunits of each LBD presents a unique mode of dimerization that is not observed in the structures of TraR (Vannini et al. 2002; Zhang et al. 2002), CviR (Chen et al. 2011), or SdiA (Yao et al. 2006). QscR has a mode of AHL recognition that is similar to the LBD of LasR, but is distinct from ligand binding observed in other QS receptors (Fig. 8.5).

Both full-length receptor structures of TraR and QscR are proposed to be in an activated state due to binding of the native activating ligands, 3OC8- and 3OC12-HSL respectively, that promote the function of the receptors such as DNA recognition for transcription (Zhang et al. 2002). The LBDs of TraR are asymmetrically configured relative to the palindromic DNA binding site. However, the DBDs are bound to the DNA with the expected dyad symmetry. QscR is not bound to DNA and has a symmetrical crossed structure, where the DBD of each monomer makes similar contacts with the LBD of the other monomer (Lintz et al. 2011). Furthermore, QscR recognizes a DNA sequence that is palindromic, and the overall symmetry of the receptor is consistent with a symmetric mode of binding DNA. In fact, the DBDs of QscR overlay with the DBDs of TraR with a small root-mean-square deviation (RMSD) value, indicating that the structural configurations of the two DBDs are folded very similarly (Lintz et al. 2011). Thus, QscR bound to 3OC12-HSL appears to be in the activated state and poised to bind DNA. On the other hand, the full-length structure of CviR is bound to the antagonist CL and the receptor is proposed to be in an inactivated state due to the DBDs being tucked away by the LBD through inter-subunit contacts (Chen et al. 2011).

The dimerization interfaces observed in the AHL receptor structures are much less similar than expected. The LBDs are all comprised of beta strands ß1–ß5 that form an alpha-ß-alpha sandwich together with alpha-helices α1–α5. The interface between the two LBDs of QscR is symmetrical, and upon comparison of the RMSD of the LBD interface with other known AHL receptors, QscR and LasR have the most similar dimerization interface. CviR had the largest RMSD value, indicating that it has the furthest divergence of the LBD structural conformations that dictate the dimerization interfaces of the LBD regions of AHL receptors. Figure 8.6a highlights the LBD interface contacts that are unique to QscR. Further examination into the interface of the QscR LBD dimer revealed that the charged residues Lys121 and Glu84 make crucial intersubunit contacts that are essential for the dimerization of the receptor LBD monomers. These residues are not found in the other AHL receptors whose structures are known. It is not clear why these LBD-LBD interfaces differ so greatly among these few AHL receptors.

QscR also has unique interactions between the LBDs and DBDs that are due to the symmetrical cross-subunit architecture of the homodimer. The LBD-DBD contacts in QscR (Fig. 8.6b) show that Arg42 and Arg79 of Chain A of QscR are in close contact with the C-terminal region in the DBD of Chain B, and that both residues serve to stabilize the carboxylate group at the C-terminus of the protein. Also, Arg42 and Arg79 of are in close proximity to 3OC12-HSL binding by the LBD of Chain A in QscR. Subsequent mutagenesis analysis revealed that these residues are important for receptor conformation and activation. In the presence of different AHLs, the activity of the receptor is still diminished, suggesting that the length of the acyl chain does not alter the LBD-DBD contacts observed in the crystallographic structure of QscR. These close inter-domain interactions in QscR and lack of these in TraR might explain why QscR adopts a tight symmetric structure, whereas the TraR dimer appears less compact and is asymmetric.

Intermolecular charged interactions bridge the QscR LBD and DBD regions together and are important for the overall configuration of the receptor (Lintz et al.

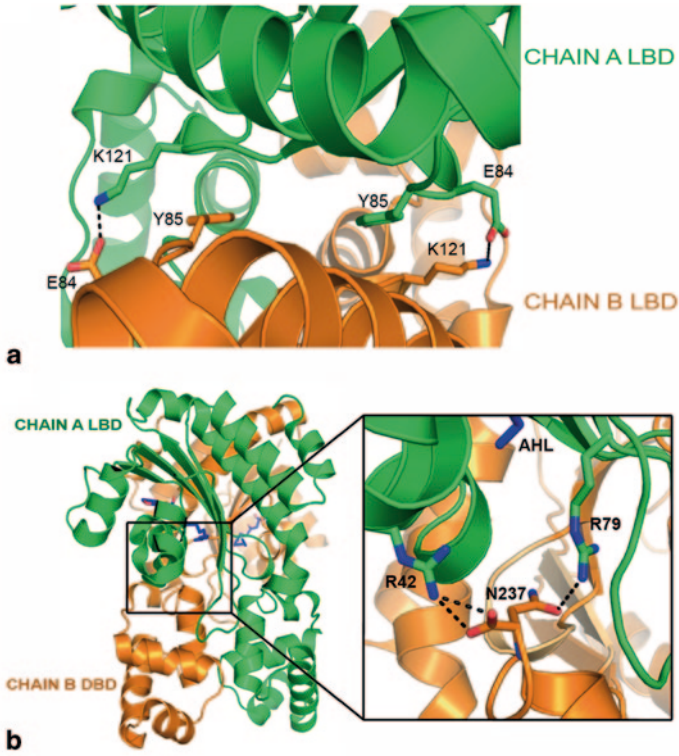

Fig. 8.6 QscR dimerization interfaces. **a** Critical contacts in the LBD-LBD interface of the QscR dimer (PDB ID: 3SZT) promote stability and subsequent DNA recognition. **b** The LBD-DBD interface between each monomer shows the sequestration of the C-terminal tail of the DBD that further stabilizes the overall receptor conformation

2011). Asn237 is only found in QscR, and serves as a crucial residue for making contacts with the LBD and DBD regions of each adjacent monomer. Asn237 is the last residue on each monomer chain of QscR at the C-terminal end of the protein, and tucks into the criss-crossed region of the dimer. Asn237 interacts with the LBD of the opposite monomer through residues Arg42 and Arg79 to further stabilize the crossed configuration of the receptor. Mutagenesis studies have confirmed that all of the residues discussed above are crucial for receptor activity (Lintz et al. 2011).

Acyl-Homoserine Lactone Recognition by QscR and Other QS Receptors

The modes of AHL recognition differ surprisingly among the various AHL receptors. Structures of only the LBD of *P. aeruginosa* LasR (Bottomley et al. 2007) and *Escherichia coli* SdiA (Yao et al. 2006) have been published, but these do not give

full insight into receptor conformation or DNA recognition. However, they contribute to better understanding how the AHL receptors can discriminate between AHLs with very similar characteristics and might be targeted with synthetic antagonists (Fig. 8.5) (Galloway et al. 2011; Stevens et al. 2011).

The QscR structure reveals binding of 3OC12-HSL that is similar to that observed in the structure of the LBD of the LasR, also bound to 3OC12-HSL (Bottomley et al. 2007; Lintz et al. 2011). The long, 12 carbon, fatty-acyl chain of 3OC12-HSL *inserts deeply into* the HSL binding pocket of the LBD. The hydrophobic chain of 3OC12-HSL is stabilized through non-polar residues that are in close proximity to the DNA binding domain (DBD) and the symmetrical dimerization interface. In addition, QscR and LasR have binding pockets of similar volumes (Lintz et al. 2011). The degree of similarity in binding modes observed for QscR and LasR is fascinating because both receptors are found in the *P. aeruginosa* quorum sensing system, recognize the same AHL, but they activate different and overlapping transcription programs.

In comparison, the full-length structures of TraR and CviR, as well as the LBD of SdiA, reveal a different mechanism of HSL recognition. All three of these structures are bound to an AHL with a shorter acyl chain length on the HSL ring (Vannini et al. 2002; Yao et al. 2006). TraR and SdiA recognize 3OC8-HSL and C8-HSL, respectively, whereas CviR is bound to the CviR antagonist of quorum sensing in *C. violaceum*, known as CL (Chen et al. 2011; Swem et al. 2008, 2009). All of these ligands *protrude out* toward the more hydrophilic portion of the binding pocket where the molecules are in contact with the surrounding aqueous environment (Lintz et al. 2011).

QscR and LasR are bound to 3OC12-HSL and the receptors further stabilize the fatty acyl chain into the binding pocket, whereas TraR, SdiA, and CviR are bound to ligands that protrude from the binding pocket towards the aqueous environment outside the LBD. These differences in AHL recognition between QscR and LasR versus TraR, SdiA, and CviR could possibly be correlated to specific amino acid residues located in the binding pocket or possibly also the balance of hydrophobic to polar character of the ligands. Figure 8.5 illustrates the differences of HSL recognition between the receptors. Interestingly though, the residues that make up the AHL-binding pocket of the LBD of QS receptors are normally conserved. The head group of 3OC12-HSL is stabilized by a network of hydrogen bonds: Trp62 binds to the carbonyl group on the lactone ring, whereas Tyr58, Ser56, and Ser38 partake in hydrogen bonding interactions with the first carbonyl group on the fatty acyl chain. Ser56 serves to stabilize a water molecule that operates as a polar bridge for a hydrogen-bonding network to stabilize the polar contacts on the carbonyl groups of the fatty acyl chain of 3OC12-HSL. Asp75 makes a hydrogen bond at the nitrogen group of the HSL. Deeper investigation into the binding pocket revealed certain hydrophobic residues that appear to stabilize the fatty-acyl chain of 3OC12-HSL through either hydrophobic or van der Waal forces: Val78, Gly40, Leu82, Tyr52, Ile125, and Arg42. Arg42 is located at the distal end of the binding pocket for 3OC12-HSL, and it is also the previous mentioned residue at the LBD-DBD interface of the crossed-subunit contacts that stabilize the overall conformation of the receptor.

Single-residue substitutions into the 3OC12-HSL binding pocket QscR revealed important residues that are crucial for AHL binding with this particular receptor.

First, amino acid substitutions were made to either disrupt or stabilize the hydrogen-bonding interactions that contact the hydrophilic head group of 3OC12-HSL. The S56G mutation disrupted the water molecule binding that made several hydrogen bonds with the polar oxo groups on 3OC12-HSL. The specificity of the receptor for 3OC12-HSL decreased, but increased for the more hydrophobic AHL C12-HSL, which is missing the second oxo group. Other mutations were made to investigate the molecule specificity of QscR for different AHLs of either shorter or longer fatty-acyl chain lengths. A G40F mutation would introduce a bulky phenylalanine group into the pocket, possibly restricting the pocket size and subjecting QscR to bind AHLs with shorter chain lengths. The results were in agreement with predictions of their functional effects; for example, the G40F substitution enabled QscR to respond better to 3OC6-HSL than the wildtype receptor. On the other hand, a V78F mutation, which would copy the function of the previous mutation, completely abolished QscR activity. This particular mutation indicates that Val78 is important for stabilization of the binding pocket of QscR. The same holds true for the L82F mutation, showing that this patch of hydrophobic residues is important for long-chain AHL stabilization in the binding pocket and possibly the cross-dimerization interface of the two QscR monomers.

The current understanding of the relationship between AHL receptor structure and function highlights the dissimilarity of ligand recognition. Furthermore, it shows that distinct modifications of AHLs on the acyl chain such as long hydrophobic tails or substitutions at the 3 position might dictate the molecular recognition pattern globally for particular receptors. The observed dependence of AHL receptors on binding to ligands for increased stability suggests that the ligands act as a scaffold for the receptors to promote proper protein folding. This appears to have a far-reaching impact on the final structural conformation of the receptors locally in the AHL binding pocket as well as in the dimerization interface and inter-domain contacts. These subtleties of AHL recognition by the AHL receptors have brought significant challenges to the development of specific synthetic agonists and antagonists.

Therapeutic Modulation of QSCR and Other Quorum Sensing Receptors

The process of quorum sensing serves an integral role in virulence factor production and biofilm formation, which further complicates the disease state of infected individuals. With the discovery of the different components of the quorum sensing pathway leading to biofilm development, the avenues for therapeutic development to target this process are abundant. A common goal of researchers has been to find novel compounds to target the AHL receptors (Geske et al. 2008; Stevens et al. 2011). Laboratories have discovered synthetic compounds that either antagonize or agonize AHL-mediated quorum sensing. Synthetic antagonists and agonists serve to interrupt native AHL binding to the LuxR-type class of receptors and either inac-

tivate or activate the receptors that result in changes in the activity of the receptors as measured in a variety of different assays (Praneenararat et al. 2009; Rasmussen et al. 2005; Steindler and Venturi 2007).

The most common class of synthetic molecules that can bind to AHL receptors consist of AHL analogs, and they have different chemical compositions varying from acyl chain and lactone ring substitutions to differing amide chemical groups conjugated to the lactone ring (Eberhard et al. 1986). In addition, chemical screens, *in vitro* assays, and virtual molecule docking using known quorum sensing receptor crystallographic structures, have been implemented to find novel AHL receptor binders that are classified as AHL analogs and non-AHL analogs (Liu et al. 2010; Müh et al. 2006; Rasmussen et al. 2005; Skovstrup et al. 2013). Some of these varieties of molecules are specific for a certain class of LuxR-type receptors, whereas others serve to target other LuxR-type receptors. Hence, there is signal overlap in different quorum sensing systems. For example, QscR recognizes a panel of molecules that are also predominantly recognized by LasR (Mattmann et al. 2011).

AHL Analogs

The AHL analog class of quorum sensing modulators differ in their acyl chain length, either having an unsubstituted or oxo group at the third position of the carbon chain. Also, different moieties can be substituted on the carbon chain. AHLs of different acyl chain lengths that are either substituted or unsubstituted at the 3 position are found in many bacterial quorum sensing systems. Hence, the first AHL analogs of quorum sensing modulators contain slight differences in their acyl chains that serve to either activate or inhibit quorum sensing. The Greenberg laboratory initially identified AHLs that activate or inactivate QscR and LasR (Lee et al. 2006). The Blackwell laboratory has demonstrated this effect using different flavors of AHLs with unique moieties on the acyl chain that were identified as potent analogs that either activate or inactivate QscR and LasR (Mattmann et al. 2011). Figure 8.7a shows a panel of synthetic AHL analogs and AHLs not native to *P. aeruginosa* that are specifically recognized by QscR and LasR (Lee et al. 2006; Mattmann et al. 2011). All of these molecules have the characteristic homoserine lactone ring, but different acyl chains that help to confer specificity for either being recognized by QscR or LasR (Mattmann et al. 2011). However, in all cases the synthetic compounds are less potent than the native AHLs, and it is important to find dedicated binders for QscR versus LasR since these receptors serve different roles in the quorum sensing process in *P. aeruginosa*.

Non-AHL Analogs

One of the first inhibitors of quorum sensing to be discovered was the natural product family of brominated furanones that are isolated from Red Algae known as

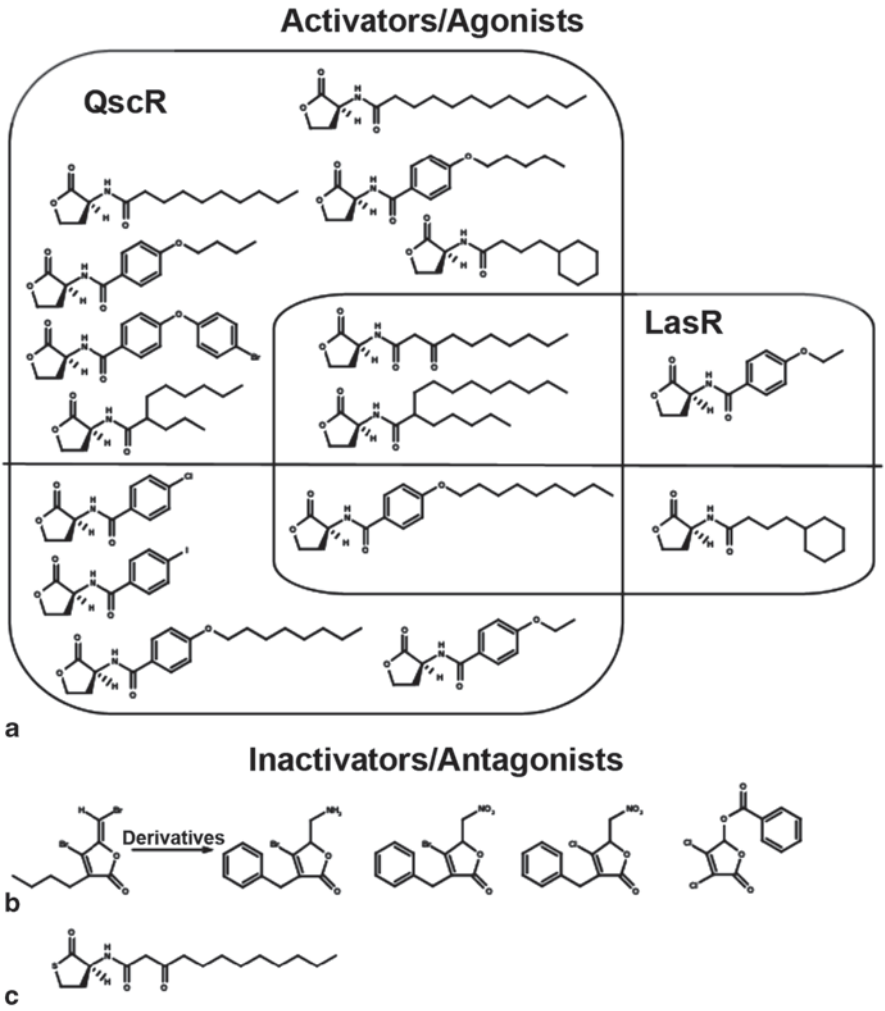

Fig. 8.7 AHL receptor modulators a Synthetic AHL analogs that activate QscR, LasR, or both receptors. **b** Non-AHL analog brominated furanone isolated from *Delisea pulchra* and its synthetic derivatives. **c** 3-oxo-C12 HTL homoserine thiolactone

Delisea pulchra, as these were found to disrupt the swarming motility patterns of the human pathogen *Proteus mirabilis* (Gram et al. 1996). Figure 8.7b illustrates the parent brominated furanone isolated from *Delisea pulchra* and other brominated furanone derivatives that have illicited inhibitory actions against QscR (Liu et al. 2010; Stevens et al. 2011). Different varieties of furanones are one possible avenue to investigate when combating quorum sensing in *P. aeruginosa*.

Acyl homoserine lactones are unstable in mammalian cells due to the hydrolytic capabilities of the lactone ring (Teiber et al. 2008; Yates et al. 2002). Therefore, thiolactone modulators (as known as homocysteine lactones) have been developed and tested to improve the potency of AHL analogs for use in a mammalian setting (Eberhard et al. 1986; McInnis and Blackwell 2011). Analogs have been initially tested in *V. fisheri* against LuxR activity, and more recent studies in have revealed modulators of QscR and LasR in *P. aeruginosa* (McInnis and Blackwell 2011; Passador et al. 1996; Schaefer et al. 1996a). Figure 8.7c illustrates the structure of acyl-homoserine-thiolactone-3-oxo-C12 HTL used to target LasR and QscR (Eberhard et al. 1986; McInnis and Blackwell 2011). These synthetic compounds have moderate to weaker potencies in comparison to native AHLs (McInnis and Blackwell 2011; Schaefer et al. 1996a).

There several other classes of synthetic compounds have promise as modulators of AHL receptor activity. These include molecules discovered from high-throughput screens, natural molecules found in nature, and other non-AHL analogs that have been tested in other quorum sensing systems (Müh et al. 2006; Stevens et al. 2011; Tan et al. 2013). Understanding which synthetic molecules work best for the similar LasR and QscR receptors and how these differ from the synthetic modulators of the AHL receptors that respond to the short-chain AHL signals will be informative for teasing apart the different mechanisms involved in the response to AHLs as well as in the development of therapeutic approaches to be used to combat bacterial pathogenicity through the quorum sensing process.

Conclusions

QscR has emerged as an excellent model for the AHL receptors that recognize AHLs with long acyl chains. QscR shares with LasR many similar properties of structure and activity, such as the sequestration of the AHL in the core of the LBD. This is despite the greater promiscuity of QscR for alternative AHLs than LasR, which is quite specific for 3OC12-HSL. In contrast, the equivalent properties of the AHL receptors that recognize AHLs with short acyl chains, such as CviR and TraR, are quite different. The acyl-chain of these AHLs protrudes into the solvent. Whereas the agonist bound form of QscR is poised to bind to DNA, the antagonist bound form of CviR is stabilized in a DNA binding incompetent form. Therefore, one major lesson learned from the study of multiple AHL receptors, including QscR, is that there is not a single uniform mechanism of activity, and therefore there will likely also be multiple approaches to modulate their activity for the purpose of antibacterial therapy.

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Part III
Soil Microbiology

Chapter 9
Understanding and Exploiting Bacterial Outer Membrane Vesicles

Brandon D. Vella and Jeffrey W. Schertzer

Abstract The bacterial outer surface defines the interface between an organism and its environment and thus plays an important role in facilitating interactions between the two. The structures comprising the cellular envelope have received considerable research interest, but an emerging area aims to understand how and why Gram-negative bacteria actively shed fragments of their outer membrane into the surrounding milieu. This phenomenon has been observed in nearly all Gram-negative organisms studied and has even been described as a dedicated secretion system. In this communication, we describe the history, structure, composition, functions and mechanism of formation of outer membrane vesicles. In addition, we discuss the promise of exploiting OMVs for pharmaceutical purposes and the early successes that have been seen with OMV vaccines.

Composition and Structure

Structure and General Features

Bacterial secretion has long been an area of research interest due to the importance of secreted factors in health and disease. In the 1960's, Work and colleagues (Bishop and Work 1965; Taylor et al. 1966) investigated factors released by nutrient-starved mutants of *Escherichia coli* and gave an early characterization of a 'lipoglycopeptide' that contained lipopolysaccharide (endotoxin), a component normally found residing in the outer leaflet of the bacterial outer membrane. Further analysis by the same group and others explained this phenomenon by postulating that the outer membrane of the organism had budded off to form vesicular structures with a

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composition similar to the outer membrane (Chatterjee and Das 1967; Knox et al. 1966; Work et al. 1966). The case for this was strengthened by electron micrographs that appeared to capture the budding and pinching event in various stages and multiple studies in many organisms have since corroborated these early findings. Although the first microscopic evidence for the production of OMVs came from nutrient-starved bacteria, production of OMVs has now been seen in nearly every Gram-negative organism that has been tested. This list includes but is not limited to *E. coli* (Gankema et al. 1980; Hoekstra et al. 1976), *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge 1995), *Haemophilus* spp. (Deich and Hoyer 1982; Kahn et al. 1982), *Neisseria* spp. (Devoe and Gilchrist 1973; Dorward et al. 1989), *Vibrio* spp. (Chatterjee and Das 1967; Kondo et al. 1993), *Salmonella* spp. (Vesy et al. 2000; Wai et al. 2003), *Shigella* spp. (Dutta et al. 2004; Kadurugamuwa and Beveridge 1999), *Helicobacter pylori* (Fiocca et al. 1999), *Bacteroides* (including *Porphyromonas*) spp. (Forsberg et al. 1981; Grenier and Mayrand 1987; Mayrand and Holt 1988), *Acinetobacter baumannii* (Jin et al. 2011), *Brucella melitensis* (Gamazo and Moriyón 1987), *Borrelia burgdorferi* (Shoberg and Thomas 1993; Whitmire and Garon 1993) and *Aggregatibacter actinomycetemcomitans* (Lai et al. 1981; Nowotny et al. 1982). OMV production has been so widely observed that it is now commonly accepted to be a characteristic feature of Gram-negative bacteria. Typically, OMVs are spherical in shape, possess a unilamellar membrane and range in diameter from 50–300 nm. They can generally be recovered from culture supernatants in the logarithmic through stationary phases of growth (Chatterjee and Das 1967; Dorward et al. 1989; Kadurugamuwa and Beveridge 1995; Mashburn and Whiteley 2005) and their release is often described as constitutive, though in *P. aeruginosa* OMV production remains very low until cells approach stationary phase (Bauman and Kuehn 2006), consistent with this process being controlled by quorum sensing molecules (Mashburn and Whiteley 2005). By comparing the amount of OM protein released into the supernatant vs. retained in cells, it has been estimated that *E. coli* releases up to 5% (Gankema et al. 1980) and *P. aeruginosa* between 0.75—2.5% of their total outer membrane as OMVs (Bauman and Kuehn 2006).

As it became more evident that OMVs were composed of fragments of the OM, it was important to be sure that they did not arise simply through lysis of some cells in the population. As stated above, electron micrographs showing various stages of OMV formation suggested a controlled process that did not compromise cellular integrity. A rigorous examination of this question was carried out by Gankema et al. (1980), who tested for cell lysis by measuring glucose-6-phosphate dehydrogenase activity in OMV-containing supernatants and found that less than 0.2% of the cytoplasmic dehydrogenase escaped the cells. Inner membrane (IM) marker enzymes NADH oxidase, D-lactate dehydrogenase and succinate dehydrogenase have also been shown by several investigators to be absent from OMV preparations (Gankema et al. 1980; Hoekstra et al. 1976; Horstman and Kuehn 2000; Renelli et al. 2004), strongly supporting the idea that OMVs originate from the OM and do not arise from lysis of the cell. In a related study, McBroom et al. characterized a set of hyper-vesiculating mutants of *E. coli* and found that leakage of cytoplasmic contents was very rare (McBroom et al. 2006). Leakage of periplasmic contents

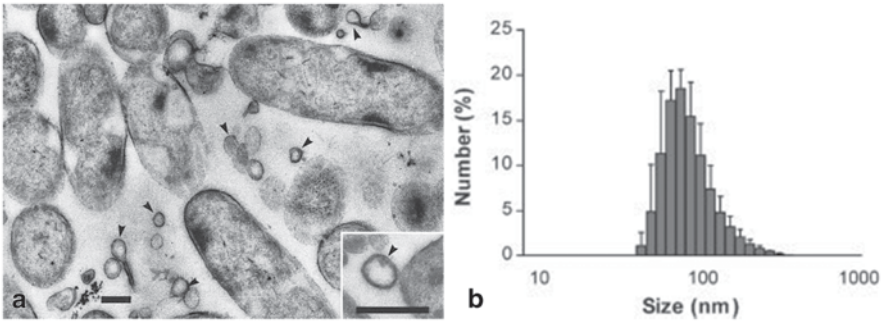

Fig. 9.1 Size and Physical Structure of OMVs. **a** Thin section electron micrograph showing OMVs in the extracellular matrix of a *P. aeruginosa* biofilm. OMVs are marked by black arrowheads. The inset shows an OMV being released from a parent cell at higher magnification. Scale bars = 250 nm. **b** Size distribution of OMVs recovered from a planktonic *P. aeruginosa* culture as measured by dynamic light scattering. Average vesicle diameter was 109 ± 49 nm. Reproduced with permission from (Beveridge et al. 1997) and (Choi et al. 2011)

was more common, but was mainly seen in mutants with disruptions in important envelope structural genes, and the strongest OMV overproducers showed little periplasmic leakage.

The earliest studies of OMVs confirmed their existence through chemical characterization and direct microscopic observation. These investigations established the ubiquity of OMV release across Gram-negative species and made the case that these structures were part of a natural secretory pathway rather than simply cellular debris. Since that time, work has largely focused on what cargo is trafficked in OMVs, how they are generated and how this mode of delivery is advantageous to the organism (Fig. 9.1).

OMVs Resemble the Outer Membrane

The first components of OMVs to be carefully analyzed were those that could help trace their origins to the OM. Lipid content differs significantly between the inner and outer membrane of Gram-negative bacteria and thus could be used to discriminate between the two. Across several species, the major phospholipids (PLs) found in OMVs include phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. These lipids are generally present in ratios that resemble the PL composition of the OM from the parent bacterium (Hoekstra et al. 1976; Horstman and Kuehn 2000; Kato et al. 2002; Tashiro et al. 2011). OMVs are also more similar to the OM than the IM in fatty acid composition, buoyant density and the ratios of phospholipid:protein and 3-Deoxy-D-*manno*-octulosonic acid (KDO):protein (Hoekstra et al. 1976; Renelli et al. 2004; Tashiro et al. 2011). KDO sugar is a marker for the presence of lipopolysaccharide (LPS), which is the signature lipid of the OM and one that has consistently been shown to be present in OMVs, whether by detection of the

component parts (e.g. KDO) or by direct observation using methods like thin layer chromatography (Kato et al. 2002). Interestingly, although all OMVs contain LPS as a testament to their OM origin, strains that express more than one type of LPS do not necessarily equally distribute these lipids into OMVs. *P. aeruginosa* can simultaneously express two types of LPS on its surface: A-band LPS (containing a shorter, neutral common antigen) and B-band LPS (containing a serotype specific, anionic O-antigen). Kadurugamuwa and Beveridge showed that naturally produced OMVs were highly enriched in B-band LPS relative to the OM, suggesting that these lipids were preferentially packaged into OMVs (Kadurugamuwa and Beveridge 1995). Similarly, in *Porphyromonas gingivalis* anionic LPS is preferentially packaged into OMVs over neutral LPS and OMV LPS may also be significantly deacylated compared to OM LPS (Haurat et al. 2011). The authors proposed that this preferential sorting of LPS into OMVs also contributes to the preferential sorting of specific proteins.

The protein content of OMVs was also examined to help gain insight into their biogenesis. Beginning with only surface-exposed proteins, Pettit and Judd performed surface-radioiodination on whole cells and purified OMVs. They discovered that the pattern of labeled proteins was nearly identical between whole cells and OMVs for several strains of *Neisseria gonorrhoeae* and that all of the major surface-labeled cellular proteins were detected in the OMV preparations (Pettit and Judd 1992a). In agreement with this, many studies using simple SDS-PAGE analysis of OMV preparations showed that OMV total-protein profiles most closely resembled the OM, even though proteins of periplasmic origin were often observed (Bauman and Kuehn 2006; Hoekstra et al. 1976; Horstman and Kuehn 2000; Kato et al. 2002). As with LPS, however, it was quickly noticed that distributions of proteins—even proteins of OM origin—were not identical between the OM and vesicles. Most notably, peptidoglycan-associated lipoproteins were found to be largely excluded from *E. coli* OMVs (Wensink and Witholt 1981). On the other hand, several proteins are unusually abundant in OMVs and many of these are virulence factors destined to be delivered to target cells. For example cytolethal distending toxin, Cytolysin A, heat-labile endotoxin and leukotoxin have been identified to be concentrated into OMVs of several species (Horstman and Kuehn 2000; Kato et al. 2002; Lindmark et al. 2009; Wai et al. 2003).

By looking at the nature of both the protein and lipid constituents of OMVs, it has been established that they originate from the OM of the parent bacterium. The analyses described here were useful for comparing gross profiles or identifying individual proteins for which there were antibodies or robust activity assays available. However, increasingly available proteomic techniques quickly became the preferred method to identify the full complement of OMV protein cargo.

OMVs in the Age of Proteomics

Protein profiles using SDS-PAGE were useful in mapping the origin of OMVs, but this process became much more comprehensive with the widespread usage of

proteomics. Protein cargo could now be described in great detail or subdivided into useful categories to examine the effects of subtle mutations in genes suspected of being involved in OMV formation or packaging, for example. Several groups have published proteomic analyses of OMVs from *P. aeruginosa* (Choi et al. 2011; Wessel et al. 2013) and other organisms (Avila-Calderón et al. 2012; Berlanda Scorza et al. 2008; Galka et al. 2008; Haurat et al. 2011; Lee et al. 2007; Moon et al. 2012; Park et al. 2011; Post et al. 2005; van de Waterbeemd et al. 2013a). The first consequence of such analysis was that it supported the consensus that OMVs were derived from the OM. In *P. aeruginosa*, OM and periplasmic proteins were seen to comprise the majority of OMV protein content by abundance and were also enriched in OMVs vs. whole cells with respect to the number of individual proteins originating from these fractions (Choi et al. 2011) (Fig. 9.2a). It is important to consider both measures of protein frequency because the appearance of small quantities (low abundance) of a large number of IM or cytoplasmic proteins, as might arise from minor amounts of cell lysis, could lead to confusion. However, a large abundance of relatively few OM proteins strongly supports the OM origin of OMVs. When broken down by predicted functional category, OMV proteins clustered toward traditional OM and exoproduct roles in transport and proteolysis rather than cellular roles like DNA metabolism and transcription (Fig. 9.2b). As more OMV proteomes are published, patterns emerge regarding common proteins and protein classes that are secreted in OMVs. For instance, OMV cargo in *E. coli* was even more heavily skewed toward OM and periplasmic proteins than in *P. aeruginosa* (Lee et al. 2007). In reviewing proteomic data available for OMVs from several Gram-negative organisms, Lee et al. organized commonly appearing proteins and protein families into functional groups likely to contribute to the many known roles of OMVs (Lee et al. 2008). Across species, proteins found in OMVs can be expected to be involved in bacterial survival, nutrient sensing & transport, killing competing bacteria, immune modulation and targeting to host cells (Fig. 9.2c).

Proteomic characterization of OMVs has implied that active cargo sorting takes place in many organisms. This arises from the fact that inclusion of specific proteins in OMVs does not appear to be a simple function of their overall abundance in the OM or periplasm. Several low abundance OM proteins have been identified in OMVs, while some of the most abundant periplasmic proteins can be excluded (Lee et al. 2008). In a proteomic analysis of 181 proteins secreted into the culture supernatant by *Legionella pneumophila*, it was seen that the soluble secreted protein pool and the OMV proteome consisted of specific members and were only partially overlapping (Galka et al. 2008). This pattern persisted when only proteins believed to be involved in virulence were considered. As a striking example of protein sorting, Haurat et al. analyzed the cargo of OMVs from *P. gingivalis* and found that nearly all of the most abundant proteins were gingipain proteases (Haurat et al. 2011). Analysis of global protein profiles can also shed light on the possible mechanisms of OMV formation and cargo sorting. In the above example of gingipain sorting (Haurat et al. 2011), it appeared that the composition of LPS in the OM directed cargo packaging. In another study, mutation of the peptidoglycan-associating *ompA* homolog in *A. baumannii* resulted in increased OMV production but also alterations in the makeup of the OMV proteome in this organism (Moon et al. 2012).

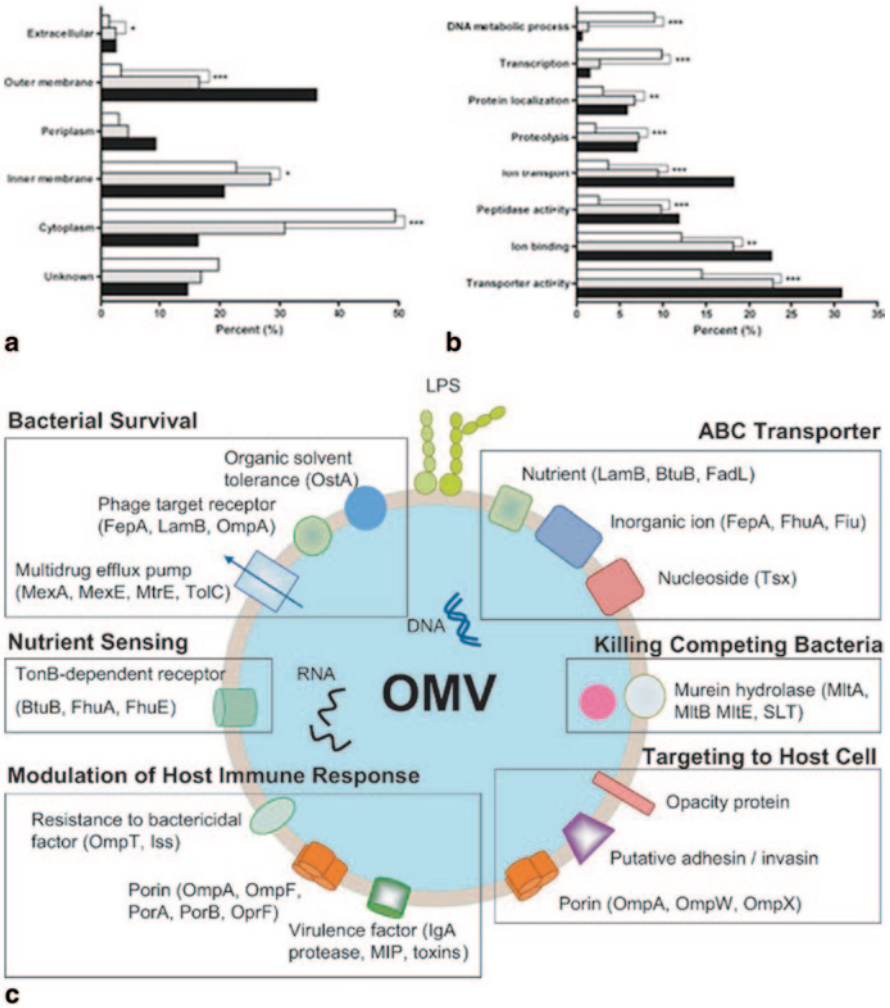

Fig. 9.2 Proteomic Analysis Sheds Light on OMV Cargo. Advancements in proteomics have allowed for detailed mapping of the proteins carried in OMVs. The proteome of *P. aeruginosa* OMVs can be subdivided on the basis of protein localization **a** or function **b** to yield useful information. *White* and *grey bars* indicate the percentage distribution of proteins in cells and OMVs, respectively, based on the number of proteins identified. *Black bars* represent the percentage distribution of proteins in OMVs by abundance. Using this analysis, it can be seen that OMVs are enriched in OM proteins, transporters and proteases while cytoplasmic proteins and those involved in core metabolism are excluded. **c** As biochemical, biological and proteomic studies from several organisms accumulate, they can be compared for general similarities. When this is done, broad categories of commonality emerge that correlate well with the observed functions of OMVs. Reproduced with permission from (Choi et al. 2011) and (Lee et al. 2008)

In addition to greatly expanding our knowledge about the identity of virulence factors secreted in OMVs (Ellis and Kuehn 2010), proteomic analysis has supported the prevailing ideas concerning the physical origin of OMVs and is proving to be a useful tool to address questions of OMV formation and protein sorting, even down to a mechanistic level.

Other Components Associated with OMVs

As important as they are, proteins are not the only interesting macromolecules found in OMVs. Dorward et al. identified nucleic acids associated with *N. gonorrhoeae* OMVs in the form of RNA and both linear and circular DNA (Dorward et al. 1989). The RNA and linear DNA were destroyed by nuclease treatment, suggesting they were only externally associated, but the circular DNA was protected within the lumen of the vesicle. Through a series of experiments, these authors concluded that OMVs were capable of delivering DNA to target cells and genetically transforming them. Since then, DNA has been detected in OMVs naturally released from a number of organisms, including *E. coli* (Kolling and Matthews 1999) and *P. aeruginosa* (Kadurugamuwa and Beveridge 1995), whose OMVs have been extensively studied. A broad survey was performed using 18 species of both Gram-negative and Gram-positive bacteria that showed only Gram-negative bacteria were capable of packaging DNA into external membrane structures (Dorward and Garon 1990). Notably, all 14 Gram-negative species tested released DNA-laden OMVs. The DNA found within OMVs is generally believed to have originated from within the parent cell, but studies on *P. aeruginosa* OMVs suggested plasmid DNA could be taken up from the environment after the OMVs had been released from the cell (Renelli et al. 2004). It was noted, however, that a small proportion of OMVs in typical preparations did rupture, and reformation of such vesicles could have contributed to the observed DNA uptake. In nature, OMVs are often found in close proximity to large amounts of extracellular DNA in the matrix of biofilms (Beveridge et al. 1997; Palsdottir et al. 2009; Schooling and Beveridge 2006) where the two components have been documented to interact (Schooling et al. 2009). In this context, horizontal gene transfer is an interesting possible role for OMVs, as are the possible physical implications of the OMV-DNA interactions in contributing to the stability of the matrix itself.

In addition to macromolecules, a number of small molecules are also packaged in OMVs. These small molecules differ somewhat from the traditional paradigm discussed so far in that their presence in OMVs has been attributed in several contexts to excretion as well as dedicated transport. Beveridge and coworkers discovered that treatment of *P. aeruginosa* with gentamicin induced the overproduction of OMVs with slightly different characteristics from natural OMVs (Kadurugamuwa and Beveridge 1995). Interestingly, vesicles released in this manner were seen to contain nearly 5 ng gentamicin/μg OMV protein, presumably captured from the periplasm during the vesiculation process (Kadurugamuwa and Beveridge 1996).

This and subsequent work showed that gentamicin-containing OMVs were more lethal to competitor cells than natural OMVs and gave an indication that OMVs could be intentionally loaded with exogenously added small molecules. A more concrete example of excretion of harmful compounds in OMVs comes from work done on a toluene-tolerant strain of *Pseudomonas putida*. The tolerance mechanism of this strain involves increased production of OMVs in the presence of toluene and concentrating more of the small molecule into OMVs than the rest of the OM, thus removing the threat in the shed OMVs (Kobayashi et al. 2000). Using a similar strain of *P. putida*, Baumgarten et al. showed that this strain responded to toxic long-chain alkanols by concentrating and excreting them in OMVs (Baumgarten et al. 2012b). Interestingly, there was no such response to 2,4-dichlorophenol, another solvent with similar physiochemical and toxicity properties, suggesting that specific aspects of the small molecule and how it interacts with the membrane are required to stimulate OMV production. This idea is consistent with work done in *P. aeruginosa* showing that the Pseudomonas Quinolone Signal (PQS) is trafficked within OMVs. Aside from its role in quorum sensing, PQS was found to physically stimulate OMV production by dissolving into the OM where it makes specific interactions with the lipid A portion of LPS, leading to the induction of membrane curvature and ultimately OMV production (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008; Schertzer and Whiteley 2012). This process concentrates PQS into OMVs through an unknown mechanism and leads to greater than 85% of the PQS in a stationary phase culture being localized in OMVs (Mashburn and Whiteley 2005).

Clearly, cargo other than lipids and proteins contribute greatly to several important roles of OMVs in bacterial physiology. As research on this topic expands, significant non-proteinaceous factors will undoubtedly continue to be identified.

Functional Roles

Threat Avoidance

It is well established that OMVs are comprised of constituents that closely resemble both the composition and arrangement of the cellular OM (see above). This feature means that by releasing OMVs, an organism can rapidly and effectively expand the surface area it presents to any array of external threats. Such reasoning is behind a number of reports that describe OMV formation as a defense mechanism against harmful agents whose modes of action require first binding to specific elements of the cellular envelope. In essence, OMVs can be viewed as decoys or molecular flak released to engage surface-active toxins in fruitless interactions while shielding the surface of the parent bacterium from attack. Pettit and Judd examined serum-resistant and serum-susceptible strains of *N. gonorrhoeae* and found qualitative and quantitative differences between the ‘elaborated blebs’ from each strain (Pettit and Judd 1992a; Pettit and Judd 1992b). Notably, several OM proteins that were

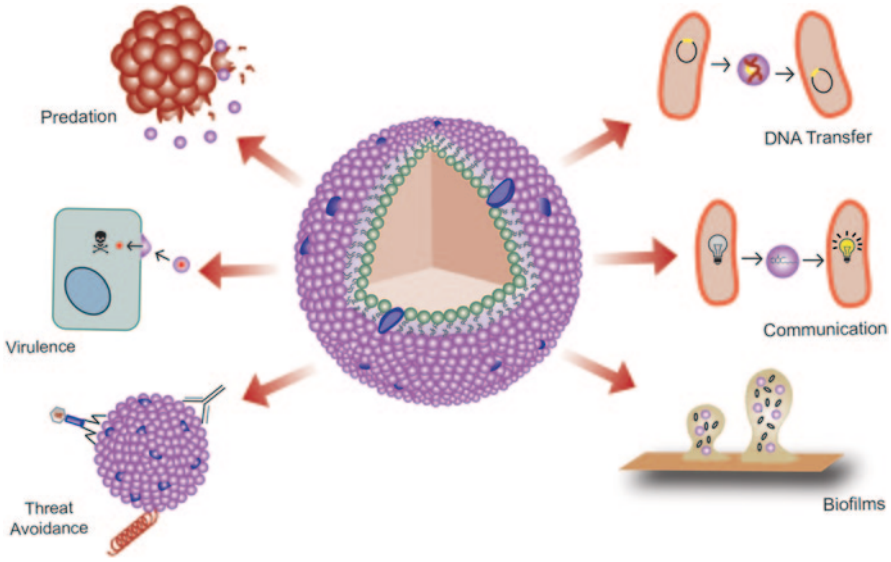

Fig. 9.3 Functions of OMVs. Outer membrane vesicles are involved in many aspects of bacterial physiology. Interspecies interactions are depicted on the *left*, while processes on the *right* are considered to be primarily intraspecies, though this is not always the case

recognized by serum immunoglobulins were over-represented in the OMVs from the resistant strain. Serum-induced killing of the bacterium was inhibited by pre-incubation of the serum with OMVs from either strain and by exogenous addition of either type of OMV to the bacteriocidal assays. It was noted that OMVs purified from the resistant strain were much more potent at reducing killing, providing evidence that OMVs can adsorb killing factors from serum to protect the parent cells and that their efficacy can be affected by the surface constituents of the vesicles. As an example of multi-species interactions in the context of infection, Tan et al. showed that *Moraxella catarrhalis* OMVs bound C3 protein and thereby disrupted complement-mediated killing of the parent strain and co-infecting *Haemophilus influenzae* (Tan et al. 2007). Working from a similar premise but examining different threat agents, Manning and Kuehn demonstrated that OMVs protect enterotoxigenic *E. coli* from killing by binding and neutralizing polymixin B, colistin and bacteriophage (Manning and Kuehn 2011).

A related mechanism by which OMVs have been proposed to protect their parent bacteria is by packaging enzymes whose activities destroy or thwart the threats themselves. A good example of this strategy is the packaging of β-lactamase into OMVs. Ciofu et al. identified the presence and activity of β-lactamase in OMVs derived from cystic fibrosis clinical isolates of *P. aeruginosa* (Ciofu et al. 2000). In *M. catarrhalis*, such β-lactamase-containing OMVs were shown to destroy amoxicillin in solution and to protect several species of bacteria when exogenously added together with the antibiotic (Schaar et al. 2011). In both cases the β-lactam antibiotic was proposed to have diffused into the lumen of the OMVs, which contained β-lactamase.

Hydrolysis of the β-lactam in the OMV lumen would then result in lowering of the effective concentration of the antibiotic in solution, thus conferring protection.

Not limited to setting up a distractionary smokescreen, OMVs can modulate the immune system in other ways as well. The *M. catarrhalis* superantigen MID, packaged into OMVs, has been shown to activate B-cells to initiate an immune response that does not target the parent bacterium (Vidakovics et al. 2010). In this way, the organism appears to use OMVs to direct the immune system away from itself, resulting in successful infection. On the other hand, OMVs are also capable of initiating a strong inflammatory response that leads to tissue damage. All Gram-negative OMVs contain large amounts of LPS, the prototypical pathogen-associated molecular pattern, and many of the most abundant proteins in OMVs are porins. Both of these are known to be potent pro-inflammatory triggers (Beutler 2000; Tufano et al. 1994), and work with *Salmonella* OMVs even lead to the suggestion that OMVs represent the native presentation of LPS that has the greatest biological activity (Munford et al. 1982). Consistent with this, OMVs have been shown to be capable of inducing sepsis in rats regardless of whether whole cells are present (Shah et al. 2012). OMVs from several species have been shown to stimulate the release of a wide range of pro-inflammatory mediators (reviewed in (Ellis and Kuehn 2010)), and this property has allowed them to be used successfully as both adjuvants and antigens in the development of vaccines (see below).

Many of the examples cited above deal with threat avoidance in the context of a host immune system or antibiotic treatment. It is important to note that OMVs have been identified as major constituents of the exopolymeric matrix of environmental biofilms isolated from diverse locations, where they have also been suggested to act as “sponges” that function to adsorb harmful substances (Schooling and Beveridge 2006). Thus the role that OMVs play in threat avoidance is likely to be important in many contexts.

Delivery of Cargo

The ability to transport cargo in discrete packages provides a number of distinct advantages over reliance on simple diffusion. Transport in OMVs offers the potential for targeted delivery depending on specific receptors or membrane composition and ensures that concentrated cargo can travel long distances while avoiding environmental hazards or detection/inactivation by the immune system. It is well understood that eukaryotic organisms exploit these advantages through the deployment of their many membrane-bound compartments, and acknowledgement of related processes in the bacterial domain is slowly growing (Saier and Bogdanov 2013). Though OMVs can clearly be used for non-targeted secretion of factors into the environment, as was seen in the example of β-lactamase described above, a substantial and growing body of evidence also shows that nucleic acids, lipids, proteins and small molecules can be trafficked via OMVs to functionally relevant target destinations.

Many early reports identified DNA associated with OMVs and this has been thoroughly corroborated in more recent studies of both planktonic and biofilm

OMVs (see above sections). In many cases the OMVs were shown not only to associate with DNA, but were capable of delivering it to other cells where it was internalized, resulting in genetic transformation of the recipient cells. Dorward et al. characterized nucleic acids on the exterior and interior of *N. gonorrhoeae* OMVs and showed genetic transfer of antibiotic resistance determinants via OMVs, even in the presence of exogenous DNAse (Dorward et al. 1989). Similar results have also been described in other species. Virulence genes were successfully transferred from *E. coli* O157:H7 to other enteric bacteria via OMVs and expression of the newly acquired genes increased the cytotoxicity of the recipient strains (Kolling and Matthews 1999; Yaron et al. 2000). In *A. baumannii*, genetic material was successfully transferred via OMVs, conferred resistance to the recipient strain and was subsequently re-packaged into OMVs released from that strain (Rumbo et al. 2011). Use of OMVs as vehicles of genetic transformation may not be universal, however, as stable transfer of genetic information was not seen to occur in *P. aeruginosa* despite the fact that DNA is known to be carried in the lumen and on the surface of OMVs from this organism (Kadurugamuwa and Beveridge 1995; Renelli et al. 2004).

In many cases, delivery of cargo from OMVs to target cells is believed to occur through fusion of the OMVs with the membrane of the recipient cell. If this is the case, OMVs should be seen to transfer lipids into recipient membranes and to release proteins directly into the periplasm or cytoplasm of target cells. OMV fusion had been hinted at in studies with DNA transformation, but electron microscopic evidence began to directly support this notion when Beveridge's group analyzed the killing of bacteria by OMVs through the action of packaged peptidoglycan hydrolases. First, thin section immunogold electron micrographs showed firm integration of *P. aeruginosa* OMVs and their distinctive LPS into the cell surface of target *E. coli* cells (Kadurugamuwa and Beveridge 1996). Delivery of hydrolases was evidenced by destruction of the underlying murein sacculus and subsequent cell lysis. These results were corroborated a couple of years later when, after incubation with OMVs from *P. aeruginosa* or *Sigella flexneri*, target *E. coli* and *Salmonella* strains displayed a surface mosaic composed of intermixed host- and OMV-derived surface antigens (Kadurugamuwa and Beveridge 1999). OMVs can also fuse with and deliver cargo to eukaryotic cells. Multiple virulence factors have been shown to be directly transferred to the cytoplasm of airway epithelial cells (Bomberger et al. 2009) and gingival fibroblasts (Rompikuntal et al. 2012) via fusion of *P. aeruginosa* and *A. actinomycetemcomitans* OMVs to lipid raft domains in the cytoplasmic membrane. In other systems, OMVs have been shown to enter mammalian cells via endocytosis (Furuta et al. 2009a; Furuta et al. 2009b; Kesty et al. 2004). Interestingly, this uptake mechanism also requires association of OMVs with lipid raft domains. By a variety of mechanisms, it is clear that OMVs are capable of protein and lipid cargo delivery to other prokaryotes and also to eukaryotic cells.

Many early studies on *P. aeruginosa* OMVs were carried out using gOMVs, or OMVs released after perturbation of the OM by gentamicin. This work helped to develop ideas about how OMVs are formed (see below), but also gave indications that they could traffic small molecules between cells. gOMVs were found to contain measurable amounts of the gentamicin used to induce their formation, and they were more effective at lysing both Gram-negative and Gram-positive bacteria

than natural OMVs (Kadurugamuwa and Beveridge 1996). Importantly, gOMVs were capable of lysing bacteria with permeability resistance to gentamicin indicating that peptidoglycan hydrolases and the antibiotic were delivered into the target periplasm. In separate experiments, gOMVs were observed to deliver gentamicin into the cytoplasm of *shigella*-infected human intestinal epithelial cells where the antibiotic was capable of reducing the number of intracellular bacteria by ~1.5 $\log_{10}$ CFU compared to exogenous treatment with soluble gentamicin, to which the epithelial cells were impermeable (Kadurugamuwa and Beveridge 1998). In a more natural context, most of the antibiotic activity attributable to *P. aeruginosa* OMVs has been shown to be extractable into ethyl acetate, suggesting that antimicrobial quinolones—small molecule cargo—are the likely active agents (Mashburn and Whiteley 2005). Not all small molecules carried by OMVs are killing agents, however. In the same work, Mashburn & Whiteley showed that PQS, a *P. aeruginosa* quorum sensing molecule, is packaged into and delivered via OMVs. Very hydrophobic in nature, PQS is believed to transit between cells dissolved into the membrane of OMVs. Consistent with this, OMVs purified from wild type bacteria are capable of rescuing PQS-controlled phenotypes in PQS biosynthetic mutant strains, indicating successful delivery of the exogenously added signal to the "mute" cells.

Studies of the composition of OMVs revealed the presence of proteins, lipids, DNA and small molecules. As the above examples show, OMVs from different species are capable of delivering each of these components to functionally relevant destinations.

Predatory OMVs

Having established that OMVs were capable of transporting cargo between cells, it was natural to then ask what the purposes of such transport might be. Even before proteomics allowed investigators to make detailed catalogs of their protein cargo, it became apparent that OMVs were enriched in certain proteins while others were excluded. Early reports noted the near absence of peptidoglycan-associated lipoproteins and the enrichment of toxins and virulence factors (see above). These observations spoke to both the formation and function of OMVs and lead Kadurugamuwa and Beveridge to refer to them as "predatory MVs" (Kadurugamuwa and Beveridge 1996). They were proposed to function as offensive weapons that allowed for enhanced competitiveness of the parent organism. Consistent with this characterization, OMVs from many species have been shown to lyse other bacteria when administered exogenously or produced in mixed culture. As described above, Kadurugamawa and Beveridge showed functional delivery of peptidoglycan hydrolase activity to the cell walls of both Gram-positive and Gram-negative organisms via *P. aeruginosa* OMVs, facilitating the lysis of both cell types (Kadurugamuwa and Beveridge 1996). The same group (Li et al. 1998) later screened OMVs purified from different organisms to test their lytic effectiveness against a partially overlapping panel of target organisms. The OMVs were most effective at lysing strains with the same peptidoglycan

chemotype as the parent organism, though in cases where OMVs were tested back against the actual parent strain there appeared to be protection mechanisms in place to mitigate damage. This supported the idea that OMVs could be used to compete with 'non-self' neighbors for limited resources. In fact, Mashburn et al. reported that *P. aeruginosa* could convert its neighbors *into* growth resources should conditions require it (Mashburn et al. 2005). Using a dialysis sac implanted into the peritoneum of a rat, it was shown that *P. aeruginosa* could lyse *S. aureus in vivo* and that such lysis resulted in *P. aeruginosa* no longer perceiving the environment as iron limited. A mutant deficient in PQS production could not relieve iron limitation in this way. Since PQS is central to OMV formation in *P. aeruginosa* (see below) and such OMVs contain the majority of observed *S. aureus* killing power (Mashburn and Whiteley 2005), it is reasonable to assume that OMVs play a role in this process. Consistent with this, both PQS biosynthetic mutants (Mashburn et al. 2005; Mashburn and Whiteley 2005) and wild type *P. aeruginosa* grown in the absence of O_2 (an essential substrate for PQS production (Schertzer et al. 2010)) showed loss of OMV production and diminished lysis of Gram-positive organisms *in vitro*.

Outside of the pseudomonads, recent reports have also confirmed the antibacterial potential of OMVs. In *Myxococcus xanthus*, OMVs were able to lyse *E. coli* by delivering a combination of proteases, phosphatases and hydrolases (Evans et al. 2012). Killing was enhanced when co-treated with glyceraldehyde-3-phosphate dehydrogenase, which acted to enhance fusion of the OMVs with *E. coli*. Similarly, *Lysobacter* OMVs were able to kill *S. aureus* and *Erwinia marcescens* by specifically packaging bacteriolytic endopeptidase L5 into OMVs and delivering it to the target cells (Vasilyeva et al. 2008). It is noteworthy that the endopeptidase was only effective against *Erwinia* when presented in OMVs. Soluble L5 had no lytic effect, highlighting the effectiveness of OMV fusion in depositing degradative enzymes inside the outer membrane of Gram-negative targets.

Across species, we see that OMVs are enriched in agents that are lytic against other bacteria. These hydrolytic "bombs" (Kuehn and Kesty 2005) likely play an important role in bacterial ecology by allowing certain species to prey on their neighbors.

Virulence

The offensive impact of OMVs is not limited to interactions between bacteria; the use of these versatile structures to deliver toxins and virulence factors across kingdom boundaries is also well documented. Advances in proteomics have lead to a wealth of studies demonstrating the presence of virulence factors in OMVs (see above). While this is certainly suggestive of a role for OMVs in virulence, recent work has confirmed that purified OMVs alone are effective at inducing virulence in animal models or cultured cell lines—sometimes more effectively than the purified virulence factors themselves. OMVs purified from *Serratia marcescens* were found to be strongly toxic in a moth killing model of virulence (McMahon et al. 2012). When injected into the haemocoel of *Galleria mallonella* larvae, the OMVs caused

100 % larval death after 24 h. The observed killing effect was attributed to virulence factors contained within the OMVs since injection of equivalent amounts of free lipopolysaccharide had a greatly reduced effect (25 % killing). Several studies have shown that OMVs are capable of inducing pathology or death in mammalian cells as well. Using rounding of Y1 adrenal cells as a readout, Horstman and Kuehn were able to show that heat-labile enterotoxin packaged into enterotoxigenic *E. coli* OMVs was active in a time and dose-dependent manner (Horstman and Kuehn 2000). Similarly, cytolethal distending toxin (CDT) secreted in OMVs from *Campylobacter jejuni* (Lindmark et al. 2009) or *A. actinomycetemcomitans* (Rompikuntal et al. 2012) was effective at inducing cell cycle arrest in human intestinal epithelial cells or cellular distension in HeLa and human gingival fibroblast cells, respectively. These effects were not observed when OMVs from mutant strains defective in biosynthesis of CDT were used.

More dramatic effects can also be seen. For example, OMVs purified from *A. actinomycetemcomitans* (Demuth et al. 2003; Kato et al. 2002), *E. coli* (Wai et al. 2003), *Kingella kingae* (Maldonado et al. 2011) and *P. aeruginosa* (Bomberger et al. 2009) have been shown to lyse cultured human cells upon exposure. Interestingly, cell-free supernatants from WT *P. aeruginosa* PA01 were more effective at killing hybridoma cells if the bacterium was grown at high vs. low oxygen tension (pO_2) before OMV harvest (Sabra et al. 2003). This effect correlated well with the increased number of OMVs that were produced under high pO_2 conditions. Conceptually similar results were seen in *P. aeruginosa* PA14 grown anaerobically, where loss of OMV production due to arrest of PQS production (substrate limitation) was correlated with reduced killing of *S. epidermidis* (Schertzer et al. 2010).

The examples above show that OMVs from various organisms carry the necessary components to elicit virulence phenotypes, but are they the true vehicle for these factors *in vivo*? One way to answer that is to look at the *effectiveness* of OMV-delivered virulence factors. In a number of cases, virulence factors delivered in OMVs have been found to be more effective than when administered directly. While studying OMVs from *E. coli* and other enterobacteria, Wai et al. showed that the OMVs (containing the ClyA toxin) caused much earlier cytotoxicity against HeLa cells and had nearly 10-fold the cytolytic activity against erythrocytes than an equivalent amount of purified ClyA (Wai et al. 2003). Similarly, the CFTR Inhibitory Factor (cif), a toxin found in OMVs from *P. aeruginosa*, was found to be strikingly more effective when delivered in OMVs (Bomberger et al. 2009). In this case, similar levels of CFTR inhibition required 17,000-fold less toxin when delivered in OMVs than when administered in soluble form (3 ng vs. 50 μg, respectively).

As was seen with their antibacterial effects, OMVs from a number of species are capable of negatively effecting eukaryotic cells as well, to the point of facilitating lysis in many cases. The fact that virulence factors are often preferentially packaged within OMVs and that a number of them have been found to be more effective when delivered that way suggest that OMVs do play a role in the virulence of many important Gram-negative pathogens. In this vein, it is noteworthy that more virulent strains of a given species tend to produce significantly more OMVs (Horstman and Kuehn 2000; Lai et al. 1981; Wai et al. 1995).

Biofilms

Biofilms are defined as structured, surface-attached communities encased in a complex, self-produced matrix of extracellular polymeric substances (EPS). This mode of growth is predominant in nature (Costerton et al. 1995) and is a feature of many bacterial infections (Costerton et al. 1999). Growth in a biofilm protects a bacterium from grazing amoeba in natural settings, biocides in industrial settings, potent antimicrobial agents in clinical settings and elements of the immune system while inside a host (Costerton et al. 1999). Biofilm maturation is a developmental process comprised of several defined steps (reversible attachment, irreversible attachment, maturation, dispersion) involving the differential expression of a large number of genes and the involvement of small molecule signals (O'Toole et al. 2000; Sauer 2003; Stoodley et al. 2002). Critical to the development of mature biofilms is the assembly of a multicomponent amalgam known as the extracellular matrix. The composition of the matrix has received considerable investigation in several species and is generally considered to consist of polysaccharides, protein filaments and extracellular DNA (eDNA) (Flemming and Wingender 2010; Karatan and Watnick 2009). Despite extensive investigation into matrix composition, one area that has not been well studied is the contribution of lipids and membrane structures/particulates to the assembly and maintenance of the biofilm. This is a conspicuous oversight considering that electron micrographs of dental biofilms taken nearly 40 years ago identified vesicles and other membranous structures as matrix constituents (Halhoul and Colvin 1975).

Freeze-substitution preparation techniques for electron microscopy allowed this issue to be revisited in greater detail. Rapid vitrification of biofilm samples allows for the avoidance of several issues associated with more traditional preparation techniques, including the reconfiguring of proteins, extraction of many lipids and collapse of the EPS matrix. The Beveridge group made use of this technique to examine thin sections of *P. aeruginosa* biofilms to confirm the presence of OMVs in the EPS matrix (Hunter and Beveridge 2005). Further investigations revealed that OMVs were abundant in *P. aeruginosa* biofilms grown in the laboratory using a variety of biofilm reactors and also in natural multispecies biofilms sampled from domestic water drains, sewage and water treatment plants, pulp and paper manufacturers, freshwater fish aquariums, water storage tanks and riverbeds (Schooling and Beveridge 2006). In each case, OMVs were seen to be heterogeneously distributed throughout the entire volume of the biofilm (mixed-species biofilms exhibited greater heterogeneity owing to the uneven distribution of Gram negative bacteria throughout the population). Interestingly, OMVs harvested from the biofilm matrix displayed a number of different characteristics from those harvested from planktonic cultures. They were seen to be more abundant, smaller in diameter, had differences in protein profiles and were more proteolytically active (Schooling and Beveridge 2006). OMVs were even more abundant in the matrix of *M. xanthus* biofilms and appeared to be distributed in a non-random manner (Palsdottir et al. 2009). There is no reason to expect that the OMV-related functions described in the above sections

should be absent in biofilms, but the observed differences and greater abundance of OMVs in biofilms suggest that they could play additional roles in this context.

One such biofilm-specific function could be in the formation or maintenance of the biofilm structure itself. Very little direct investigation has been carried out on this subject but it was observed in *H. pylori* that biofilm formation correlated strongly with the amount of OMVs present and that exogenous addition of OMVs enhanced biofilm formation (Yonezawa et al. 2009). In agreement with this, increased OMV formation in strains of *P. putida* was correlated with enhanced biofilm formation (Baumgarten et al. 2012a). The discovery that the Pseudomonas Quinolone Signal (PQS) physically stimulates OMV formation in *P. aeruginosa* (Mashburn and Whiteley 2005) now allows us to connect a number of related observations and offers a possible handle for future studies in this area. Several studies revealed that mutants in PQS biosynthesis failed to form mature, differentiated biofilms and instead remained as thin, flat structures reminiscent of the early irreversibly-attached stage of development (Allesen-Holm et al. 2006; Müsken et al. 2010; Yang et al. 2009). Further, exogenous addition of PQS has been shown to enhance biofilm formation (Diggle et al. 2003). A number of explanations for the involvement of PQS in biofilm formation have been proposed, including simple loss of PQS-mediated QS signaling or the mobilization of a prophage resulting in cell lysis and release of eDNA for matrix formation (Allesen-Holm et al. 2006; D'Argenio et al. 2002). In light of the OMV-related observations above and the multiple independent roles of PQS (in QS and OMV formation) it is possible that the OMVs themselves may play a role in the maturation of biofilms and that the PQS-related observations stem from the loss or gain of OMV formation rather than QS gene regulation or phage-mediated cell lysis. Of course, these models need not be mutually exclusive as OMVs are known to possess lytic activity (Kadurugamuwa and Beveridge 1996) that could contribute to the release of eDNA and documented interactions between biofilm OMVs and eDNA that alter the characteristics of both (Schooling et al. 2009) suggest cooperative interactions between these two matrix components.

Mechanism of Formation

Loosening of the Outer Membrane

Since it was established that OMV production is not the result of cell lysis (see above), many hypotheses have been proposed to explain how intact cells can safely and predictably release portions of their OM as vesicles. The simplest explanation for the elaboration of OMVs from growing cells would be that construction of the OM or its connection to the underlying PG might periodically become misregulated, resulting in shedding of unnecessary and/or poorly attached segments of the OM. Electron micrographs of dividing *E. coli* revealed the frequent presence of OM blebs localized to the region that would become the septum (Burdett and Murray 1974a, b). Large septal blebs were also documented in *Salmonella* strains

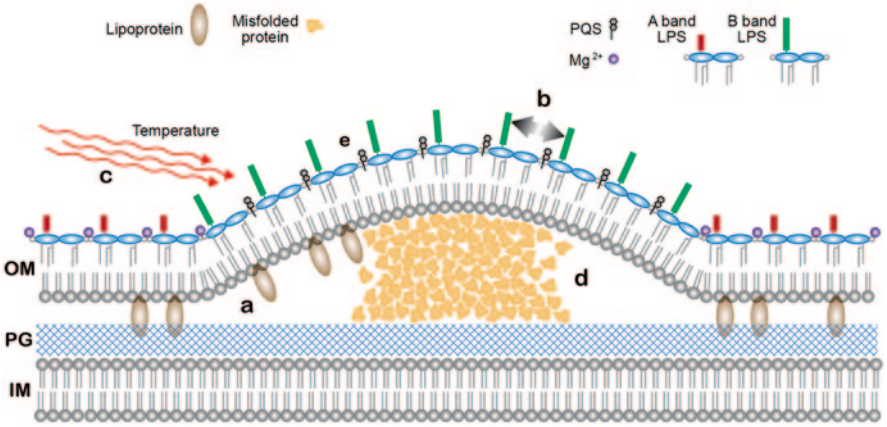

Fig. 9.4 Factors Associated with OMV Formation. Observations from many Gram-negative bacterial species have prompted the development of several models to describe OMV formation. The illustration depicts major features that are included in one or more of these models. **a** Loss of contacts between the OM and PG contribute to loosening of the OM. **b** Charge repulsion between OM constituents force them apart, inducing membrane curvature in areas where they are concentrated. **c** Temperature changes affect the fluidity of the OM, altering its characteristics and impacting OMV formation. **d** The accumulation of misfolded proteins in the periplasm (or other envelope stresses) initiate genetic responses that promote the release of OMVs. **e** Intercalation of membrane-active molecules, like PQS, into primarily one leaftlet of the OM causes asymmetric membrane expansion and induces the curvature necessary to initiate OMV formation. Reproduced with permission from (Schertzer and Whiteley 2013)

(Deatherage et al. 2009). Initial interpretation of these results was to propose that the OM had become "loosened" from the remaining constituents of the cell envelope to allow for invagination of the septum.

Though the elaboration of large vesicles was commonly seen at the septal plane, it was clear from the electron micrographs presented that OMVs could also originate at sites along the cell body (Burdett and Murray 1974a, b; Deatherage et al. 2009). Thus a septum-specific mechanism could not explain all natural vesiculation. Clues to a more general model were obtained by investigating the lipoprotein content of released OMVs. Several groups reported that purified *E. coli* OMVs contained very little murein lipoprotein when compared to isolated OM and also had reduced levels of a number of structural proteins responsible for tethering the OM to PG (Hoekstra et al. 1976; Wensink and Witholt 1981). Further, lipoprotein that was seen in OMVs was predominantly in the unbound form, which would presumably not have contributed to OM attachment before liberation of the OMV (Wensink and Witholt 1981). The absence of such tethering proteins in OMVs lead to the idea that, regardless of their position on the cell, sites of low OM-PG attachment would be prime locations for OMV formation to begin (Fig. 9.4a). The driving force of OMV formation was proposed to be mismatched growth between the OM and PG, where excess OM could then be shed from these low-attachment sites. Disruption of many genes coding for specific tethering proteins has resulted in overproduction of OMVs. The importance of PG-bound Braun's lipoprotein was confirmed (Bernadac

et al. 1998; Weigand et al. 1976; Yem and Wu 1978), but other important contributors were also identified. Loss of a protein homologous to *E. coli* OmpA frequently elicits hyper-vesiculation (Moon et al. 2012; Song et al. 2008; Sonntag et al. 1978; Wessel et al. 2013). Interestingly, though increased production of OMVs can often be complemented by overexpression of other OM-PG tethering proteins, this was not the case for OmpA (Deatherage et al. 2009). Large, multicomponent structures such as the Tol-PAL complex also contribute to maintaining OM stability (Bouveret et al. 1995). Accordingly, mutants that disrupt this complex have been associated with increased OMV formation (Bernadac et al. 1998; Cascales et al. 2002).

Connections between the OM and PG could also be compromised by unbalanced remodeling of PG by autolytic enzymes. Zhou et al. identified the presence of PG turnover products (most likely low molecular weight muramyl peptides) in OMVs from *P. gingivalis*, suggesting that PG remodeling takes place at or near the sites of OMV formation. Accumulation of these PG intermediates was proposed to generate periplasmic turgor that encouraged the formation of OMVs by pushing the OM outwards at localized areas of low OM-PG connectivity (Zhou et al. 1998). OMV formation was also connected to cell wall turnover using an autolysin mutant of the same organism (Hayashi et al. 2002). Disruption of the *ami* autolysin gene resulted in increased release of OMVs compared to wild type, as would be consistent with the buildup of partially degraded PG intermediates or imbalance between the rates of growth of PG vs. OM.

In Gram-negative bacteria, the OM is connected to the cell wall through an extensive network of protein contacts. It makes sense that release of OM fragments as vesicles would involve loss or remodeling of these connections and this feature has been assumed to participate to a greater or lesser extent in all subsequent models of OMV biogenesis.

Physical Properties of Outer Membrane Components

A second group of OMV-formation models focuses on the physical characteristics of and interactions between lipids in the OM. The OM provides a significant permeability barrier in Gram-negative cells, owing largely to the differences in physical properties between LPS (particularly lipid A) and phospholipids. Additionally, ionic cross-bridging by divalent cations contribute to strong lateral interactions between LPS molecules that can substantially raise the melting temperature of the membrane (reviewed in (Nikaido 2003)). Consequently, phenomena that impact the physical characteristics of LPS are expected to contribute to OM stability and OMV biogenesis. It is well established that mutant strains lacking OM-PG connections are strongly sensitive to divalent cation concentration and that treatment with chelating agents compromises the integrity of these cells (Sonntag et al. 1978; Yem and Wu 1978). In wild type *E. coli*, EDTA treatment induced shedding of greater than 10% of cellular LPS into the culture supernatant together with OM proteins commonly found in OMVs (Marvin et al. 1989). The released LPS was believed to have originated from patches of the OM that had blebbed and sheared off from

the cell. As a corollary, lipoprotein mutants were stabilized by addition of excess Mg^{2+}, and the amount of OMVs produced was reduced (Fung et al. 1978; Suzuki et al. 1978). Aminoglycoside antibiotics that disrupt OM integrity while stimulating OMV formation have also been reported to displace Mg^{2+} from the cell surface (Kadurugamuwa and Beveridge 1995, 1996, 1997; Walker and Beveridge 1988), suggesting that weakening interactions between surface lipids and disrupting LPS packing order could be one route to OMV formation.

Interactions between the O-antigen portions of LPS were implicated in OMV formation when Beveridge's group documented the preferential packaging of B-band LPS into *P. aeruginosa* OMVs (Kadurugamuwa and Beveridge 1995). Because the highly anionic B-band LPS appeared to be concentrated at the sites of OMV formation in the OM, these authors proposed a mechanism involving electrostatic repulsion between the O-antigen portions of LPS that would force the underlying lipid portions to press together and generate localized membrane curvature (Fig. 9.4b) (Kadurugamuwa and Beveridge 1996, 1997). This curvature induction would then serve to initiate the formation of B-band-rich blebs that would be released as OMVs. However, further work by the same group showed that *P. aeruginosa* mutants lacking B-band LPS (or lacking both A- and B-band LPS simultaneously) remained capable of liberating OMVs and thus emphasized the contributions of ionizable groups in the lipid and core oligosaccharide regions of LPS (Nguyen et al. 2003). Similarly, the O-antigen portion of LPS in *P. gingivalis* does not appear to be a determining factor in OMV generation (Haurat et al. 2011). Nevertheless, alterations in the identity and potential interactions between LPS molecules in *P. aeruginosa* did affect the amount and physical properties of OMVs produced (Nguyen et al. 2003).

The properties of the OM can also be affected by temperature (Fig. 9.4c). In general, membranes transition from a rigid gel-like structure to a more fluid liquid crystalline structure as the temperature is increased. Interestingly, OMV production can be induced or inhibited by higher temperatures. In *E. coli* and *P. putida*, shocking cells by rapidly heating to 55°C quickly resulted in increased production of vesicles (Baumgarten et al. 2012a, b; Katsui et al. 1982). Analysis of the phospholipid, fatty acid and protein content of the released vesicles confirmed that they originated from the outer membrane. The severity of the heat shock was critical, as rapidly heating cells from 0 to 37 °C did not induce OMV production in *E. coli* (Katsui et al. 1982), nor did temperature shifting from 25 to 39 °C in *P. aeruginosa* (Macdonald and Kuehn 2013). Conversely, McMahon et al. observed that fewer OMVs were naturally produced in *S. marcescens* culture supernatants when cells were grown at 37 °C versus either 22 or 30 °C (McMahon et al. 2012). Indeed, increased temperature even worked against EDTA-induced OMV formation when the two stressors were combined (Marvin et al. 1989). In this case, the authors proposed that temporary disruptions in OM structure induced by the EDTA treatment were quickly reversed by redistribution of OM components and that this redistribution was favored at elevated temperature. This explanation reinforces the idea that membrane fluidity is important in OMV formation, as has been suggested for *P. aeruginosa* (Mashburn-Warren et al. 2008; Tashiro et al. 2011).

Envelope Stress Response

So far only physical aspects that contribute to the biogenesis of OMVs have been discussed. Evidence exists that formation of OMVs may also be under regulatory control and associated with envelope stress. McBroom et al. conducted a screen of *E. coli* transposon mutants with the aim of identifying genes involved in OMV formation (McBroom et al. 2006). Overproduction mutants were plentiful and often corresponded to disruptions of genes involved in maintaining envelope structure, but few underproducing mutants were identified. Interestingly, several of the gene disruptions resulting in strong overproduction of OMVs were involved in the σ^E envelope stress pathway. Because some of the disruptions would seemingly affect the σ^E pathway in opposite directions (*degS*/*degP* vs. *rseA*), the authors noted that whether one caused impairment or hyperactivation of the σ^E pathway, both would result in increased protein in the periplasm. Misfolded proteins would accumulate in an impairment mutant, and σ^E effectors would be overproduced in a hyperactivation mutant. Following up on this work, the same group showed that the extent of OMV production correlated with the level of protein accumulation in the cell envelope and that a peptide engineered to resemble a misfolded protein was selectively expelled via OMVs (McBroom and Kuehn 2007). This led to the proposal that release of OMVs by Gram negative bacteria is a novel envelope stress response (McBroom and Kuehn 2007) (Fig. 9.4d). This idea is supported by the finding that the *Vibrio cholerae* small RNA *vrrA*, which impacts OMV formation by repressing *ompA* translation, requires σ^E activation for its own expression (Song et al. 2008). Similarly, disruption of *S. marcescens* enterobacterial common antigen synthesis resulted in a hypervesiculation response that appeared to be regulated through the Rcs phosphorelay system (McMahon et al. 2012). Thus, a number of regulatory pathways have been implicated in OMV biogenesis.

Some observations conflict with the idea of OMV formation as a transcriptionally regulated response to external stress. In one study, the kinetics of OMV formation induced by heat shock or small molecule exposure was too rapid to be explained by transcriptional regulation (Baumgarten et al. 2012a). Studies have also shown that OMV release is induced by similar stressors in the presence of a number of different protein synthesis inhibitors, demonstrating an independence from *de novo* protein synthesis (Baumgarten et al. 2012a; Mashburn and Whiteley 2005). Together, the above observations suggest that stress-induced OMV formation may be a strictly physical process in some cases, or may involve purely biochemical regulation of existing enzyme systems. Accordingly, it has been noted that multiple mechanisms of OMV formation likely exist (Macdonald and Kuehn 2013; Tashiro et al. 2009) and that stress-induced OMV formation may occur by a mechanism that differs from that of the 'typical' vesiculation process (McBroom et al. 2006).

Insertion of Small Molecules into the Outer Membrane

It is important to understand how envelope stresses, whether caused by loss of OM-PG contacts or periplasmic calamities, can influence OMV formation. However, these models for OMV formation have difficulty explaining how wild type Gram-negative organisms reproducibly generate OMVs under a wide array of conditions, including those where stress to the organism appears to be minimal. Thus, there must be a 'natural' mechanism for generation of OMVs that operates either separately or with integration into any stress-induced paradigms that may also exist.

While much research in other species has focused on stress responses and other genetic programs, the emphasis in *P. aeruginosa* has remained largely on physical mechanisms. Important early work suggesting that small molecules could influence OMV formation by interacting with the OM was performed using the aminoglycoside antibiotics amikacin and gentamicin (Kadurugamuwa and Beveridge 1995; Walker and Beveridge 1988). These antibiotics were observed to disrupt the normal packing of the OM and interfere with cation cross-bridging between LPS molecules (see above). The recent discovery that the Pseudomonas Quinolone Signal (PQS) physically stimulates OMV formation in *P. aeruginosa* (Mashburn and Whiteley 2005) demonstrated that a self-produced small molecule can direct OMV formation and suggested a mechanistic route to controlled OMV formation in the absence of stress. As PQS is much more hydrophobic than other *P. aeruginosa* QS signals (clogP = 4.34 ± 0.6, (Schertzer and Whiteley 2012)), it was hypothesized that it might traffic between cells dissolved into the membrane of OMVs. It was confirmed that greater than 85 % of PQS in *P. aeruginosa* culture is localized within OMVs and that purified OMVs are capable of delivering PQS to recipient cells (Mashburn and Whiteley 2005). An unexpected finding from this study was that PQS stimulates the formation of the very OMVs into which it is packaged. Since PQS is a QS signal in *P. aeruginosa*, its detection by a specific receptor is known to affect gene expression in this organism (Pesci et al. 1999). To be sure that PQS-induced OMV formation was not the result of a QS-responsive genetic program, Mashburn and Whiteley showed that exogenously added PQS stimulated OMV formation in both tetracycline-treated WT cells and in a PQS receptor mutant strain (*pqsR*$^-$) (Mashburn and Whiteley 2005). Further, PQS has been shown to stimulate OMV formation in other Gram-negative organisms and even in Gram-positive *Bacillus subtilis* (Mashburn-Warren et al. 2008; Tashiro et al. 2010). These organisms are unlikely to possess specific receptors for PQS or to participate in Pseudomonas-specific QS pathways, lending support to the idea that PQS induces OMV formation through a strictly physical mechanism.

The first insights into how this occurs were gained by examining the interactions of PQS with LPS. PQS was found to strongly interact with the acyl chains and 4'-phosphate of lipid A in LPS, as well as lower membrane fluidity (Mashburn-Warren et al. 2009; Mashburn-Warren et al. 2008). Importantly, no such interactions occurred between LPS and HHQ, the immediate precursor to PQS and a molecule known not to induce OMV formation in *P. aeruginosa* or other bacteria. Using the red blood cell model of Sheetz and Singer (Sheetz and Singer 1974), PQS was

shown to have an inherent ability to bend membranes into which it has dissolved (Schertzer and Whiteley 2012). The proposed mechanism involves accumulation of PQS into only one leaflet of the membrane, which causes expansion of that leaflet relative to the other and ultimately induces membrane curvature to alleviate the resulting physical strain (Fig. 9.4e). The strong interactions between PQS and LPS are believed to play an important role in reducing interleaflet flip-flop of PQS and therefore promoting this 'bilayer-couple' mechanism (Schertzer and Whiteley 2012).

In contrast to models that cast OMV formation as a reaction to external events, a mechanism involving induction by self-produced small molecules could explain baseline or 'natural' vesiculation that may operate in parallel to other pathways. Such a system can easily be generalized to other organisms, though so far PQS remains the only molecule known to have such function. Further, the basic 'bilayer-couple' mechanism may be relevant in explaining some stress-induced responses, such as the packaging and expulsion of membrane perturbing toxins in *P. putida* OMVs (Baumgarten et al. 2012b; Kobayashi et al. 2000).

Manipulation and Applications of OMVs

Production

OMV biogenesis is a fundamental bacterial secretion process that is involved in virulence and many other cell-cell interactions. Accordingly, much of the research effort in this area has focused on biological aspects and their impact on bacterial physiology and disease. However, being small cargo packages with surface characteristics nearly identical to live cells, OMVs also present exciting possibilities in applied science as delivery vehicles and vaccine agents. Whether for use in research or other applications, OMVs must first be produced and purified. Several approaches can be used for this, depending on need.

The OMVs best suited for fundamental research are those naturally produced by the organism itself, often termed "natural OMVs" or nOMVs. Production of these vesicles requires only growing the organism to late-logarithmic or stationary phase and collecting the cell-free supernatant. However, depending on the strain, growth conditions or end use of the OMVs, simply waiting for the organism to produce them spontaneously may not yield sufficient material. For this reason, several techniques have been developed to increase OMV yield, though vesicles produced by these methods often have different characteristics from nOMVs and must be considered "non-native" (Guthrie et al. 2004; Kulp and Kuehn 2010). The most disruptive preparation techniques use detergent extraction and/or physical shearing of cells to generate vesicles (Claassen et al. 1996; Drabick et al. 2000; Fredriksen et al. 1991). This produces scrambled vesicles containing material from all compartments of the cell and is routinely used only in vaccine development (for reasons specific to this end—see below). Milder treatments can be used to increase the yield of OMVs that

more closely resemble nOMVs. Some of the earliest analyses of OMVs were done on material overproduced by lysine-starved *E. coli* (Bishop and Work 1965; Knox et al. 1966; Taylor et al. 1966) and recent work has shown that cysteine depletion can induce the onset of stationary growth, oxidative stress and increased OMV production in *N. meningitidis* (van de Waterbeemd et al. 2013b). OMVs overproduced by treatment with chelating agents (Marvin et al. 1989) and heat shock (Baumgarten et al. 2012a, b; Katsui et al. 1982) have been well characterized and these procedures have also been combined to increase yield (Saunders et al. 1999). As a byproduct of trying to understand how they are formed, it has been established that OMVs can be used by some bacteria to concentrate and expel membrane-perturbing agents. For example, treatment of *P. aeruginosa* and *P. putida* with gentamicin (Kadurugamuwa and Beveridge 1995) and toluene (Kobayashi et al. 2000), respectively, result in increased OMV production. If such compounds are desired or can be tolerated in downstream applications, these methods can be used effectively. A similar but more general approach could include treatment of cells with exogenous PQS, a secondary metabolite involved in natural OMV formation in *P. aeruginosa* that has also been shown to induce OMV production in a range of other organisms (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008; Tashiro et al. 2010).

Though technically straightforward, external treatments are not the only means to overproducing OMVs. The identification of numerous overproduction mutants in *E. coli* (McBroom et al. 2006), for example, presents the option of using engineered strains to generate OMVs at high-yield that may avoid problems associated with specific chemical treatment options. With such a diverse selection available, it is likely that a suitable route to OMV production can be found for nearly any undertaking.

Purification and Quantification

Regardless of how OMVs are produced, they nearly always must be separated from cells and other secreted products before they can be used. Depending upon the level of purity required, several methods can be employed; but all make use of the inherent size and/or buoyancy of OMVs. The most straightforward approach involves differential centrifugation to separate OMVs from parent cells and soluble secreted proteins (Wai et al. 1995). Intact cells are first removed from the culture in a low-speed spin. The OMVs are then sedimented from the culture supernatant in a high-speed ultracentrifugation step. Often the cell-removal centrifugation is accompanied by filtration steps to further ensure the OMV preparation is not contaminated by whole cells (Kadurugamuwa and Beveridge 1995). In addition, OMVs can be concentrated at various steps by precipitation with ammonium sulfate (Bauman and Kuehn 2006; Wai et al. 1995), though this runs the risk of artificially inducing non-specific binding of extracellular proteins to the OMVs (Kulp and Kuehn 2010). Simple differential centrifugation can result in sedimentation of large protein structures such as pili and flagella along with OMVs (Allan et al. 2003; Bauman and Kuehn 2006; Lee et al. 2007), so more involved protocols have been developed for sensitive applications like OMV proteomics (Lee et al. 2008).

The most widely used of these approaches is to float OMVs in a density gradient (usually sucrose) after differential centrifugation (Allan et al. 2003; Horstman and Kuehn 2000; Lee et al. 2007). Because of their composition, OMVs have a buoyant density different from the cytoplasmic membrane and protein structures like flagella. Therefore density gradient separation is effective at separating OMVs from large macromolecular contaminants and also cell fragments that may have survived filtration and differential centrifugation. Another lesser-used but interesting approach is to purify OMVs by size exclusion chromatography (Post et al. 2005). This method is particularly effective when the OMVs to be purified are relatively homogeneous in size.

Once OMVs have been produced and purified, they can be quantified in a number of ways that vary in effectiveness depending on the presence or absence of contaminants. Early methods included quantification of OMVs by weight (Kadurugamuwa and Beveridge 1995) or protein abundance (Kadurugamuwa and Beveridge 1995; Mashburn and Whiteley 2005). These methods are straightforward but protein abundance measurements can be greatly affected by the method of preparation since the most common method, differential centrifugation, may sediment large protein structures in additions to OMVs (see above). To avoid this problem, many groups have quantified OMVs on the basis of recovered lipid (Schertzer et al. 2010; Tashiro et al. 2009). Though the method employed was originally designed to quantify phospholipids (Stewart 1980), which are not specific to OMVs or even the OM, this remains an effective tool. The most effective method might be to quantify OMVs on the basis of the amount of LPS recovered in the preparation (Allan et al. 2003; McMahon et al. 2012). This is typically done by quantifying the KDO sugar that is unique to LPS (Karkhanis et al. 1978; Marolda et al. 2006). Though a very specific method to quantify OMVs, this technique does require knowledge of the structure of LPS from the organism of interest to ensure KDO will be effectively liberated by the chemical treatments involved.

Tailoring OMVs for Specific Applications

OMVs are fascinating biological structures and purified nOMVs have many natural properties that may be of use in applied science. For instance, it has be known for many years that OMVs possess strong lytic activity and this has prompted the suggestion that simply purifying nOMVs could provide a conceptually new form of antibiotic (Kadurugamuwa and Beveridge 1996). As more is learned about how cargo is selected and packaged into OMVs and how the vesicles are then targeted and fuse to target cells, such a proposition may become feasible. However, to be a truly attractive platform OMVs would need to be customizable or at least allow for flexibility in their properties. To this end, several groups have shown that both the lipid and protein content of OMVs can be modified by chemical or genetic means.

The most firmly established application of OMVs has been in the development of vaccines. However, a significant hurdle to their successful use was overcoming the toxic abundance of endotoxin (LPS) in the vesicle membranes themselves.

Table 9.1 Select studies showing immunogenic properties of OMVs and their potential for vaccine use

Organism	Route	Recipient	Result	Reference
Serogroup B *Neisseria meningitis*	Intra-muscular	Humans	Immunity conferred	(Sierra et al. 1991) (Fredriksen et al. 1991) (Bjune et al. 1991) (Boslego et al. 1995)
Haemophilus influenzae (NTHi)	Intranasal	BALB/c mice	Cross-protective immunity conferred	(Roier et al. 2012)
Vibrio Cholerae	Multiple routes	BALB/c mice	Immunity conferred and transferred to offspring	(Schild et al. 2008)
Bordetella Pertussis	Multiple routes	BALB/c mice	Immunity conferred	(Roberts et al. 2008)
Shigella Flexneri	Multiple routes	BALB/c mice	Immunity conferred	(Camacho et al. 2011)
Burkholderia pseudomallei	Sub-cutaneus	BALB/c mice	Immunity conferred	(Nieves et al. 2011)
Acinetobacter baumannii	Intra-muscular	C57BL/6 mice	Immunity conferred	(McConnell et al. 2011)
Edwardsiella tarda	Intra-peritoneal	Olive Flounder Paralichthys olivaceus	Immunity conferred	(Park et al. 2011)
Porphyromonas gingivalis	Intranasal	BALB/c mice	Humoral Immune Response	(Nakao et al. 2011)
Enterotoxigenic Escherichia coli	Intranasal	CD-1 mice	Humoral Immune Response, Reduced Intestinal Colonization	(Roy et al. 2011)
Brucella melitensis	Intra-peritoneal	BALB/c mice	Reduction in Bacterial Load (Spleen)	(Avila-Calderón et al. 2012)

Removal of this endotoxin was one of the major driving forces behind the development of detergent extraction as an OMV preparation technique. Extraction with deoxycholate was found to reduce endotoxin content in OMVs and render them nonpyrogenic while still retaining several major surface antigens (Fredriksen et al. 1991). Such lipid-modified OMVs have been successfully used in human vaccination trials (Bjune et al. 1991; Claassen et al. 1996; Peeters et al. 1996). Though it reduced toxicity, preparation of OMVs in this manner often led to vesicle aggregation and a narrowing of the immune response (Claassen et al. 1996; van de Waterbeemd et al. 2010). Rather than chemically extracting LPS from OMVs, another strategy has been to genetically engineer strains whose endotoxin is attenuated. *N. meningitidis* is especially tolerant to modifications of the Lipid A biosynthetic pathway and mutants in *lpxL1* and *lpxL2* have been shown to produce partially de-acylated

LPS that stimulates a much weaker TLR4 response but maintains strong immunogenicity (van der Ley et al. 2001; van der Ley and van den Dobbelsteen 2011). Thus OMVs naturally purified from these strains achieve the desired goals without the need for detergent extraction.

Selective alteration of OMV protein cargo has also been an area of great interest. To further enhance *N. meningitidis* OMV vaccine coverage, strains have been engineered to express multiple alleles of immunodominant outer membrane proteins that are packaged into OMVs and then combined with OMVs from other multivalent strains (van den Dobbelsteen et al. 2007; Kaaijk et al. 2013; van der Ley et al. 1995). Other groups have specifically studied the packaging of heterologous proteins into OMVs. Kuehn and colleagues successfully targeted the *Yersinia enterocolitica* Ail adhesion/invasin to *E. coli* OMVs and showed that the engineered vesicles were internalized by human colorectal HT29 cells (Kesty and Kuehn 2004). In the same work periplasmic-targeted GFP was incorporated into OMVs, demonstrating that heterologous proteins can be targeted to both the membrane and lumen of OMVs. Non-meningococcal OMVs have also been shown effective at facilitating antibody responses to target antigens, even those not native to the host bacterium. Salmonella-derived OMVs engineered to contain the pneumococcal protein PspA elicited a protective serum antibody response to PspA in mice that was absent when the protein was injected alone (Muralinath et al. 2011). This enhancement of immunogenicity is a further illustration of the benefit OMVs provide by acting as a delivery vehicle and also an "internal" adjuvant. In a similar study, the poorly-immunogenic protein GFP was made to elicit a strong antibody response by fusing it to the *E. coli* hemolysin ClyA and delivering it to mice in OMVs (Chen et al. 2010). Using a combination of detergent extraction, genetic engineering and natural production, OMVs have been used successfully to combat epidemics in a number of countries (Holst et al. 2009). Though the bulk of investigation in this area has focused on *N. meningitidis*, Table 9.1 lists several studies that show promise for the use of OMVs as vaccines against a number of other organisms as well.

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Chapter 10
Events in Root Colonization by *Pseudomonas putida*

Paloma Pizarro-Tobías, Zulema Udaondo, Amalia Roca and Juan L. Ramos

Abstract Plant growth-promoting rhizobacteria (PGPR) constitute a fraction of the bacterial population favouring seed germination and plant growth and development by increasing the availability of nutrients or producing specific ones. Naturally, one of the most important traits for PGPR strains to be effective is their capacity to colonize and survive in the rhizosphere once they are released in the environment.

Pseudomonas putida is a natural inhabitant of the rhizosphere, it exhibits chemotactic response towards plant root exudates and forms biofilms on root surfaces. Through solubilisation of nutrients, chelation of iron and inhibition of growth of pathogens *Pseudomonas* favour plant growth.

Keywords Pseudomonas · Lap proteins · biofilms · PGPR · rhizosphere · pangenome · colonisation

Introduction

The rhizosphere, defined as the area influenced by the root system (Hiltner 1904), is a complex environment with longitudinal and radial gradient variations within its physico-chemical and biological properties (Brimecombe et al. 2007; Ahmad et al. 2011). It's there where exudation and nutrient and water uptake take place and where microbial population activity has been reported to be higher than in bulk soil (Molina et al. 2000; Morgan et al. 2005). Root exudates comprise amino acids, organic acids, sugars, vitamins, fatty acids and sterols, nucleotides, proteins and volatile compounds such as carbon dioxide and molecular hydrogen (Ahmad et al. 2011). Although nutrient-rich, the rhizosphere is a changing environment in which

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the inhabitants are exposed to multiple carbon sources, signalling molecules, decomposing aromatic compounds derived from lignins, and secondary metabolites derived from plants and microbes—all of which exert selective pressure over the living organisms inhabiting this niche (Matilla et al. 2007; Attila et al. 2008). The diversity of microbial populations colonizing the root vicinity has been reported to differ significantly from that of bulk soil (Marilley and Aragno 1999; Weisskopf et al. 2005; García-Salamanca et al. 2012 depending on the composition imposed by local soil traits, plant species and root area (Chen et al. 2006). However, microorganisms inhabiting the rhizosphere can act either beneficially or detrimentally on d plant growth.

Plant growth-promoting rhizobacteria (PGPR) constitute a fraction of the bacterial population favouring seed germination and plant growth and development by increasing the availability of nutrients or producing specific ones. Naturally, one of the most important traits for PGPR strains to be effective is their capacity to colonize and survive in the rhizosphere once they are released in the environment.

Pseudomonas putida, which are considered ubiquitous inhabitants of the environment, have been reported as one of the most effective root-colonizing bacteria (Lugtenberg et al. 2001). Members of this genus often live in mutual association with plants in the rhizosphere, where they produce phytohormone precursors, solubilise non bioavailable nutrients like iron and phosphate, and produce compounds with antifungal activity and chemicals that promote systemic resistance. Some Pseudomonads strains with PGPR properties are suitable for agriculture and environmental uses (Roca et al. 2013).

We examined the available literature to determine the genes required for competence, proliferation and stress adaptation of *Pseudomonas putida* to the rhizosphere. We identified 180 genes involved in rhizosphere fitness (Table 10.1). Identified traits included 25 proteins related to the ability of the bacteria to attach to biotic surfaces and biofilm genesis; 13 related to flagella biosynthesis, 2 involved in chemotaxis response; 14 regulators and sensor proteins; 64 proteins involved in stress adaptation and detoxification; 8 proteins involved in DNA repair, 8 proteins involved in transport and secretion processes, 30 related to metabolism, 7 involved in protein synthesis and 9 hypothetical proteins of unknown function.

Attachment, Biofilm Formation and Flagella

A critical issue in root colonization is the ability of *Pseudomonas putida* to recognize plant-root exudates. Using video and microscopy techniques Espinosa-Urgel et al. (2002) showed that *Pseudomonas* cells recognize corn seed exudates and move towards the seeds. Later, a research article by Lacal et al. (2010) revealed that all TCA cycle intermediates present in root exudates are recognized by the McpS chemoreceptor, and that these intermediates are among the signalling molecules that promote motility towards the plant roots. Many approaches have been used to identify genes involved in chemotaxis and attachment of microbes to plant cells;

Table 10.1 Pseudomonas putida genes encoding traits involved in rhizosphere competence. In silico pairing with reciprocal genes found in P. putida BIRD-1 genome

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
Adhesion biofilm formation	*lapD*	PP_0165	PP_0164–PP_0165	0195	Diguanylate cyclase	98.92	0.0	Yousef-Coronado et al. 2008
	lapC	PP_0166	PP_0166–PP_0167	0196	HlyD family type I secretion membrane fusion protein	99.78	0.0	Yousef-Coronado et al. 2008
	lapB	PP_0167		0197	Secretion ATP-binding protein	99.86	0.0	Yousef-Coronado et al. 2008
	lapA	PP_0168	–	0199	Surface adhesion protein	99.97	0.0	Duque et al. 2013; Matilla et al. 2011b; Reva et al. 2006; Roca et al. 2013; Yousef-Coronado et al. 2008
	lapH	PP_0805	PP_0803–PP_0806	0851	TolC family type I secretion outer membrane protein	99.13	0.0	Duque et al. 2013; Roca et al. 2013
	lapF	PP_0806		0852	Putative surface protein	96.67	0.0	Duque et al. 2013; Martínez-Gil et al. 2010; Roca et al. 2013
	algD	PP_1288	PP_1281–PP_1288	4273	GDP-mannose 6-dehydrogenase	99.77	0.0	Reva et al. 2006
	–	PP_1789	PP_1789–PP_1791	0454[d]	HAD superfamily Hydrolase	22.17	0.009	Matilla et al. 2011b; Nilsson et al. 2011
	–	PP_1790		3811[d]	Acylneuraminate cytidylyltransferase	43.48	1.1	Matilla et al. 2011b; Nilsson et al. 2011

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	–	PP_1791		0016[d]	Aldolase	20.81	1e-04	Matilla et al. 2011b; Nilsson et al. 2011
	–	PP_1792	PP_1792–PP_1795	1485	Glycosyl transferase family protein	30.18	2e-23	Matilla et al. 2011b; Nilsson et al. 2011
	–	PP_1793		2597[d]	Glycosyl transferase family protein	26.55	3e-08	Matilla et al. 2011b; Nilsson et al. 2011
	–	PP_1794		–	Hypothetical protein	–	–	Matilla et al. 2011b; Nilsson et al. 2011
	–	PP_1795		0194[d]	Hypothetical protein	22.78	0.21	Matilla et al. 2011b; Nilsson et al. 2011
	bcsQ	PP_2634	PP_2629–PP_2638	3057	Cellulose synthase containing putative MinD/ParA-binding domain	98.72	1e-132	Matilla et al. 2011b; Nilsson et al. 2011
	bcsA	PP_2635		3056	Cellulose synthase catalytic subunit	98.21	0.0	Matilla et al. 2011b; Nielsen et al. 2011; Nilsson et al. 2011
	bcsB	PP_2636		3054	Cellulose synthase regulator protein BscB	98.55	0.0	Matilla et al. 2011b; Nielsen et al. 2011; Nilsson et al. 2011
	bscC	PP_2637		3053	Endo-1,4-D-glucanase/cellulase	99.19	0.0	Matilla et al. 2011b; Nielsen et al. 2011; Nilsson et al. 2011
	peaG	PP_3139	PP_3133–PP_3141	2593	Glycoside hydrolase family protein	96.50	0.0	Matilla et al. 2011b; Nielsen et al. 2011; Nilsson et al. 2011

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	peaH	PP_3140		2592	Glycosyl transferase family protein	98.73	0.0	Matilla et al. 2011b; Nielsen et al. 2011; Nilsson et al. 2011
	peaI	PP_3141		2591	WecB/TagA/CpsF family glycosyl transferase	95.82	7e-14	Matilla et al. 2011b Nielsen et al. 2011; Nilsson et al. 2011
	galU	PP_3821	–	1959	UTP-glucose-1-phosphate uridylyltransferase	99.64	0.0	Yousef-Coronado et al. 2008
	hemN	PP_4264	–	1592	Coproporphyrinogen III oxidase	99.78	0.0	Yousef-Coronado et al. 2008
	–	PP_4934	–	4717	Bifunctional heptose 7-phosphate kinase/ heptose 1-phosphate adenyltransferase	100	0.0	Cheng et al. 2009
	ndvB	PputW619_2133	PputW619_2132–PputW619_2133	3043	Glycosyltransferase - Cellobiose phosphorylase	79.49	0.0	Wu et al. 2011

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
Flagella	*fliA*	PP_4341	PP_4341-PP_4344	1510	DNA-directed RNA polymerase specialized sigma subunit	100	5e-164	Yousef-Coronado et al. 2008
	flhB	PP_4352	PP_4352-PP_4361	1506	Flagellar biosynthesis protein FlhB	99.74	0.0	Duque et al. 2013; Roca et al. 2013
	fliO	PP_4356		1502	Flagellar assembly protein FliO	100	2e-64	Ramos-González et al. 2005
	fliN	PP_4357		1501	Flagellar motor switch protein	99.38	6e-87	Yousef-Coronado et al. 2008
	fliL	PP_4359		1499	Flagellar basal body protein FliL	100	1e-84	Matilla et al. 2007
	fliI	PP_4366	PP_4365–PP_4367	1493	Flagellum-specific ATP synthase	99.78	0.0	Yousef-Coronado et al. 2008
	fliF	PP_4369	PP_4368–PP_4370	1490	Flagellar MS-ring protein—flagellar hook	99.49	0.0	Duque et al. 2013; Roca et al. 2013
	fleQ	PP_4373	–	1470	Fis family transcriptional regulator	100	0.0	Yousef-Coronado et al. 2008
	fliD	PP_4376	–	1467	Flagellar cap protein FliD	46	5e-96	Duque et al. 2013; Roca et al. 2013
	fliC	PP_4378	PP_4377–PP_4378	1465	Flagellin FliC	65.09	1e-57	Duque et al. 2013; Roca et al. 2013
	flgL	PP_4380	PP_4380–PP_4386	1462	Flagellar hook-associated protein FlgL	67.95	0.0	Yousef-Coronado et al. 2008

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	flgD	PP_4389	PP_4387–PP_4391	1453	Flagellar basal body rod modification protein	99.57	2e-132	Yousef-Coronado et al. 2008
	flgB	PP_4391		1451	Flagellar basal-body rod protein FlgB	93.43	2e-81	Matilla et al. 2007
Chemotaxis	–	PP_4331	PP_4331–PP_4334	1520	Hypothetical protein	99.24	9e-73	Matilla et al. 2007
	–	PP_4988	PP_4987–PP_4992	4775	Chemotaxis protein CheA	98.91	0.0	Matilla et al. 2007
Regulators sensor proteins	–	PP_0700	–	0741	FecR anti-FecI sigma factor	99.39	0.0	Matilla et al. 2007
	colS	PP_0902	PP_0901–PP_0902	0953	Integral membrane sensor signal transduction histidine kinase	99.33	0.0	Barret et al. 2011; Ramos-González et al. 2005
	rpoN	PP_0952	PP_0948–PP_0952	1005	RNA polymerase factor sigma 54	99.80	0.0	Duque et al. 2013; Roca et al. 2013
	–	PP_1066	PP_1065–PP_1070	1116	Fis family transcriptional regulator	100	0.0	Matilla et al. 2007
	gacS	PP_1650	PP_1647–PP_1650	3966	Sensor protein GacS—multi-sensor hybrid histidine kinase	99.35	0.0	Duque et al. 2013; Roca et al. 2013
	–	PP_2070	–	3583	AraC family transcriptional regulator	90.25	2e-141	Ramos-González et al. 2005
	–	PP_2127	PP_2126–PP_2127	3526	Sensor histidine kinase (signal transduction)	97.44	0.0	Matilla et al. 2007
	–	PP_3640	–	2149	AraC family transcriptional regulator	92.08	0.0	Matilla et al. 2007

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	–	PP_4295	–	1561	TetR family transcriptional regulator	99.51	1e-137	Matilla et al. 2007
	pfrI	PP_4424	–	4559[d]	AsnC family transcriptional regulator	34.21	1e-25	Ramos-González et al. 2005
	–	PP_4508	–	2668	AraC family transcriptional regulator	99.71	0.0	Matilla et al. 2007
	–	PP_4959	PP_4957–PP_4959	4743	Response regulator receiver modulated diguanylate cyclase/phosphodiesterase	98.87	0.0	Matilla et al. 2007; Matilla et al. 2011b
Regulators sensor proteins	–	PP_4966	PP_4966–PP_4967	4750	ArsR family transcriptional regulator	100	0.0	Ramos-González et al. 2005
	phoR	PP_5321	PP_5320–PP_5321	5113	PAS/PAC sensor signal transduction histidine kinase	98.85	0.0	Matilla et al. 2007
Stress adaptation detoxification transporters	–	PP_0063	PP_0062–PP_0063	0092	Lipid A biosynthesis lauroyl acyltransferase	99.32	0.0	Reva et al. 2006
	–	PP_0109	PP_0103–PP_0111	0137	Cytochrome oxidase assembly	98.33	1e-175	Matilla et al. 2007; Yousef-Coronado et al. 2008
	cyoE-1	PP_0110		0138	Protoheme IX farnesyltransferase—polyprenyltransferase	98.57	2e-131	Matilla et al. 2007; Yousef-Coronado et al. 2008

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	–	PP_0196	PP_0196–PP_0197	0225	ABC transporter ATP-binding protein	99.37	0.0	Matilla et al. 2007
	–	PP_0296	–	0325	Glycine/betaine ABC transporter substrate-binding protein	99.31	5e-172	Cheng et al. 2009
	–	PP_0368	–	0405	Acyl-CoA dehydrogenase	100	0.0	Reva et al. 2006
	–	PP_0373	–	0401	Hypothetical protein	100	3e-19	Matilla et al. 2007
	–	PP_0383	PP_0382–PP_0383	0418	Amine oxidase	99.82	0.0	Ramos-González et al. 2005
	–	PP_0486	–	0523	Transcriptional regulator, GntR family	98.52	0.0	Reva et al. 2006
	–	PP_0670	–	0710	Bile acid/Na+ symporter family protein	95.58	3e-166	Matilla et al. 2007
	–	PP_0721	PP_0719–PP_0725	0767	50S ribosomal protein L25	92.31	0.0	Ramos-González et al. 2005
	cyoE-2	PP_0816	PP_0809–PP_0816	0860	Protoheme IX farnesyltransferase	100	5e-177	Reva et al. 2006
	–	PP_0906	PP_0906–PP_0907	0957	Multidrug efflux protein	99.50	0.0	Matilla et al. 2007
	phoP	PP_1186	PP_1186–PP_1187	1227	Winged helix family two component transcriptional regulator	98.67	1e-115	Reva et al. 2006
	oprD	PP_1206	–	1242	Porin	82.20	0.0	Cheng et al. 2009
	–	PP_1271	PP_1271–PP_1273	4290	Major facilitator super-family transporter	98.93	0.0	Matilla et al. 2007; Reva et al. 2006

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	aapJ	PP_1297	PP_1297–PP_1300	4263	Amino acid ABC transporter substrate-binding protein	99.68	0.0	Cheng et al. 2009
	fpr	PP_1638	–	3978	oxidoreductase FAD/NAD(P)-binding domain-containing protein	100	5e-177	Cheng et al. 2009
	relA	PP_1656	PP_1654–PP_1656	3961	(p)ppGpp synthetase I SpoT/RelA	99.73	0.0	Reva et al. 2006
	–	PP_1874	PP_1873–PP_1874	3741	Glutathione peroxidase	99.38	3e-108	Matilla et al. 2007
	oprF	PP_2089	–	3563	OmpF family protein	100	0.0	Cheng et al. 2009
Stress adaptation detoxification transporters	*lolD*	PP_2155	PP_2154–PP_2156	3499	Lipoprotein releasing system ATP-binding protein	100	2e-152	Matilla et al. 2007
	speB	PP_2196	PP_2195–PP_2196	3455	Agmatinase	99.69	0.0	Reva et al. 2006
	fepA	PP_2242	PP_2242–PP_2243	3412	Outer membrane receptor FepA	98.52	0.0	Reva et al. 2006
	cti	PP_2376	PP_2376–PP_2378	3309	Fatty acid cis/trans isomerase	98.56	0.0	Matilla et al. 2007
	azlC	PP_2385	PP_2383–PP_2385	3299	AzlC family protein)	99.57	2e-131	Matilla et al. 2007
	–	PP_2543	–	3134	Amino acid ABC transporter permease	99.36	0.0	Ramos-González et al. 2005
	–	PP_2561	–	0199[d]	Heme peroxidase	50.00	1e-12	Matilla et al. 2007; Matilla et al. 2010
	–	PP_2669	PP_2667–PP_2669	3007	Hypothetical protein	98.48	0.0	Matilla et al. 2007

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	mexC	PP_2817	PP_2817–PP_2819	2337[d]	Multidrug efflux RND membrane fusion protein	50.15	6e-76	Matilla et al. 2007
	proP	PP_2914	–	2820	Proline/glycine betaine transporter	99.60	0.0	Reva et al. 2006
	–	PP_3132	–	2601	Polysaccharide biosynthesis protein	99.34	0.0	Matilla et al. 2007
	–	PP_3183	PP_3183–PP_3184	2306[d]	Electron transport protein SCO1/SenC	37.18	8e-29	Matilla et al. 2007
	–	PP_3210	PP_3208-PP_3201	2543	ABC transporter permease	98.88	1e-133	Matilla et al. 2007
	–	PP_3223	PP_3220–PP_3226	0936[d]	ABC transporter substrate-binding protein	25.10	1e-18	Matilla et al. 2007
	ggt-1	PP_3535	PP_3534_PP_3535	2258	Gamma-glutamyltransferase	96.81	0.0	Matilla et al. 2007
	–	PP_3583	PP_3582–PP_3583	2209	Acriflavin resistance protein	99.71	0.0	Matilla et al. 2007
	–	PP_3668	–	2060	Catalase/peroxidase HPI	99.07	0.0	Cheng et al. 2009
	gloA	PP_3766	–	1996	Lactoylglutathione Lyase	98.29	4e-117	Ramos-González et al. 2005
	–	PP_3802	PP_3797–PP_3804	1979	Cation ABC transporter ATP-binding protein	98.39	4e-160	Matilla et al. 2007
	–	PP_4002	PP_3999–PP_4004	1812	Recombination factor protein RarA	100	0.0	Reva et al. 2006

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	–	PP_4034	PP_4034–PP_4035	1786	Allantoate amidohydrolase	98.13	0.0	Reva et al. 2006
	treZ	PP_4051	PP_4050–PP_4054	1774	Malto-oligosyltrehalose trehalohydrolase	97.59	0.0	Roca et al. 2013
	nuoL	PP_4129	PP_4119–PP_4131	1721	NADH dehydrogenase subunit L	99.50	0.0	Reva et al. 2006
	–	PP_4305	–	1552	Sulfate ABC transporter substrate-binding protein	99.10	0.0	Ramos-González et al. 2005
	–	PP_4483	PP_4483–PP_4485	1418	Basic amino acid ABC transporter ATP-binding protein	99.61	1e-162	Matilla et al. 2007
	–	PP_4615	–	4309	Phosphate-starvation -inducible E	99.36	3e-90	Ramos-González et al. 2005
Stress adaptation detoxification transporters	–	PP_4646	–	4342	Oxidoreductase FAD/NAD(P)-binding domain-containing protein	99.61	4e-173	Reva et al. 2006
	cbrA	PP_4695	PP_4695–PP_4696	4396	Multi-sensor signal transduction histidine kinase	99.90	0.0	Reva et al. 2006
	cbrB	PP_4696		4397	Fis family transcriptional regulator	99.58	0.0	Reva et al. 2006
	pcnB	PP_4697	PP_4697–PP_4698	4398	Poly(A) polymerase	98.91	0.0	Reva et al. 2006
	omlA	PP_4731	–	4434	SmpA/OmlA domain-containing protein	99.36	6e-87	Reva et al. 2006
	vacB	PP_4880	PP_4879–PP_4880	4669	Ribonuclease R	99.88	0.0	Reva et al. 2006

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	putP	PP_4946	–	4729	Sodium/proline symporter	99.80	0.0	Ramos-González et al. 2005; Vílchez et al. 2000
	putA	PP_4947	–	4730	Pproline dehydrogenase/ pyrroline-5-carboxylate dehydrogenase	99.62	0.0	Vílchez et al. 2000
	pstP	PP_5145	PP_5145–PP_5146	4936	Protein PtsP	99.87	0.0	Reva et al. 2006
	–	PP_5278	–	5072	Aldehyde dehydrogenase	100	0.0	Cheng et al. 2009
	–	PP_5297	PP_5297–PP_5299	5090	Amino acid transporter	99.81	0.0	Matilla et al. 2007
	spoT	PP_5302	PP_5301-PP_5304	5095	(p)ppGpp synthetase I SpoT/RelA	99.72	0.0	Reva et al. 2006
	–	PP_5311	–	5104	Signal transduction protein	99.79	0.0	Wu et al. 2011
	–	PP_5322	–	5114	Metal ion transporter, putative	99.55	0.0	Reva et al. 2006
	pstA	PP_5327	PP_5325–PP_5328	5119	Phosphate ABC transporter permease	99.82	0.0	Reva et al. 2006
	pstS	PP_5329	–	5121	Phosphate ABC transporter substrate-binding protein	98.79	0.0	Duque et al. 2013; Reva et al. 2006; Roca et al. 2013
	atpA	PP_5415	PP_5412–PP_5418	0061	ATP synthase F0F1 subunit alpha	99.61	0.0	Cheng et al. 2009

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
DNA replication recombination repair	*recJ*	PP_1477	PP_1476–PP_1477	4082	Single-stranded-DNA-specific exonuclease RecJ	98.95	0.0	Matilla et al. 2007
	–	PP_2565	–	3114	UvrD/REP helicase	99.16	0.0	Matilla et al. 2007
	xthA	PP_2890	–	2845	Exonuclease III	98.89	0.0	Cheng et al. 2009
	–	PP_3966	PP_3964–PP_3966	0990[d]	ISPpu14, transposase Orf1	33.33	0.011	Matilla et al. 2007
	hsdM	PP_4741	PP_4741–PP_4742	4450	Type I restriction-modification system, M subunit	99.80	0.0	Ramos-González et al. 2005
	purA	PP_4889	PP_4889–PP_4890	4676	Adenylosuccinate synthetase	100	0.0	Cheng et al. 2009
	parC	PP_4912	PP_4912–PP_4915	4699	DNA topoisomerase IV subunit A	99.73	0.0	Ramos-González et al. 2005
	–	PputW619_2699	PputW619_2699-PputW619_2670	2563	TraX family protein	72.49	4e-96	Wu et al. 2011
Secretion	*yidC*	PP_0006	PP_0006–PP_0008	0071	Inner membrane protein translocase component YidC	100	0.0	Ramos-González et al. 2005
	hlpA	PP_1449	PP_1449–PP_1450	4110	Filamentous hemagglutinin	97.45	0.0	Molina et al. 2006
	hlpB	PP_1450	PP_1449–PP_1450	4109	TPS family activation/secretion protein	97.88	2e-131	Molina et al. 2006; Reva et al. 2006
	–	PP_4514	–	1386	Patatin	99.20	0.0	Barret et al. 2011
	secB	PP_5053	PP_5053–PP_5055	4841	Preprotein translocase subunit SecB	99.38	1e-106	Ramos-González et al. 2005

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	exbB	PP_5306	PP_5306–PP_5308	5099	Ferric siderophore transport system protein ExbB	95.68	6.0E-154	Molina et al. 2005
	exbD	PP_5307		5100	Biopolymer transport protein ExbD	100	1.0E-88	Molina et al. 2005
	tonB	PP_5308		5101	TonB family protein	100	7.0E-53	Molina et al. 2005
Metabolism	*gabT*	PP_0214	–	0237	4-aminobutyrate aminotransferase	100	0.0	Cheng et al. 2009; Espinosa-Urgel and Ramos 2001; Revelles et al. 2004
	soxG	PP_0326	PP_0322-PP_0327	0357	Sarcosine oxidase subunit gamma	99.52	3e-139	Matilla et al. 2007
	aceE	PP_0339	–	0372	Pyruvate dehydrogenase subunit E1	99.77	0.0	Ramos-González et al. 2005
	glcB	PP_0356	–	0392	Malate synthase G	99.59	0.0	Cheng et al. 2009
	pykA	PP_1362	–	4200	Pyruvate kinase	100	0.0	Cheng et al. 2009
	bglX	PP_1403	–	4156	Beta-glucosidase	99.18	0.0	Matilla et al. 2007
	lecA	PP_1661	PP_1659-PP_1661	3956	LecA - dehydrogenase subunit	98.80	0.0	Duque et al. 2013; Roca et al. 2013
	serC	PP_1768	PP_1678-PP_1679	3845	Phosphoserine aminotransferase	99.45	0.0	Cheng et al. 2009
	–	PP_2009	–	3642	1-aminocyclopropane-1-carboxylate (ACC) deaminase	93.60	3e-149	Cheng et al. 2009
	–	PP_2624	PP_2616-PP_2624	3066	Hypothetical protein	98.20	2e-106	Matilla et al. 2007

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	phaE	PP_3279	–	2493	Phenylacetate-CoA ligase	99.09	0.0	Matilla et al. 2007
	phaB	PP_3283	PP_3280-PP_3284	2489	Enoyl-CoA hydratase	98.48	2e-163	Matilla et al. 2007
	–	PP_3352	–	0106[d]	Arylsulfatase	24.90	1e-21	Matilla et al. 2007
	hpd	PP_3433	PP_3433-PP_3434	2355	4-hydroxyphenylpyruvate dioxygenase	99.44	0.0	Cheng et al. 2009
Metabolism	*ilvE*	PP_3511	–	2284	Branched-chain amino acid aminotransferase	99.41	0.0	Cheng et al. 2009
	glcE	PP_3746	PP_3745-PP_3749	2016	Glycolate oxidase FAD binding subunit	96.86	0.0	Matilla et al. 2007
	–	PP_3786	PP_3781-PP_3788	0861[d]	Aspartate/tyrosine/aromatic aminotransferase	26.23	1e-34	Matilla et al. 2007
	–	PP_3854	–	4480[d]	Endolysin - Muramidase	43.48	1.3	Ramos-González et al. 2005
	–	PP_3923	–	1884	Phosphoglycerate mutase	98.58	7e-145	Matilla et al. 2007
	icd	PP_4011	–	1803	Isocitrate dehydrogenase	99.28	0.0	Cheng et al. 2009
	gnd	PP_4043	PP_4041-PP_4043	1777	6-phosphogluconate dehydrogenase	99.39	0.0	Ramos-González et al. 2005
	aceA	PP_4116	–	1734	Isocitrate lyase	99.55	0.0	Ramos-González et al. 2005
	sucC	PP_4186	PP_4185-PP_4186	1666	Succinyl-CoA synthetase subunit beta	100	0.0	Cheng et al. 2009; Reva et al. 2006
	–	PP_4588	–	1310	Nitroreductase	99.49	1e-131	Matilla et al. 2007
	thiD	PP_4782	PP_4782-PP_4784	4565	Phosphomethylpyrimidine kinase	99.25	5e-151	Matilla et al. 2007

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
	fda	PP_4960	–	4744	Fructose-1,6-bisphosphate aldolase	100	0.0	Cheng et al. 2009
	hutU	PP_5033	–	4821	Urocanate hydratase	99.64	0.0	Barret et al. 2011; Cheng et al. 2009
	pgm	PP_5056	–	4844	Phosphoglyceromutase	100	0.0	Ramos-González et al. 2005
	gltB	PP_5076	–	4865	Glutamate synthase subunit alpha	99.80	0.0	Matilla et al. 2007
	ubiF	PP_5197	PP_5197-PP_5202	4992	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	99.75	0.0	Matilla et al. 2007
Protein synthesis folding degradation	*surA*	PP_0403	PP_0401-PP_0403	0441	Survival protein SurA	99.09	0.0	Cheng et al. 2009
	tyrS	PP_0436	–	0473	Tyrosyl-tRNA synthetase	99.75	0.0	Cheng et al. 2009
	clpB	PP_0625	–	0677	ATP-dependent Clp protease	99.77	0.0	Cheng et al. 2009
	–	PP_0893	PP_0887-PP_0893	0944	Pfpi family intracellular protease	99.48	2e-118	Cheng et al. 2009
	prc	PP_1719	PP_1718-PP_1719	3938	Carboxyl-terminal protease	99.72	0.0	Cheng et al. 2009
	gltX	PP_1977	–	3692	Glutamyl-tRNA synthetase	100	0.0	Ramos-González et al. 2005
	typA	PP_5044	–	4833	GTP-binding protein TypA	99.83	0.0	Cheng et al. 2009; Reva et al. 2006

Table 10.1 (continued)

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPU-BIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference
Other	*fstA*	PP_1341	PP_1328–PP_1342	4222	Cell division protein FtsA	99.77	0.0	Cheng et al. 2009
	–	PP_1633	–	3983	Hypothetical protein	100	9e-77	Duque et al. 2013; Roca et al. 2013
	–	PP_2076	PP_2075–PP_2076	2172[d]	Hypothetical protein	24.52	1e-04	Matilla et al. 2007
	–	PP_2298	–	3387	Hypothetical protein	97.32	3e-67	Ramos-González et al. 2005
	–	PP_2550	–	3127	Hypothetical protein	100	7e-144	Cheng et al. 2009
	–	PP_3855	–	4549[d]	Hypothetical protein	56.25	2.9	Ramos-González et al. 2005
	–	PP_4306	PP_4306–PP_4307	1546	Hypothetical protein	98.74	0.0	Ramos-González et al. 2005
	–	PP_4634	–	4330	Hypothecical protein	96.82	0.0	Ramos-González et al. 2005
	–	PP_5390	PP_5389–PP_5392	3657[d]	Hypothetical protein	40	0.96	Ramos-González et al. 2005

The % identity column corresponds to the degree of identity obtained after pairing the protein sequence of the reference strain to the nucleotide sequence of *Pseudomonas putida* BIRD-1 using TBlastn

[a]Predicted operons

[b]Orthologues

[c]Products according to Pseudomonas Genome Database (http://www.pseudomonas.com/) (Winsor et al. 2009)

[d]Non predicted as an orthologue

the most successful being the generation of random mini-Tn*5* mutants and the identification of mutants deficient in attachment to seeds and root surfaces. Espinosa-Urgel et al. (2000) obtained eight *Pseudomonas putida* KT2440 random transposon mutants with diminished ability to attach to corn seeds. Sequencing of the DNA adjacent to the insertions, and later characterization of the mutants suggested that the initial steps in attachment and biofilm formation in *Pseudomonas putida* share similarities with pathogenic microorganisms infecting host tissues.

Regarding the 13 genes related to flagella biosynthesis and assembly, which were described above as being determinant for attachment and rhizosphere colonization in *Pseudomonas putida* (Yousef-Coronado et al. 2008; Duque et al. 2013), it was found that all of them had a relatively good degree of conservation in the sequenced strains (Table 10.2) except for the *fliD* gene, with < 50 % of identity in the 9 *P. putida* strains compared to KT2440.

The two genes found to be involved in rhizosphere chemotaxis processes (PP_4331 and PP_4988) (Table 10.1) were also found to be highly conserved in most of the screened strains (75 to 92 % identity), except for UW4 (66 and 58 % identity, respectively) and Idaho (38 % for PP_4331) (Table 10.2).

The analysis of these mutants (Yousef-Coronado et al. 2008; Martínez-Gil et al. 2010; Duque et al. 2013) revealed that two large surface adhesion proteins influencing plant colonization were essential for attachment and biofilm formation (Table 10.1): (i) LapA, and genes related to its synthesis and transport (LapB, LapC, LapD), which is the most essential component in the initial steps of attachment to plant surfaces, biofilm formation, and the subsequent success of the bacteria in the rhizosphere; and (ii) LapF, which plays a significant role at later stages in the maintenance of the biofilm structure during the sessile growth stage of the colonizing *Pseudomonas* strain (Martínez-Gil et al. 2010). Further analysis of mutants revealed that flagella and the sigma factors which control flagella biogenesis, FliA and RpoN, were also important in the early stages of attachment. These results are in agreement with a study on early biofilm formation in *Escherichia coli* (Domka et al. 2007), and a more recent visual analysis of the role of flagella in surface colonization (Wood, 2013). Reciprocal adhesion genes (Table 10.1) with a high degree of identity (≈ 90 %) were also identified in the PGPR strain *Pseudomonas putida* BIRD-1 (Roca et al. 2013), which is an efficient colonizer of several plants of agronomical and environmental interest (Roca et al. 2013; Pizarro-Tobías et al., unpublished results).

Moreover, Reva et al. (2006) when assessing the important features required by *Pseudomonas putida* to cope with environmental stresses, using transcriptomic analysis of random transposon mutants, found that LapA (Table 10.1) was involved in coping with urea-induced stress (chaotropic solute). LapA functions by providing a hydrophilic micromilieu suitable for the incorporation of urea into its mesh, avoiding the water stress.

An important trait for PGPR bacteria is their ability to produce siderophores, which increase iron uptake by microbes and also by the host plants (Gamalero and Glick 2011); iron constitutes an important element involved in attachment and biofilm formation (O'Toole and Kolter 1998). It has been shown that *Pseudomonas*

Table 10.2 Identity (%) of rhizosphere competence determinants identified in sequenced strains of *Pseudomonas putida*

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Adhesion attachment biofilm formation	*lapD*	PP_0165	100	98	93	70	86	93	89	91	90	93
	lapC	PP_0166	100	99	97	78	87	96	94	95	94	97
	lapB	PP_0167	100	99	92	78	88	92	92	91	92	92
	lapA	PP_0168	100	99	82	39	36	70	68	72	48	86
	lapH	PP_0805	100	99	87	24	80	91	23	84	23	87
	lapF	PP_0806	100	87	86	44	65	87	38	80	29	88
	algD	PP_1288	100	99	100	85	97	100	96	98	97	100
	–	PP_1789	100	22	22	22	25	22	75	22	22	22
	–	PP_1790	100	43	21	26	24	50	92	22	50	29
	–	PP_1791	100	20	26	28	27	26	93	20	27	27
	–	PP_1792	100	30	36	28	64	61	78	29	25	62
	–	PP_1793	100	26	27	29	60	59	75	27	28	60
	–	PP_1794	100	0	0	35	49	74	74	38	22	74
	–	PP_1795	100	22	23	25	84	97	96	34	23	97
	bcsQ	PP_2634	100	98	90	24	38	90	38	79	28	88
	bcsA	PP_2635	100	98	95	29	27	95	27	90	27	95
	bcsB	PP_2636	100	98	93	34	28	91	45	87	45	91
	bscC	PP_2637	100	99	96	24	35	96	31	88	31	92
	peaG	PP_3139	100	96	91	34	79	91	87	89	87	92
	peaH	PP_3140	100	98	78	31	58	78	67	73	66	78
	peaI	PP_3141	100	95	92	50	68	93	80	86	80	40
	galU	PP_3821	100	99	100	92	96	100	97	91	97	100

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	hemN	PP_4264	100	99	100	83	94	100	98	97	99	100
	–	PP_4934	100	100	97	89	96	97	96	96	96	96
	ndvB	PputW619_2133	41	79	42	61	100	31	36	22	36	0
Flagella	*fliA*	PP_4341	100	100	100	90	96	100	99	99	99	100
	flhB	PP_4352	100	99	78	62	77	77	80	81	78	92
	fliO	PP_4356	100	100	79	70	90	78	96	74	95	78
	fliN	PP_4357	100	99	90	88	85	90	88	87	87	91
	fliL	PP_4359	100	100	82	64	76	81	79	81	79	91
	fliI	PP_4366	100	99	99	87	96	99	99	99	99	100
	fliF	PP_4369	100	99	88	77	84	88	87	87	86	90
	fleQ	PP_4373	100	100	100	93	96	100	97	99	98	100
	fliD	PP_4376	100	46	38	37	40	35	54	40	36	50
Flagella	*fliC*	PP_4378	100	65	62	61	57	65	54	66	68	44
	flgL	PP_4380	100	67	46	46	51	45	90	64	45	77
	flgD	PP_4389	100	99	54	54	54	54	82	84	55	90
	flgB	PP_4391	100	93	93	80	95	93	97	99	97	100
Chemotaxis	–	PP_4331	100	99	89	66	81	87	75	75	76	38
	–	PP_4988	100	98	91	58	80	91	87	88	87	92
Regulators sensor proteins	–	PP_0700	100	99	86	27	63	89	81	80	80	86
	colS	PP_0902	100	99	88	67	83	88	86	86	87	88
	rpoN	PP_0952	100	99	98	89	96	98	97	97	97	98
	–	PP_1066	100	100	100	87	97	100	98	98	98	100
	gacS	PP_1650	100	99	90	69	82	91	86	88	86	91
	–	PP_2070	100	90	97	36	34	98	38	36	38	35

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	–	PP_2127	100	97	97	61	72	96	89	91	89	100
	–	PP_3640	100	92	92	29	25	92	87	92	88	91
	–	PP_4295	100	99	100	86	96	100	99	100	99	100
	pfrI	PP_4424	100	34	34	33	34	34	35	40	35	34
	–	PP_4508	100	99	96	79	92	29	93	94	29	98
	–	PP_4959	100	99	98	70	88	98	92	94	92	99
	–	PP_4966	100	100	89	69	83	89	86	87	86	89
	phoR	PP_5321	100	98	97	81	92	97	95	96	95	97
Stress adaptation detoxification transporters	–	PP_0063	100	99	100	82	98	100	98	99	97	100
	–	PP_0109	100	98	77	57	72	78	78	76	78	78
	cyoE-1	PP_0110	100	98	77	67	74	77	76	76	76	78
	–	PP_0196	100	99	95	82	92	95	94	94	93	95
	–	PP_0296	100	99	91	75	88	91	89	90	89	90
	–	PP_0368	100	100	100	94	98	100	99	99	99	100
	–	PP_0373	100	100	7675	100	77	76	77	77	77	76
	–	PP_0383	100	99	97	88	92	97	94	94	94	100
	–	PP_0486	100	98	93	32	81	92	87	31	87	94
	–	PP_0670	100	95	84	24	75	84	76	80	77	85
	–	PP_0721	100	92	86	75	86	86	93	86	93	86
	cyoE-2	PP_0816	100	100	92	72	90	91	91	91	91	92
	–	PP_0906	100	99	91	79	86	91	89	90	89	91
	phoP	PP_1186	100	98	81	72	79	81	81	80	81	82

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Stress adaptation detoxification transporters	*oprD*	PP_1206	100	82	96	44	82	95	93	80	93	96
	–	PP_1271	100	98	86	61	83	87	85	85	85	87
	aapJ	PP_1297	100	99	96	85	94	96	95	95	96	25
	fpr	PP_1638	100	100	100	97	99	100	99	98	99	100
	relA	PP_1656	100	99	100	90	99	100	99	100	99	34
	–	PP_1874	100	99	97	71	90	98	94	93	94	100
	oprF	PP_2089	100	100	97	83	81	97	83	96	83	97
	lolD	PP_2155	100	100	100	84	98	100	99	99	99	100
	speB	PP_2196	100	99	99	84	44	100	97	98	97	100
	fepA	PP_2242	100	98	98	24	88	98	95	98	94	99
	cti	PP_2376	100	98	99	74	93	99	96	98	95	99
	azlC	PP_2385	100	99	91	71	86	90	88	89	88	90
	–	PP_2543	100	99	98	55	95	98	97	97	97	98
	–	PP_2561	100	50	88	78	52	88	49	83	81	88
	–	PP_2669	100	98	93	90	29	94	89	91	89	94
	mexC	PP_2817	100	50	48	44	48	48	48	75	91	98
	proP	PP_2914	100	99	94	37	90	93	91	92	91	94
	–	PP_3132	100	99	81	0	67	81	75	79	77	88
	–	PP_3183	100	37	98	65	36	96	90	94	90	99
	–	PP_3210	100	98	34	28	83	86	33	30	30	87
	–	PP_3223	100	25	25	26	26	25	26	37	26	97
	ggt-1	PP_3535	100	96	95	63	84	95	88	90	88	34
	–	PP_3583	100	99	89	74	86	89	87	89	87	90
	–	PP_3668	100	99	94	76	89	94	88	92	88	94

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	gloA	PP_3766	100	98	99	80	93	99	95	98	96	99
	–	PP_3802	100	98	99	79	91	99	94	94	96	99
	–	PP_4002	100	100	100	91	96	100	99	99	99	100
	–	PP_4034	100	98	96	82	89	96	91	94	93	77
	treZ	PP_4051	100	97	94	63	84	95	89	91	88	95
	nuoL	PP_4129	100	99	94	86	93	94	93	94	93	93
	–	PP_4305	100	99	94	87	92	94	93	93	93	95
	–	PP_4483	100	99	96	90	95	96	94	94	94	96
	–	PP_4615	100	99	92	65	65	92	88	91	88	92
	–	PP_4646	100	99	99	79	95	99	98	99	98	100
Stress adaptation detoxification transporters	*cbrA*	PP_4695	100	99	92	85	91	92	91	91	91	95
	cbrB	PP_4696	100	99	93	84	90	93	91	91	91	93
	pcnB	PP_4697	100	98	93	80	90	93	90	92	91	93
	omlA	PP_4731	100	99	87	62	83	83	82	86	81	83
	vacB	PP_4880	100	99	93	76	90	93	90	91	90	95
	putP	PP_4946	100	99	92	80	88	92	90	88	89	92
	putA	PP_4947	100	99	96	87	94	96	95	96	95	96
	pstP	PP_5145	100	99	98	91	96	98	97	97	97	38
	–	PP_5278	100	100	96	84	94	96	95	96	95	96
	–	PP_5297	100	99	85	33	79	84	81	83	81	86
	spoT	PP_5302	100	99	98	92	97	98	97	97	97	98
	–	PP_5311	100	99	100	80	90	100	96	97	96	100
	–	PP_5322	100	99	91	79	87	91	88	90	88	91
	pstA	PP_5327	100	99	86	72	82	86	83	84	84	86

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	pstS	PP_5329	100	98	89	82	88	89	89	89	89	90
	atpA	PP_5415	100	99	97	93	96	98	96	97	96	98
DNA replication recombination repair	*recJ*	PP_1477	100	98	92	82	90	92	91	91	91	92
	–	PP_2565	100	99	93	65	85	94	91	92	91	94
	xthA	PP_2890	100	98	99	90	96	99	96	96	96	99
	–	PP_3966	100	33	29	29	99	27	89	34	31	58
	hsdM	PP_4741	100	99	89	44	27	30	23	28	28	25
	purA	PP_4889	100	100	100	88	99	100	99	100	99	100
	parC	PP_4912	100	99	96	85	93	97	94	95	94	97
	–	PputW619_2699	28	72	29	37	100	48	69	27	69	33
Secretion	*yidC*	PP_0006	100	100	94	77	92	94	90	93	90	94
	hlpA	PP_1449	100	97	96	23	72	96	81	84	81	96
	hlpB	PP_1450	100	97	90	30	65	91	75	33	76	93
	–	PP_4514	100	99	97	33	93	97	94	97	94	98
	secB	PP_5053	100	99	100	87	97	100	97	98	97	100
	exbB	PP_5306	100	95	77	78	73	79	75	77	75	79
	exbD	PP_5307	100	100	100	83	99	100	99	99	99	100
	tonB	PP_5308	100	100	73	62	87	73	72	72	72	73
Metabolism	*gabT*	PP_0214	100	100	100	85	97	100	99	99	100	100
	soxG	PP_0326	100	99	100	80	95	100	96	98	97	100
Metabolism	*aceE*	PP_0339	100	99	99	93	98	99	98	98	98	100
	glcB	PP_0356	100	99	96	81	93	96	96	95	96	97
	pykA	PP_1362	100	100	100	87	98	100	99	99	99	100
	bglX	PP_1403	100	99	99	76	95	100	97	98	98	28

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	lecA	PP_1661	100	98	97	22	94	97	27	26	27	98
	serC	PP_1768	100	99	99	81	97	99	96	97	97	100
	–	PP_2009	100	93	79	53	67	78	74	75	74	80
	–	PP_2624	100	98	99	42	39	98	34	89	84	99
	phaE	PP_3279	100	99	98	24	94	98	96	98	96	100
	phaB	PP_3283	100	98	93	33	81	93	90	89	91	95
	–	PP_3352	100	24	99	42	88	46	25	23	25	99
	hpd	PP_3433	100	99	99	90	98	99	97	99	97	99
	ilvE	PP_3511	100	99	100	84	98	99	99	99	99	37
	glcE	PP_3746	100	96	94	69	82	94	86	89	87	95
	–	PP_3786	100	26	26	27	26	26	26	26	26	26
	–	PP_3854	100	43	45	26	28	59	91	39	72	74
	–	PP_3923	100	98	92	62	81	93	88	89	88	94
	icd	PP_4011	100	99	100	90	98	99	99	98	99	100
	gnd	PP_4043	100	99	96	69	92	96	95	94	94	96
	aceA	PP_4116	100	99	100	95	98	100	99	98	99	100
	sucC	PP_4186	100	100	97	93	97	97	97	95	97	97
	–	PP_4588	100	99	99	85	25	99	96	99	95	99
	thiD	PP_4782	100	99	91	83	88	90	89	89	89	44
	fda	PP_4960	100	100	100	98	99	100	100	99	100	100
	hutU	PP_5033	100	99	95	88	94	95	94	95	95	95
	pgm	PP_5056	100	100	94	81	89	94	91	92	91	95
	gltB	PP_5076	100	99	100	89	97	100	98	99	98	100
	ubiF	PP_5197	100	99	99	86	92	99	98	97	98	100

Table 10.2 (continued)

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Protein synthesis folding degradation	*surA*	PP_0403	100	99	96	80	92	96	92	95	92	96
	tyrS	PP_0436	100	99	89	81	86	89	87	87	87	89
	clpB	PP_0625	100	99	92	86	91	100	91	91	91	43
	–	PP_0893	100	99	93	78	88	93	89	92	91	93
	prc	PP_1719	100	99	97	85	95	97	95	95	95	96
	gltX	PP_1977	100	100	100	91	98	99	99	99	99	100
	typA	PP_5044	100	99	96	91	95	96	92	95	92	40
Other	*fstA*	PP_1341	100	99	98	92	97	98	97	98	97	98
	–	PP_1633	100	100	99	63	88	100	95	93	95	100
	–	PP_2076	100	24	89	35	31	86	38	81	45	25
	–	PP_2298	100	97	98	45	94	98	94	95	93	100
	–	PP_2550	100	100	92	71	86	92	86	90	86	92
	–	PP_3855	100	56	28	43	68	70	42	35	36	28
	–	PP_4306	100	98	99	66	94	100	96	95	95	99
	–	PP_4634	100	96	97	65	86	98	90	90	91	99
	–	PP_5390	100	40	74	0	74	41	44	74	44	40

The % identity columns corresponds to the degree of identity obtained after pairing the amino acid sequence of the reference strain (*P. putida* KT2440) to the amino acid sequence of other sequenced *Pseudomonas putida* using BlastP

putida strains have multiple iron acquisition systems which enable them to acquire iron complexes through self-produced and exogenous siderophores. This acquisition enables allows bacteria to become efficient rhizosphere colonizers (Martínez-Bueno et al. 2002). To elucidate up to what extent iron is involved in seed attachment/colonization and, consequently in *Pseudomonas putida* KT2440 rhizosphere fitness, Molina et al. (2005) performed corn-seed attachment assays. Since corn seeds are rich in ferrous iron, the assay was carried out using seeds pre-treated with iron chelators; the result was a reduction in the number of bacterial cells attached to the seeds. The result points towards iron having a potential role as a chemoattractant and/or in the establishment of bacteria on the surface of the seed. To further assess what the role of seed-borne iron could be in attachment and biofilm formation, the authors tested mutants in the TonB gene cluster (*exbB/exbD/tonB*, PP_5306–5308) (Table 10.1), which is essential for siderophore iron uptake. Data showed that mutation of the TonB cluster reduced attachment of bacterial cells to corn seeds. Competition assays between the mutant and the wild-type strain in non-sterile corn rhizospheric soil resulted in the displacement of the mutant, pointing out the importance of the TonB system in bacterial fitness in the rhizosphere. An *in silico* analysis performed on the *Pseudomonas putida* BIRD-1 genome revealed that it has 25 iron receptors with over 95 % identity to their reciprocal *Pseudomonas putida* KT2440 gene products (Roca et al. 2013). This finding is consistent with the capacity of this strain to attach to seeds and survive and colonize the rhizosphere of maize and several plants of agronomical and environmental importance (Roca et al. 2013; Pizarro-Tobías et al. unpublished results). The TonB/ExbD/ExbB cluster was found to be highly conserved in all *Pseudomonas putida* strains whose genome has been sequenced, with > 73 % identity (Table 10.2).

Of the 25 genes identified as being involved in adhesion, attachment and biofilm formation processes, *lapA*, *lapF* and *lapH* have been identified as key determinants of attachment in *P. putida* (Yousef-Coronado et al. 2008; Martínez-Gil et al. 2010; Duque et al. 2013). We found that these three genes are highly conserved in most of the sequenced *P. putida* strains with > 70 % identity, except for UW4 and PC9 (< 48 %). The LapA and LapF proteins were found to be less conserved in W619 (36 and 65 % identity, respectively) when compared to LapH (80 % identity) (Table 10.2). The LapBC cluster was found to be conserved in all 10 strains, with over 78 % identity in all cases.

Exopolysaccharides (EPS), such as alginate, are molecules involved in biofilm formation and stability, and have been found to play an important role in the ability of *Pseudomonas putida* to cope with desiccation (Ramos et al. 2001). A gene involved in alginate biosynthesis, *algD* (PP_1288), was identified by Ramos-González et al. (2005) as being induced in the rhizosphere. Interestingly *algD* had previously been shown to be involved in water stress and involved in root colonization accomplishment (van de Mortel and Halverson, 2004). An *algD* orthologue with a 99.8 % degree of identity of was found in *Pseudomonas putida* BIRD-1 (Table 10.1) and this strain showed an enhanced capacity to overcome water stress in soil, when compared to *P. putida* KT2440 (Roca et al. 2013). This gene was also found to be highly conserved in all sequenced *Pseudomonas putida* strains, with > 85 % identity

(Table 10.2). Chang et al. (2007) reported that alginate is involved in biofilm structural maintenance in water scarcity conditions, increasing the survival capacity of *P. putida* cells within the matrix and stressing the importance of exopolysaccharides in *Pseudomonas* environmental fitness and stress tolerance. Nielsen et al. (2011) studied the role of two other putative exopolysaccharides found in *Pseudomonas putida* mt2, namely, the Pea exopolysaccharide (*pea*) and a cellulose-like polymer (*bcs*) (Table 10.1), their involvement in biofilm formation and environmental stress tolerance using site-specific mutagenesis and subsequent phenotypic assays. The study revealed the importance of Bcs and alginate in maize rhizosphere colonization and that both, Bcs and Pea, promote the retention of water within the biofilm matrix during water shortages. Nielsen et al. (2011) also reported the existence of an additional *P. putida* exopolysaccharide gene cluster (*peb*) (PP_1789—PP_1791, PP_1792—PP_1795) (Table 10.1) that is also involved in biofilm stability. The *bcs* and *pea* clusters are conserved with a degree of identity of 95% in *Pseudomonas putida* BIRD-1 (Table 10.1). In other sequenced *Pseudomonas putida* strains, we found that the *bcs* cluster was less conserved, with low identify in UW4, W619, S16 and PC9 strains (<45% identity) (Table 10.2); the *pea* cluster was also found to be less conserved in UW4 (<50% identity) (Table 10.2). In the case of the *peb* cluster, the orthologue genes showed a low degree of identity (<30%), and within the cluster only the ORF PP_1792 was predicted to act as an orthologue for *P. putida* BIRD-1 (Table 10.1). This cluster was also found to be relatively non-preserved among the sequenced *Pseudomonas putida*, except for *P. putida* S16 (>74% identity) (Table 10.2).

By inserting a plasmid harbouring *rup4959* (locus PP_4959) in miniTn*5* derivatives of *P. putida* KT2440, Matilla et al. (2011a) identified two gene clusters coding for surface polysaccharides: (i) *P. putida* exopolysaccharide (*pea*, PP_3133–3141) (Nielsen et al. 2011; Nilsson et al. 2011), and (ii) *P. putida* exopolysaccharide (*peb*, PP_1789–1791) (Nilsson et al. 2011), related to increased levels of cyclic diguanylate.

The *algD*, *galU*, *hemN* and PP_4934 genes were also found to be conserved in all the strains (>85, >91, >83 and >89% identity, respectively) (Table 10.2). The KT2440 gene clusters associated with the synthesis of surface exopolysaccharides, Pea and Bcs, were found to be reciprocal in the BIRD-1, F1, DOT-T1E, GB-1 and Idaho strains (Table 10.2). All of the KT2440 adhesion determinants that were not found to be common in BIRD-1 (PP_1789, PP_1790, PP_1791, PP_1793 and PP_1795) (Table 10.1), were only found to be conserved in the S16 strain; a microbe that has the ability to degrade nicotine and which was isolated from plant roots (Wang et al. 2007). Nonetheless, a gene identified in *Pseudomonas putida* W619 to be involved in the attachment of endophytes to higher plants (Wu et al. 2011), was found to have an orthologue only in BIRD-1 (73% of identity) (Tables 10.1 and 10.2). Further research would be necessary to determine whether this strain behaves as an endophyte, although it is unlikely for a single gene to be the sole determinant of such a complex process as endophytism.

Regulators and Sensor Proteins

Regulator proteins involved in fitness in the rhizosphere have been identified through the analysis of mutants with enhanced sensitivity to stress conditions, as well as through transcriptomic analysis. The work of Reva et al. (2006) identified the CbrAB two-component response regulator system to be involved in coping with abiotic stress. The authors found that *cbrAB* mutants were more sensitive than the parental strain to cold stress (4 °C). Amador et al. (2010) also reported the importance of CbrB to *Pseudomonas putida* rhizosphere fitness. They identified a variety of functions unrelated to carbon metabolism which are subject to CbrB regulation, such as chemotaxis, stress tolerance and biofilm formation. The CbrAB two-component response regulator system is well conserved in all sequenced *P. putida* strains (Table 10.2), and *P. putida* BIRD-1 has reciprocal genes, with identity over 99 % (Table 10.1).

Some global stress response genes were identified in the study by Reva et al. (2006) (PP_0063, PP_0816, PP_4646, PP_5322) (Table 10.1), and orthologues with identity of over 98 % were found in the *P. putida* BIRD-1 genome. We also found that these are highly conserved in the rest of the sequenced Pseudomonads (> 72 % identity) (Table 10.2).

A gene in *P. putida* KT2440 coding for a response regulator (PP_4959; Table 10.1) and being involved in the regulation of the second messenger c-di-GMP (cyclic diaguanylate) was found to be induced in the rhizosphere (Matilla et al. 2007). Cyclic diguanylate has been reported to be involved in bacterial shifting from the planktonic to the sessile state (Römling and Amikam, 2006). Matilla et al. (2011a) used transcriptional fusions to reporter genes to confirm the activation of this gene (PP_4959) in the rhizosphere of maize. The authors also found that it was activated under microaerobiosis conditions in the presence of maize root exudates; conditions often found in the vicinity of roots.

Metabolism

Another method used for identifying bacterial promoters induced in the rhizosphere is *in vivo* expression technology (IVET), which allows the identification of upregulated genes in defined conditions through the generation of transcriptional fusions of genomic libraries to a reporter gene encoding a specific enzymatic activity. In a non-saturating IVET screenning of *Pseudomonas putida*, Ramos-González et al. (2005) found 28 genes that were induced in the maize rhizosphere. The genes were found to be involved in the biosynthesis of cell envelope components, nutrient acquisition, energy metabolism, DNA metabolism and defence mechanisms, stress and detoxification, chemotaxis and motility, among others (Table 10.1). Several genes associated with amino acid acquisition where induced, including *putP* a proline permease previously reported to be induced in the presence of maize exudates (PP_4946) (Vílchez et al. 2000), a gamma-aminobutyric acid (GABA) permease

(PP_2543), and an amino oxidase involved in lysine catabolism (PP_0383) (Revelles et al. 2004) (Table 10.1). Reciprocal genes for these three KT2440 proteins were found in the *Pseudomonas putida* BIRD-1 genome, with over 98% identity, confirming that the ability for amino acid metabolism is conserved in this strain; as well as being highly conserved for the rest of the sequenced *P. putida* (>79% identity), with the exception of the GABA permease in *P. putida* UW4 (55% identity) (Table 10.2). As expected, an essential gene in central metabolism, *aceE* (PP_0339), encoding a pyruvate dehydrogenase, was induced; this gene was also found in the genome of all sequenced *P. putida* strains (>93% identity) (Table 10.2).

The study of protein expression profiles in bacteria in the presence of root exudates constitutes an interesting approach for identifying essential functions required for rhizosphere colonization and fitness. Cheng et al. (2009) performed functional studies with *Pseudomonas putida* UW4 comparing the wild-type strain with an ACC deaminase mutant in the presence of *Brassica napus* (canola) root exudates to identify key proteins involved in bacterial interactions in the root-influenced environment. They found that a large proportion of the expressed proteins were required for the utilization of nutrients present in root exudates (Table 10.1), which is entirely in agreement with what was previously reported by Mark et al. (2005) for *Pseudomonas aeruginosa* PA01. Cheng et al. (2009) also identified several proteins involved in cell envelope component biosynthesis, exopolysaccharides, chaperones, and proteins involved in DNA repair or modification (Table 10.1).

Stress Adaptation

One of the most realistic and complete approaches for unravelling gene expression in rhizospheric bacteria was accomplished with *Pseudomonas putida* KT2440 in the maize rhizosphere by performing a genomic-wide analysis using microarrays, and later validating the results by performing real time RT-PCR (Matilla et al. 2007). To obtain a better picture of the specific genes induced in the rhizosphere, controls in rich medium with planktonic cells growing exponentially, planktonic cells in stationary phase, and sessile cells in sand microcosms were used. Over 90 genes were found to be differentially induced in the rhizosphere. notably most of them were involved in the uptake of specific carbon and nitrogen sources, such as amino acids, catabolic pathways for the degradation of aromatic compounds, stress response and detoxification, signal transduction sensors and response regulators. It is worth mentioning the up-regulation of the sensor histidine kinase PhoR (PP_5321) (Table 10.1). We found that *phoR* is highly conserved in all sequenced strains of *Pseudomonas putida* (>81% identity) (Table 10.2). The induction of this Pho system has been rpreviously eported to be modulated by the cell redox state (Schau et al. 2004), which implies that terminal oxidases are required for systems induction. As expected, two genes involved in cytochrome biosynthesis were found to be induced in the study performed by Matilla et al. (2011a, b) (PP_0109 and PP_0110). Reciprocal genes were found for PP_0109 and PP_0110 in all sequenced *P. putida* strains (>72% identity), except for UW4 (<67% identity) (Table 10.2).

The sensor histidine kinase, PhoR, involved in the response to inorganic phosphate limitation as described above (Matilla et al. 2007), ColS (Ramos-González et al. 2005; Barret et al. 2011), and GacS (Duque et al. 2013), was found to be relatively conserved in all of the 9 sequenced *P. putida* strains (Table 10.2). The transcriptional regulators encoded by PP_2070, PP_4424 (Ramos-González et al. 2005), and PP_3640 (Matilla et al. 2007), were found to be less conserved among the *P. putida* strains (Table 10.2).

A screening of the genome from *Pseudomonas putida* BIRD-1 for genes involved in oxidative stress led to the identification of 16 possible candidates, including several superoxide dismutases, catalases, alkylhydroperoxidases, cytochrome peroxidases and glutathione peroxidases (Roca et al. 2013). The redundancy of genes to deal with oxygen reactive species points out the importance of these enzymes for rhizosphere fitness. These genes can be relevant in the colonization of other niches. Two of the genes involved in coping with oxidative stress found in the BIRD-1 genome (PPUBIRD1_3741 and PPUBIRD1_2060) were reported by Matilla et al. (2007) (PP_1874) and Cheng et al. (2009) (PP_3668) to be induced in the rhizosphere (Table 10.1). These genes were also found to be highly conserved among sequenced *P. putida* strains, with >71 % identity and >76 % identity, respectively.

Matilla et al. (2007) also constructed *Pseudomonas putida* KT2440 mutants in 9 of the genes from the 90 up-regulated genes they found to be induced in the rhizosphere. When assayed in maize rhizosphere in competition with the wild-type strain, the mutants were less efficient colonizers than the wild type. The results indicated that the essential traits for rhizosphere fitness are stress adaptation mechanisms and the ability to metabolize a variety of nutrients available in root exudates.

As presented above, numerous studies with varied approaches have been performed to elucidate the molecular basis of the essential traits involved in rhizosphere fitness of bacterial strains in mutualistic relationship with different plant species. Barret et al. (2011) reviewed the topic, pointing out that, in the case of the *Pseudomonas* genus, the essential features induced in different mutualistic interactions within the rhizosphere encompass stress responses and secretion systems.

As far as the 14 regulators and sensor proteins found to be involved in rhizosphere fitness (Table 10.1) are concerned, Duque et al. (2013) reported the importance of the RNA polymerase sigma factor, RpoN, along with the transcriptional regulator, FleQ, fin the expression of *fliD*, encoding for a flagellar cap protein. In other microorganisms, such as *Pseudomonas aeruginosa*, *rpoN* mutants were shown to be hampered in adhesion. The RpoN sigma factor was found to be conserved in all sequenced *P. putida* strains (>89 % identity) (Table 10.2).

One third of the proteins described to be involved in rhizosphere competence are related to stress adaptation and detoxification mechanisms (Table 10.1), which is consistent with the results presented by Matilla et al. (2007), who described nutrient acquisition and stress response as the major forces driving bacterial adaptation in the rhizosphere. Only 4 of the genes identified in *Pseudomonas putida* KT2440 lacked orthologues in the BIRD-1 genome (Table 10.1) indicating that these strains have similar machinery to overcome rhizosphere stress. In fact, this could also be applied to the rest of the sequenced *Pseudomonas putida* strains (Table 10.2) since most of the described genes were relatively conserved among them.

Eight proteins were found to be involved in DNA replication, recombination, and repair and defence mechanisms (Table 10.1). Among them, it is interesting to note that a gene named *traX,* encoding a protein involved in conjugal DNA transfer/uptake in *Pseudomonas putida* W619, which was induced in the rhizosphere (Wu et al. 2011), is relatively well conserved (69% identity) only in BIRD-1, S16 and PC9 strains (Table 10.2). The *hsdM* gene encoding a protein with endodeoxyribonuclease activity was found to be well conserved only in BIRD-1 and F1 strains, with 100 and 89% identity, respectively (Table 10.2).

Eight transport and secretion proteins were described to be involved in rhizosphere fitness of *Pseudomonas putida* (Table 10.1). It is worth mentioning that the *tonB* gene cluster (Table 10.1) involved in iron uptake and adhesion to seeds in *Pseudomonas putida* KT2440 (Molina et al. 2005), is found to be highly conserved in all the sequenced *P. putida* strains (Table 10.2). The rest of genes are well conserved in most of the strains, except for UW4 in which the *hlpAB* cluster and PP_4541 encoding an esterase have less than 33% identity (Table 10.2).

A significant proportion of the genes involved in rhizospheric competence (17%), are involved in metabolism (Table 10.1). When compared to *Pseudomonas putida* KT2440, BIRD-1 has reciprocal genes for all of the described proteins, except for an arylsulfatase (PP_3352), an aminotransferase (PP_3786) and an endolysin (PP_3854) (Table 10.1). It is worth noting that the aminotransferase was not conserved in any of the other sequenced *P. putida* strains (Table 10.2). The rest of the metabolism-related genes were well conserved in all of the strains, which is consistent with the metabolic versatility commonly attributed to the *Pseudomonas* genus (Wu et al. 2011).

Seven genes required for protein synthesis, folding and degradation were found to be induced in the rhizosphere (Table 10.1); all of them are highly conserved among the sequenced *P. putida* strains (Table 10.2).

Of the 9 hypothetical proteins, with unknown function, described as putatively involved rhizosphere competence for *Pseudomonas putida* (Table 10.1), only 3 didn't have an orthologue in BIRD-1 (PP_2076, PP_3855 and PP_5390).

Conclusions

Overall it can be said that the genomic machinery necessary for *Pseudomonas putida* to accomplish survival and maintenance in the rhizosphere when a plant-bacteria mutualistic relationship is on-going, is well conserved within the species *P. putida*. This is consistent with *Pseudomonas putida* being a frequent rhizosphere colonizer and, in some cases, a plant growth-promoting bacterium (Wu et al. 2011; Roca et al. 2013).

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Part IV
History

Chapter 11
My Memories of *Pseudomonas* in the Twentieth Century

Bruce Holloway

The palest ink is better than the best memory (Chinese proverb)

Abstract The genus *Pseudomonas* is highly significant for health sciences, agriculture, microbiology, biotechnology and environmental science. This was not always so. An historical and personal perspective or this rise from microbiological obscurity in the mid twentieth century is provided by a microbial geneticist who spent fifty years working on this genus. When microbial science began to expand in the late 1940's, *Pseudomonas* had only one well defined species, *P. aeruginosa*, and a confused taxonomy of other isolates assigned to the genus. The extraordinary metabolic variation of these isolates had been recognised, but there was little biochemical knowledge by way of explanation. This situation had the virtue of attracting scientists of many disciplines who in the next ten to twenty years demonstrated novel biochemical pathways, constructed a sound taxonomic basis for the genus and established a system of genetic analysis. In turn, this groundwork enabled the potential of *Pseudomonas* in human disease, plant disease, biotechnology and aspects of the environment to be realised and exploited. The importance of *P. aeruginosa* in cystic fibrosis has stimulated an understanding of pathogenicity and virulence for human bacterial pathogens in general. All aspects of *Pseudomonas* research have been greatly enhanced following the introduction of genomic techniques in more recent years.

Keywords Historical perspective · *Pseudomonas* genetics · *Pseudomonas* taxonomy · *Pseudomonas* variability · *Pseudomonas* plasmids · *Pseudomonas* patents · Cystic Fibrosis · Pre-genomic era · Genomic era · R68.45

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In the Beginning…….

When I decided to work on the genetics of *Pseudomonas* in 1953, I was aware that *P. aeruginosa* infected wounds and other species of *Pseudomonas* were associated with plant disease. I was not to know that by the end of the century, the genus *Pseudomonas* would be recognised as one of the most widespread living organisms on the planet and would be declared one of the six "top priority, dangerous microbes" (Talbot et al. 2006). When I started to study the genetics of bacteria, it was not known how genetic information was stored in living organisms. DNA was known to be involved but it seemed too simple a molecule to store the amount of information needed and there was a view that proteins played a part in this essential biological function. In 1953 Watson and Crick published their classic paper on DNA structure, but it took time for biologists to appreciate the many implications of this discovery. Microbial genetics would play a major role in defining the molecular basis of biological information.

During the twentieth century a number of issues drove research on *Pseudomonas*. Its biological variation, unusual substrate use and novel biochemistry were always and continue to be great attractions. The rise of microbial genetics provided new tools and insights to investigate bacterial function. The importance of bacteriophages and extrachromosomal elements such as plasmids added to the genetic complexity and enabled new genetic concepts and techniques to be developed. Finally, the disease aspect of *P. aeruginosa*, particularly its importance in cystic fibrosis has been a major factor. Interest in phytopathogenic pseudomonads has become more widespread. The genomic era of biology would totally change how all these issues would be studied and my involvement covered both the pre-genomic and genomic periods. As John W. Gardner remarked "History never looks like history when you are living through it".

My Introduction to *Pseudomonas*

I completed my Ph.D. at the California Institute of Technology in October 1952. My supervisor was Nobel Laureate George Beadle and the thesis topic I had selected was "Heterocaryosis in *Neurospora crassa*". As it turned out, I was Beadle's last graduate student and I learnt a lot about genetics at Caltech from exciting people. In addition to Beadle, another two of my teachers at Caltech would go on to win Nobel Prizes—Edward Lewis and Max Delbrück. I returned to Australia to take up an offer of a Research Fellowship in Microbial Genetics by Frank Fenner, Professor of Microbiology at the newly established Australian National University in Canberra. I was the only bacteriologist and the only geneticist in a department of virologists. Frank gave me complete freedom to choose my own research topic provided it did not involve *N. crassa!*

While passing through New York on my way home from Pasadena, I had heard the first public seminar by Norton Zinder on transduction in *Salmonella typhimurium* (Zinder and Lederberg 1952) and this stimulated my interest in studying the genetics of bacteria. While there had been little interest in the genetics of bacteria while I was at Caltech, Max Delbruck's phage group had demonstrated the value of studying genetics in organisms other than those then in vogue namely *Drosophila melanogaster*, *N. crassa* and *Zea mays*. In 1945 Edward Tatum and Joshua Lederberg had published ground breaking papers on conjugation in *Escherichia coli* and the significance of this work was beginning to be widely recognised. I appreciated the difficulties of competing with the larger and well-funded bacterial genetic groups working on *E. coli* in the USA, so I looked for an alternative bacterium. To begin with I studied the role of bacteriophage in toxin production in *Corynebacterium diphtheria* which had just been discovered by Neil Groman at the University of Washington, but I made little progress so I decided to work with another bacterium. I prepared a list of essential characteristics which included ready availability of strains, the ability to obtain auxotrophic mutants, the presence of bacteriophages, low pathogenicity and given my work location, of medical interest. I searched through the major microbiological tome of the time, Topley and Wilson's Principles of Bacteriology and Immunity published in 1946 for a suitable candidate and *P. aeruginosa* fitted all these requirements. As well, to my knowledge at that time (incorrect as it turned out) no one else was working on the genetics of this organism.

John Loutit had commenced his doctoral studies at the University of Adelaide in 1947 under the supervision of Nancy Atkinson, well known for her work on *Salmonella*. His thesis topic was genetic recombination in *P. aeruginosa.* Using one strain, he isolated a range of auxotrophic mutants, being the first to do so for this organism (Loutit 1952). By growing mixtures of different auxotrophs of another strain, Loutit found that prototrophic derivatives occurred, suggesting that some sort of genetic exchange was occurring at low frequency (Loutit 1955).

It is possible that another search for gene exchange mechanisms in *Pseudomonas* was initiated by André Lwoff at the Pasteur Institute in 1950. François Jacob in his Nobel Laureate address (Jacob 1965) wrote:

> Having come to prepare a doctoral thesis with Andre Lwoff, I was assigned the study of lysogeny in *Pseudomonas pyocyanea*. Thus I conscientiously set out to irradiate this organism. However, it soon became apparent that the problem of lysogeny was primarily that of the relationship between the bacterium and bacteriophage, in other words, a matter of genetics.

There is no evidence to suggest that Jacob actively sought to find a genetic system in *P. aeruginosa* but he did publish a series of papers on lysogeny and bacteriocins in that organism. Most microbial research at the Pasteur Institute at that time was with *E. coli*, so perhaps Lwoff was seeking to extend the knowledge of genetics to other bacteria.

In the second half of 1953 I obtained strains of *P. aeruginosa* from Melbourne, Adelaide and South Africa. I made auxotrophs of two strains which I called Strain 1 (later to be PAO1) and Strain 2 (later to be PAT2) using manganous chloride

as the mutagen. I isolated a range of auxotrophs for both strains in the very first experiment. Influenced by my work with *N. crassa* which requires two strains to be mated, I mated auxotrophs of strains 1 and 2, using the technique pioneered by Lederberg and Tatum which selects for prototrophic recombinants. The first mating produced prototrophs at frequencies similar to that found in *Escherichia coli* K-12. Further matings, which included auxotrophs of two other strains, showed linkage of unselected markers and all the data was consistent with a system of conjugation in *P. aeruginosa* (Holloway 1955).

Using PAO1, as it will be referred to, a series of multiple marker derivatives were isolated and an infectious sex factor, FP2, isolated from Strain 2. Conjugation in *E. coli* had been greatly helped by the use of high frequency donor strains known as Hfr variants, in which the F factor had become inserted into a site on the chromosome and chromosome transfer from the donor Hfr strain to a recipient or F- strain commenced at that site. No such Hfr strains were ever found with FP despite efforts to do so over many years.

The discovery of transduction in *S. typhimurium* stimulated me to study bacteriophages in *P. aeruginosa*. It was already known that lysogeny in *P. aeruginosa* was common and I had no difficulty in isolating bacteriophages from my strains. Indeed one strain, Strain 3, was lysogenic for four different bacteriophages (Holloway et al. 1960) and one of these, B3, was found to be transducing (Holloway and Monk 1959). I later isolated phage F116 which proved to be more efficient in transduction and has been widely used for fine structure mapping in PAO1. With Phage D3 we found that when PAO1 was made lysogenic for this strain the bacterial surface antigenic structure was changed, the first example of lysogenic conversion in *P. aeruginosa* (Holloway and Cooper 1960). Prophages have since been shown to be a significant component of the *P. aeruginosa* genome and be important for virulence properties of this organism. By contrast, they are less common in *P. putida* and fluorescent pseudomonads but do occur in some phytopathogenic pseudomonads.

In early 1957 I moved to the University of Melbourne to take up the offer of a tenured Senior Lectureship offered by Sydney Rubbo, Professor of Bacteriology in the Medical School. This gave me the opportunity to recruit graduate students to the *Pseudomonas* project. In 1958 at the invitation of Syd Rubbo, Joshua and Esther Lederberg spent about 4 months in the Department of Bacteriology. It was an amazing experience for me to work with them on both teaching and research. What better way to learn about *E. coli* genetics?

A Greater Research Profile for *Pseudomonas*

As part of the major expansion of scientific research which occurred in the United States after the Second World War, more scientists worked on genetics and the use of a wider range of experimental organisms enabled advances in the biochemical implications of genetic function. *N. crassa* and *E. coli* became more commonly

used. Both organisms had a significant experimental advantage compared to organisms such as fruit flies, corn and mice in that they were haploid, which meant that mutations were expressed phenotypically without further genetic manipulation. *E. coli* already had an established reputation as a genetics research tool through the work of Emory Ellis who studied bacteriophages at Caltech from 1936. Max Delbrück met Ellis in 1938 and was intrigued by the possibilities of phage biology. Further collaboration with Salvador Luria and Alfred Hershey led to The Phage Group using *E. coli* B as the host. *E. coli* had been a popular and effective subject for metabolic studies since the mid nineteen thirties. It was easy to grow in a chemically defined medium and did not present any safety issues for biochemists untrained in microbiological techniques. The isolation of auxotrophic mutants was first achieved by R. Roepke and his colleagues in 1944 (Roepke et al. 1944). Shortly afterwards Tatum confirmed these results which enabled he and Lederberg to demonstrate conjugation in *E. coli* K-12 (Tatum and Lederberg 1947).

By contrast, *Pseudomonas* had a more diffuse history. While the type species, *P. aeruginosa,* had been isolated and studied extensively during the early part of the twentieth century, this was principally due to its importance as a human pathogen. Other species of *Pseudomonas* were isolated from a wide variety of environmental locations, but the taxonomy was imprecise so that there was no single species or strain which was accepted and identified for study by microbiologists in different laboratories as had been the case with *E. coli*.

During the 1940's and early 1950's a group of highly distinguished microbiologists joined the Department of Bacteriology at the University of California in Berkeley. Michael Doudoroff arrived in 1940, followed after the war by Roger Stanier, Edward Adelberg, Germaine Cohen-Bazire, Norberto Palleroni and Bill Sistrom. This group would have a profound effect on the understanding of the metabolism of *Pseudomonas* and the taxonomy of this genus. This period has been well documented by Roger Stanier (Stanier 1980) and Patricia Clarke (Clarke 1986). During the 1950's and 1960's, research interest in *Pseudomonas* increased particularly in the USA. A range of different Pseudomonads isolated from different environments were shown to have a remarkable capacity to use a wide range of carbon and nitrogen sources, a range unparalleled by any other bacterial genus. The taxonomic and biochemical implications were apparent immediately.

In 1950, Roger Stanier graphically described the current state of knowledge of bacterial metabolism (Stanier 1950):

> …if one is hardy enough to attempt its construction, (bacterial metabolism) resembles a seventeenth century geographer's map of North America. The shape of the continent is roughly outlined, some small local areas already penetrated by the pioneers are correctly shown, but vast regions are either simply left bland or filled in as fancy dictates.

Two major issues dominated *Pseudomonas* research at this time. First there was the question of identity. What was a *Pseudomonas*? How was a species of *Pseudomonas* defined? The second was the ability of many isolates to grow on a wide range of chemical substrates. The goal of bacterial physiologists at this time was to identify as many pathways of assimilation and dissimilation as possible and to determine

their regulation. However, some of the essential biochemical techniques were yet to be introduced. Fragmentation of cells for the extraction of enzymes was a problem, as the French Press and the Raytheon oscillator were yet to be commonly found in microbiological laboratories. However, major discoveries began to emerge.

Doudoroff investigated sucrose utilisation by *P. saccharophila (*now reclassified as *Pelomonas saccharophila* but his results are valid for other species of *Pseudomonas*), leading to the discovery of a previously unknown major pathway of glucose degradation in bacteria, the Entner-Doudoroff pathway (Entner and Doudoroff 1952). Stanier discovered that fluorescent *Pseudomonas* cells grown on aromatic substrates oxidised a range of other similar substrates much quicker than cells grown on non-aromatic compounds, identifying the principle of simultaneous adaptation which is not found in most other bacteria. This became a major tool in elucidating the pathways by which aromatic compounds were metabolised. It took more than 10 years to identify all those pathways and the enzymes involved. For the first time, genetics contributed to this work with the isolation of mutants lacking specific enzymatic function which were used to identify metabolic pathways, emphasising the value of genetics to the solution of biochemical problems.

From the early days of this work, particular interest was focussed on how *Pseudomonas* metabolised aromatic compounds—a property not found in *E. coli.* Beginning in Stanier's laboratory in the early 1960's, a cascade of work on the β-ketoadipate pathway has resulted in a broader understanding of gene arrangement, gene regulation, evolutionary mechanisms, enzymatic structure and function which has extended beyond *Pseudomonas* to other species of bacteria and even to Eucaryotes (Ornston 2010; Harwood and Parales 1996).

The work at Berkeley resulted in the classic paper by Stanier et al. (1966) in which 267 strains were examined and 146 compounds were screened as carbon and nitrogen sources. This was an approach first developed by den Dooren de Jong (1926), but his results had been unavailable to microbiologists generally because it was published as a doctoral thesis written in Dutch. As it happened, Roger Stanier had a copy of this paper. The Berkeley paper provided a firm basis in *Pseudomonas* taxonomy available to the world community of microbiologists. Working both independently and in collaboration with Stanier, Irwin Gunsalus at the University of Illinois made a significant contribution to characterising the enzymology and regulation of *Pseudomonas* metabolic pathways. He has written a very modest account of his long term, major contributions to *Pseudomonas* biochemistry and genetics (Gunsalus 1996).

The Foundations of Pseudomonad Taxonomy

Once the Berkeley group had decided to focus on understanding the metabolic behaviour of *Pseudomonas* the identity of independent isolates of apparently the same species became important, if only for comparison and validation of results. Establishing a taxonomic framework for the genus *Pseudomonas* was always going to be

a major challenge. Norberto Palleroni, a major participant in the taming of *Pseudomonas* taxonomy has written the definitive history from the inside perspective of a journey that has taken over 50 years. (Palleroni 2003, 2010). Palleroni came to the Department of Bacteriology at the University of California at Berkeley in 1953 to work with Mike Doudoroff. Over the next 20 years, with the active collaboration of Roger Stanier and others in the Department of Bacteriology—The *Pseudomonas* Group as it came to be known (Palleroni 1978)—the biology, metabolic activities and variation of hundreds of *Pseudomonas* isolates were studied. This work clearly established the basis for a definitive approach to the taxonomy of this genus.

It soon became clear that with such a biologically diverse group of organisms, multiple taxonomic criteria would be necessary. Palleroni recognised the value of DNA/DNA hybridisation techniques which had become available but at first this approach did not provide any additional taxonomic criteria. Independently, a study of ribosomal RNA had demonstrated high sequence conservation in bacteria and this provided a new and accurate means by which *Pseudomonas* isolates could be distinguished (Palleroni et al. 1973). This was the first time that RNA sequence homology had been used for taxonomic purposes in bacteria and it soon became widely used for other genera. Palleroni and his colleagues (Palleroni et al. 1973) showed that by using ribosomal ribonucleic acid homologies, the genus *Pseudomonas* consisted of five distinct groups. In 1992, Yabuuchi and his co-workers (Yabuuchi et al. 1992) in Osaka suggested a new genus, *Burkholderia,* to differentiate some *Pseudomonas* isolates. This was followed in 1995 (Yabuuchi et al. 1995) with a further proposal to include some *Burkholderia* species in a new genus, *Ralstonia*. There has been an ongoing reassignment of *Pseudomonas* species to these and other new genera as additional taxonomic criteria have become available. Much remains to be done to classify the hundreds of isolates in the literature named *Pseudomonas*. Given the rate of technologically development in determining genome sequences, it is likely that genomic analysis, combined with bioinformatics will provide the ultimate taxonomic criteria for pseudomonads.

Regulation of Microbial Metabolism

The characterisation of the lactose operon in *E. coli* by the group at the Pasteur Institute in Paris led by François Jacob was the first correlation of genetic organisation and enzyme regulation in any organism. The was made possible solely by the ability to identify and precisely map genes in *E. coli*, assay the relevant enzymes and relate the role bacteriophages play to these activities (Jacob et al. 1960; Ames and Martin 1964). A further example of an operon was discovered in *Salmonella typhimurium*. In 1959, a transduction analysis by Milislav Demerec and Phil Hartman at Cold Spring Harbor showed that genes of the biosynthetic pathway of histidine were clustered and probably contiguous (Demerec and Hartman 1959). As well, the coordinate expression of histidine biosynthetic enzymes was demonstrated by Bruce Ames and his colleagues (Ames and Garry 1959).

For those working with fluorescent and other Pseudomonads, this work would be a stimulus to find the genetic basis of enzymatic regulation in those organisms. The necessary equivalent tools would not become available for some years to come for the fluorescent Pseudomonads. Developments in the genetics of *P. aeruginosa* did enable a comparison of this species with enteric bacteria. The first mapping data for *P. aeruginosa* had shown that not all the genes for the tryptophan biosynthetic pathway are closely linked, in contrast to the situation in *E. coli* where they are all contiguous. In 1960, Barbara Fargie and I, followed independently by others, extended this type of gene arrangement to include nine biosynthetic pathways (Holloway and Fargie 1960; Holloway 1969).

In 1962, I spent a year with Salvador Luria at the Massachusetts Institute of Technology. During this time, I learnt a lot about science in general and microbial genetics in particular. He proposed that I work on one of his projects involving *E. coli* and lambda phage, but soon after I started, I did an experiment which demonstrated that the theory underlying the project was questionable. Surprisingly to me, Salva was pleased with this outcome, I found he was always more interested in data than his own theories. By this time, I had established interactions with other MIT staff and Salva suggested that I collaborate with them. This gave me experience in isolating and characterising DNA as well as in electron microscopy. I used *Pseudomonas* phages for this work so it had ongoing value to our overall *Pseudomonas* program (Davison et al. 1964; Slayter et al. 1964).

During my time at MIT, I attended the 1963 Cold Spring Harbor Symposium, a memorable experience. While there I met Gunny Gunsalus for the first time, and this was the beginning of a long and productive collaboration. During the 1960's I went to Urbana, Illinois several times to work with Gunny's group which at that time included Al Chakrabarty. Shortly after returning to Melbourne, I had my first overseas visitor—Ned Shrigley from Indiana University, which I had arranged during my time at MIT. He was interested in "autoplaque" formation in *P. aeruginosa* (Berk 1963). When some strains of this organism are grown on solid media, they develop what looks like phage plaques, but no phage can be isolated from these manifestations and the plaques are a manifestation of autolysis. We had hoped that a genetic approach to this problem might be successful but that did not happen. D'Argenio et al. (2002) suggested that extracellular signalling may be involved in this phenomenon but there is still no satisfactory explanation of this autolysis despite the best efforts of many workers over the years.

Pseudomonad Genetics in the Pre-Genomic Era

Genetic analysis for other species of *Pseudomonas* became possible in the nineteen sixties. Using a transduction system in *P. putida*, Gunsalus and his colleagues demonstrated a similar gene arrangement for biosynthetic pathways to that found in *P. aeruginosa* (Chakrabarty et al. 1968). They went on to study gene arrangement in the mandelate pathway and found a mixture of closely linked and unlinked

structural genes (Gunsalus et al. 1968). George Hegeman and his co-workers at Berkeley studied the genetics and regulation of the β-keto adipate pathway, a pathway that was to figure largely in the understanding of gene regulation in pseudomonads (Hegeman 1975). Overall, the results showed that *P. aeruginosa* and *P. putida* were similar in the distribution of genes for most of the pathways studied.

The regulation of many biosynthetic pathway genes in *P. aeruginosa* was shown to be quite different for the same enzyme in enteric bacteria. While coordinate repression is the predominant mode for *E. coli,* this is not the case in *P. aeruginosa*. In this organism, if such genes are clustered they show repression, but if unlinked they are constitutive in expression. This was also found to be the case for *P. putida*. Hegeman demonstrated the coordinate induction of enzymes in *P. putida* converting D-mandelate to benzoate and subsequently Nicholas Ornston demonstrated the supra-operonic genetic control of genes in the aromatic pathway in *P. putida* (reviewed in Holloway 1969).

As indicated above, much of the seminal work on bacterial metabolism and genetics was done using *E. coli* K-12 and its bacteriophages. There were good logistical reasons for this but it did lead to a proprietorial attitude by some *E. coli* workers as described in Nick Ornston's highly entertaining autobiographical article describing his work at both Berkeley and Yale (Ornston 2010). He relates that microbiologists who were not working with *E. coli* were asked "*Why* aren't you working with *E. coli*?" Millard Sussman's 1970 review entitled "General Bacterial Genetics" (Sussman 1970) was devoted entirely to *E. coli* genetics. Jacques Monod (Friedman 2004) wrote that "Anything found to be true for *E. coli* must also be true for elephants". However, the genetics and biochemistry of pseudomonads have demonstrated significant differences which are important for microbiology and biochemistry generally.

In 1968 I accepted an offer by the recently established Monash University, located in the suburbs of Melbourne, to be Foundation Professor of Genetics and as was the custom in those days, I was also Chairman of the Department of Genetics until 1992. Monash had taken its first students in 1961 so this was a rare opportunity to start a Department of Genetics from the ground up. I expanded the microbial genetics group, but I also appointed staff experienced in other aspects of genetics. I brought Jill Isaac and Vilma Stanisich with me from Melbourne University and I appointed Viji Krishnapillai and Tony Morgan. We attracted more graduate students and our grant funding increased. Jill Isaac and I continued to study regulation of biosynthetic pathways in *P. aeruginosa* focussing on the pyrimidine biosynthetic pathway (Isaac and Holloway 1968). We found a completely different pattern of regulation for genes in this pathway to that shown in *E. coli*.

A major research goal was to expand the *P. aeruginosa* chromosomal map. Vilma Stanisich and I published the first substantial paper on conjugation mapping in *P. aeruginosa* in 1969 (Stanisich and Holloway 1969). We tried to find Hfr versions of FP2 without success and looked for other fertility factors. We looked for other FP plasmids and found FP39, which had a different site of origin to FP2 but we were not able to demonstrate circularity of the *P. aeruginosa* chromosome at this time.

In 1972 spent 6 months with Patricia Clarke at University College, London. Pat had introduced an entirely new system for studying genetic regulation of enzyme function in *P. aeruginosa* using the aliphatic amidases. This work has contributed extensively to this area of *Pseudomonas* biology and has enabled a closer comparison to the situation in enteric bacteria (Clarke 1980).

The Genomic Era Begins

Since the mid 1970's, the ability to analyse and manipulate genetic material has totally changed the overall approach to biological and medical research. The two key technologies involved were the use of restriction enzymes for cloning and the sequencing of DNA bases. Bacterial genetics provided the research base for the first of these and again, the interaction of bacteria and bacteriophage was an important part of the experimental history. It was shown in the early 1950's that the ability of a bacteriophage to replicate in a given bacterial strain was dependent on the previous strain in which it was propagated. Two enzymes were shown to be involved—a restriction endonuclease and a modification methylase (Boyer 1971). Further work revealed multiple restriction endonucleases which cut double stranded DNA at a variety of base sequence recognition sites. Combined with the characterisation of plasmids, this led to the technique of cloning DNA (Morrow et al. 1974). While *E. coli* cloning vectors were widely used for many years, subsequently vectors better suited to *Pseudomonas* were constructed (Bagdasarian and Timmis 1982)

The biology and genetics of restriction and modification were studied in a variety of bacteria including *P. aeruginosa* (Holloway 1968). The features of these characteristics were similar to those found in *E. coli* with one exception. If *P. aeruginosa* was grown at 43 °C, the restriction and modification phenotypes were suppressed, so that foreign DNA could enter these bacterial cells without being degraded. This change in phenotype persisted for about 60 generations when bacteria previously grown at 43 °C were subsequently grown at 37 °C after which they reacquired the original restriction and modification phenotypes (Holloway 1965). This curious type of inheritance was named "the 43° effect" but a satisfactory explanation has never been found. However it became a valuable experimental tool in allowing foreign DNA to enter and function in *P. aeruginosa*. This was particularly useful when the wide host range Inc P1 plasmids became vectors of foreign DNA. I now think it possible that the 43° effect is due to epigenetic inheritance, made more likely as it involves changes in DNA methylation, but this hypothesis has never been tested.

The second key technique of the genomic era was DNA sequencing (Sanger et al. 1977). The mechanisms involved have advanced in ease, affordability, cost and analysis of the resulting data to the point where now it is a major source of biological information with even greater future potential.

More Genetic Complexity

Research in other aspects of medical microbiology indicated that the bacterial genome had other, novel ways of acquiring additional genetic information in nature. The discovery and use of antibiotics enabled the successful treatment of infections previously considered intransigent. An unexpected outcome was the development of infectious bacterial strains resistant to the widely used antibiotics, and from the early days of bacterial genetics, this has always been an important research topic. It needed to be established whether bacterial resistance mutants arise independently or is their formation a result of the presence of the antibiotic in the environment? While in today's idiom, this may seem like a no brainer, there were vigorous proponents for each view at the time but eventually the former view was established experimentally.

The genetics of antibiotic resistance became even more interesting in the light of clinical experience in Japan after the war. From 1945 onwards, *Shigella* strains with high resistance to sulphonamide began to appear in isolates from patients. However, from 1957 *Shigella* strains with multiple antibiotic resistance appeared, the antibiotics involved being sulphonamide, chloramphenicol, tetracycline and streptomycin. A few years later, it was demonstrated that these multiple resistances could be transferred from *Shigella* to *E. coli* in mixed culture by genetic elements similar to the fertility factor F of *E. coli*. These elements were christened plasmids, episomes or resistance transfer factors (RTF) (Watanabe 1963). They are now a significant problem in the treatment of infectious disease worldwide. The characterisation of plasmids carrying antibiotic resistance genes became a major area of research. This area attracted major funding from governmental agencies and the pharmaceutical industry because these plasmids had the potential for rendering antibiotics ineffective with major impacts on how infections were treated clinically.

Antibiotic Resistance in Pseudomonas

Antibiotic treatment of *P. aeruginosa* infections has always been difficult, given the high innate resistance of this organism to most of the commonly used antibiotics. Edward Lowbury, a surgeon in Birmingham, England was the first to isolate drug resistance plasmids in *P. aeruginosa* and which conferred resistance to a number of antibiotics including carbenicillin. More important, these plasmids could be transferred to other gram negative bacteria (Lowbury et al. 1969). This landmark discovery of wide host range plasmids created difficult clinical issues but provided a singular genetic opportunity.

Vilma Stanisich and I obtained some of these plasmids from Lowbury and tested them for their ability to transfer chromosome in *P. aeruginosa*. The first experiments were highly successful (Stanisich and Holloway 1972). This was the origin of

the work which resulted in the development of the chromosome mobilising plasmid R68.45 which would have a substantial impact on the chromosomal mapping of *P. aeruginosa* and other gram-negative bacteria.

I looked for plasmid variants that were more efficient in chromosomal transfer. I argued that as recombinants, usually selected as prototrophic recombinants were rare, these recombinants themselves might carry the plasmid variant that had caused their formation. I chose the Inc P1 plasmid R68, and set up crosses to select for recombinants prototrophic for one marker, but still carrying another auxotrophic marker so that I could test such variants in a mating to detect chromosomal transfer ability. My prediction turned out to be true and one such variant, R68.45, produced chromosomal recombinants at a frequency a thousand fold greater than that obtained with FP2 in *P. aeruginosa*.

Dieter Haas, then working at the ETH in Zurich came to Monash in 1975 and we collaborated to characterise R68.45 (Haas and Holloway 1976, 1978). This plasmid was instrumental in expanding and refining the chromosome map of PAO1. As well, with its wide host range properties it was used by other workers to study conjugation and map the chromosomes in other organisms. The circular map of *Rhizobium* was constructed by Andy Johnstone at the John Innes Institute, Norwich using R68.45, an achievement reached even before we had shown a circular map for *P. aeruginosa*. R68.45 was subsequently shown to be able to promote chromosome transfer and enable genetic analysis in a range of gram negative bacteria including *Agrobacterium, Azospirillum, Erwinia, Escherichia, Klebsiella, Methylophilus, Rhizobium, Rhodopseudomonas* and *Zymomonas* (Holloway 1993).

The fact that R68.45 transfers the chromosome of PAO1 from multiple sites located in different chromosomal regions makes it a much better mapping tool than FP2 and it can be used to measure map distances in regions of the chromosome where other sex factors do not produce enough recombinants for accurate measurement. Used as a "large" generalised transducing phage, R68.45 proved to be valuable in the construction of PAO strains with desired phenotypes. We had many requests for the plasmid and we distributed it to all who asked. It was widely used in Japan as that country was slow in permitting *in vitro* recombinant DNA experiments in bacteria to be undertaken in laboratories. R68.45 was used for transfer of genetic material between unrelated bacteria as it met the Japanese Government regulatory requirement of natural DNA transfer. Subsequently, I isolated R prime plasmids with R68.45, analogous to the F prime plasmids of *E. coli*, which expanded the intergeneric transfer capabilities of this plasmid.

In 1981 we published the first circular chromosome map of *P. aeruginosa* PAO1 (Royle et al. 1981) and began to identify the molecular structure that made R68.45 so interesting, our first venture into the genomic era. We collaborated with Alf Puhler's group in Bielefeld, Germany to show that there was a tandem duplicated region on the R68.45 chromosome (Riess et al. 1980), and when Neil Willetts came from Edinburgh to spend a year at Monash, we demonstrated that this region was an insertion sequence, IS21 (Willetts et al. 1981). There was one copy of IS21 in R68 and two tandem copies in R68.45.

We regularly received many requests for our mutant strains of *P. aeruginosa* and this activity, along with the need to have a well-documented strain collection, resulted in the appointment of Elspeth Carey, as our Strain Collection Curator. She made a major contribution to *Pseudomonas* genetics by her meticulous attention to detail and techniques in maintaining stocks, checking their genotype and ensuring that all requests for strains by other laboratories were met. This also meant that other laboratories were willing to share their strains with us, so everyone benefited. When I retired in 1993 and support for the maintenance of this culture collection ceased at Monash, most of the collection was transferred to the Department of Microbiology at the University of East Carolina in Greenville, North Carolina, thanks to the enthusiasm and generosity of Paul Phibbs. This was one of the benefits from my long standing and rewarding association with Paul. He worked in my laboratory at Monash for a while and in 1986 I went to Greenville, North Carolina to work with him. After he retired, despite the continued efforts of other staff members in Greenville to maintain the collection and distribute strains on request to other laboratories, the Greenville collection ceased to exist except for some strains which were sent to the American Type Culture Collection. Sadly, this is the fate of a number of similar culture collections in the twenty first century.

Cystic Fibrosis and Pseudomonas Aeruginosa

Cystic fibrosis is of the most frequent genetic diseases in humans and *P. aeruginosa* infection is now a major factor affecting the longevity of patients with cystic fibrosis. It was not always like that. The increasing importance of cystic fibrosis has been one of the major drivers for research on *P. aeruginosa* which in turn has had a major impact on knowledge of other species of *Pseudomonas*. The first definitive clinical description cystic fibrosis was provided by Dorothy Anderson in 1938. Upper respiratory infections of these patients were always part of the disease syndrome but while they consisted predominantly of gram positive organisms, they were controlled clinically by antibiotic treatment. This resulted in a shift in the composition of the microbial flora in the lungs of cystic fibrosis patients so that now in the twenty first century, by their late teens most have *P. aeruginosa* infections and the overall treatment of these patients has become more difficult.

A significant development was the detection in 1964 of a colonial variant called "mucoid" in isolates of *P. aeruginosa* from cystic fibrosis patients (Doggett et al. 1964). Of particular interest is that this variant is hardly ever isolated from patients with other types of *P. aeruginosa* infections, for example burns or cancer cases. Appearance of the mucoid variant in the sputa of cystic fibrotics is associated with deterioration in the prognosis. There has been a sustained and expanded study of the causes of *P. aeruginosa* infections associated with cystic fibrosis and this has been largely funded by Cystic Fibrosis associations in various countries. A major source is the American Cystic Fibrosis Association which has funded many research projects unable to access US government sources of funding. It is now

known that mucoid strains result from the formation of alginate and the biosynthesis and regulation of alginate has been intensively studied. Alginate is not the sole virulence factor. Others include pyocyanin, the blue phenazine pigment so characteristic of *P. aeruginosa*, biofilm formation, the overarching regulatory mechanism of quorum sensing, small regulatory RNA molecules, exotoxins, and a variety of other genes including those for motility and attachment. Understanding the functions and regulation of such a broad genetic structure has been and remains a major challenge to *Pseudomonas* workers and the treatment of cystic fibrosis (Govan and Deretic 1996).

The ecology of *P. aeruginosa* in the lungs of cystic fibrosis patients is complex. The majority of bacterial cells are in a biofilm which is largely composed of a polysaccharide matrix. As well there are free living planktonic cells. The biofilms enhance the emergence of antibiotic resistant variants of *P. aeruginosa* which in turn complicates the clinical management of this disease. The understanding of the infectious process of *P. aeruginosa* in cystic fibrosis has served as an important model for other bacterial infections, particularly how antibiotic resistant variants arise in the host.

I had only a limited involvement in the microbial genetic aspects of cystic fibrosis. John Govan came to work in my laboratory at Monash for 6 months and that collaboration continued after he went back to Edinburgh. Later, the cosmid bank of *P. aeruginosa* we constructed (see below) was used in a very effective collaboration with Vojo Deretic then at San Antonio, Texas to map genes involving alginate formation.

Pseudomonas and the Environment

It was a reality of life in the 1950's that there was a general lack of scientific and public interest in what is now referred to as "the environment". The catalyst for what is now a global concern in the topic was the book "Silent Spring" written by Rachel Carson and published in 1962. The literature on multidisciplinary scientific studies of the environment as well as the general public and Government interest has escalated since the nineteen sixties and seventies.

Many studies have shown that *Pseudomonas* survives in a wide variety of predominantly aqueous environmental conditions and niches. Given the range of aromatic compounds which are now common pollutants, most of industrial origin, it was inevitable that *Pseudomonas* isolates would be of interest for bioremediation studies given the innate ability of this organism to degrade so many organic molecules. Chakrabarty's seminal work (see below) on genetically modified strains of this organism attracted workers to this topic. Since then, there have been extensive studies by Ken Timmis and his colleagues in Germany and Victor de Lorenzo's group in Spain which have maintained a wider interest. However, the widespread, commercially viable and effective use of genetically modified *Pseudomonas* or other microorganisms for bioremediation is still to be achieved (Cases and de Lorenzo 2005). Nevertheless, an active search for this goal continues.

Pseudomonas Meets Industry and the Law

The broad characteristics of the genetics and metabolism of pseudomonads attracted considerable commercial research interest during the 1970's and 1980's. In addition to the need to understand the biochemical diversity of this group of organisms, the possibility of commercial outcomes loomed larger as research organisations, funding agencies and corporate entities became more involved with the genetic renaissance of biotechnology.

Once again, plasmids played an important role, as they had with developments in antibiotic resistance. Plasmids were found in strains of *Pseudomonas* isolated from particular environments which could enable these microorganisms to degrade specific hydrocarbons. The major plasmids were CAM (camphor), SAL (salicylates), NAH (naphthalene), OCT (n-alkanes), XYL (xylene) and TOL (xylene). While in most cases the strains of pseudomonads involved carried these plasmids as replicons separate from the chromosome, in other strains, the plasmids were integrated into the chromosome. This finding has special significance for studies on the evolution of the biochemical diversity of pseudomonads (Chakrabarty 1976).

In 1971, Ananda (Al) Chakrabarty started work at the General Electric Research and Development Center in Schenectady, New York. Shortly afterwards, he developed a variant of *P. putida* which was stable in culture, contained hydrocarbon degrading genes from four different plasmids and was effective in digesting organic compounds found in crude oil spills.

In 2005, Douglas Robinson and Nina Medlock wrote (Robinson and Medlock 2005):

> Chakrabarty is not well known outside the intellectual property community—the average person probably has never heard the name. Yet, Chakrabarty has affected the lives of virtually everyone in the United States, having contributed to a revolution in biotechnology that has resulted in the issuance of thousands of patents, the formation of hundreds of new companies and the development of thousands of bioengineered plants and food products

How did this come about? In 1972, Chakrabarty filed a patent application assigned to the General Electric Corporation claiming that he had constructed in the laboratory a genetically engineered bacterial strain that was man-made and possessed properties not found in naturally occurring microorganisms. The original patent was approved in part, but two claims relating to the bacterial strain were rejected on the grounds that microorganisms are products of nature and not patentable under US law.

Chakrabarty appealed this decision to the Patent Office Board of Appeals but the original decision was upheld. Next Chakrabarty took his case to the Court of Customs and Patent Appeals who reversed the previous decision declaring "…… the fact that microorganisms are alive is without legal significance". Sidney Diamond, the US Commissioner of Patents and Trademarks appealed this decision to the US Supreme Court. On June 16th, 1980 that Court, in a split 5–4 decision, upheld the view of The Court of Customs and Patent Appeals, confirming that genetically engineered microorganisms could be patented, creating the famous "Diamond v. Chakrabarty" case which has achieved worldwide recognition and acceptance (Diamond v. Chakrabarty 1980).

As a result, many biological inventions and discoveries, previously thought to be without legal protection, have been patented. This in turn has created a solid economic and legal basis for the international biotechnology industry and has added further distinction to the role that *Pseudomonas* has played in the history of microbiology. Of particular importance is the legal basis that this patent has provided to the patenting of DNA sequences.

Pseudomonas was to figure in another important legal and biotechnological event. Michael Vandenbergh, in an article on the regulation of the release of genetically modified microorganisms wrote (Vandenburgh 1986):

> When the Environmental Protection Agency (EPM) first approved a field test of a bioengineered microbe, one EPA official remarked: "We're not expecting this to the rutabaga that ate Pittsburgh".

The organism in question was *P. syringae*. Some bacteria have the ability of initiate ice formation and this was first demonstrated with *P. syringae* (Maki et al. 1974). Other species of *Pseudomonas* will also carry out this function as do some other bacterial genera (Gurion-Sherman and Lindow 1993). Such bacteria have a single protein—the "ice plus" protein on their cell surface which can provide a focus for ice crystals to form. While this is a very interesting interaction between biological and physical activities, it also has practical applications. The presence of such bacteria on the surface of plants can result in frost damage with significant economic effects on agricultural production.

Naturally occurring variants of *P. syringae* which lack the ability to catalyse ice formation were isolated. Working at Berkeley, Steve Lindow used recombinant DNA technology to create stable "ice minus" variants of *P. syringae* and sought to use them to minimise frost damage to potatoes and strawberries in California. Field tests for this purpose were given United States Government approval as was required with every release of genetically modified organisms. A legal challenge to the release was at first successful. However, the legal basis on which this challenge was based preventing field trials did not apply to commercial organisations. In 1983, Advanced Genetic Sciences obtained United States Government approval to conduct field trials using ice minus variants of *P. syringae* and *P. fluorescens* developed by Lindow. Further legal and public objections delayed final approval until 1987 when the release went ahead. Hence *P. syringae* achieved the distinction of being the first living genetically modified organism to be granted approval for release into the environment.

I have had two major interactions with industry. The first resulted from an error in microbial taxonomy. In the nineteen seventies, Imperial Chemical Industries (ICI) in the United Kingdom developed a new process for converting North Sea hydrocarbon gas into methanol and hence the need to find new markets for methanol. One was the use of microorganisms to convert methanol to biomass which could then be used as a dried animal feed called Pruteen. ICI purpose built a 6 million L stainless steel fermenter at Billingham in the North of England. They sought my advice on the genetics of the organism they had selected for this process and sent the strain to me in the belief that it was a species of *Pseudomonas*. We found, as

did other scientists at ICI, that it was not a pseudomonad, and was subsequently identified as *Methylophilus methylotrophus*, an obligate methylotroph. However, this did get my group working on the genetics of methylotrophs. ICI supported this work for some years and it was productive to transfer the genetic techniques we had developed in *Pseudomonas* to a completely different organism. The Pruteen project was closed down in the late 1980's when it became non-competitive with soybean protein.

My second industrial venture was with the Celanese Corporation in 1979. They proposed to manufacture specialty chemicals by bacterial fermentation from low cost substrates and had identified the β-ketoadipate pathway of pseudomonads and other organisms as a starting point. Together with Nick Ornston from Yale University and Howard Dalton from the University of Warwick in the United Kingdom, we provided a team of consultant advice to Celanese, and later a spin off biotechnology corporation, Celgene, in microbial applications to specialty chemical production from about 1980 to 1993. We made regular visits to the Corporation headquarters in New Jersey and they also funded research in each of our laboratories. In my case, this also involved sending members of the Monash team to New Jersey to pass on techniques to Celgene staff and in their staff coming to Monash for training. It was a highly productive association with the added benefit for me of working with two outstanding scientists as co-consultants. Friendships were established which have lasted long after the commercial activity ended.

Phytopathogenic *Pseudomonads*

Interest in bacterial phytopathogens generally became more pronounced in the period after World War II and the role of Pseudomonads in plant disease became to be more appreciated (Starr 1959). Issues of nomenclature and taxonomy became more important. The taxonomic criteria in vogue at the time did not fit well with the biological characteristics of the many organisms being isolated particularly if host range had to be considered. The phytopathogenic pseudomonads did not show any significant differences compared to saprophytic isolates but minor differences were used for differentiating host range. This was especially true for some species particularly *P. solanacearum* (Hayward 1991). In *P. syringae*, the problem was solved by use of the term pathovar, abbreviated to pv, to distinguish isolates causing disease on different hosts. In some isolates other criteria could be used to further differentiate pathovars (Hirano and Upper 1990).

For phytopathologists, the important question was how do bacteria cause disease and how do they differ from saprophytic bacteria? For Pseudomonads most of the work to address this problem has been done in two species, *P. solanacearum* and *P. syringae*. It has been shown that specific compounds produced by phytopathogenic bacteria play a role in pathogenicity, although other virulence factors are involved.

The availability of genetic approaches in bacteria has helped to define the basis of pathogenicity particularly through the use of mutants affecting disease producing

gene products. The use of rRNA homology groups has redefined the taxonomy of this group of organisms. This is particularly true in *P. solanacearum*, cause of Bacterial Wilt which affects major food crops including potato, tomato and banana. It is a particularly variable species for host range encompassing many species of 44 plant genera, ecological niches ranging over tropical, sub-tropical and temperate regions, and multiple disease characteristics. A system of races and biovars has been developed to catalogue this variability. It has been the subject of taxonomic revision, being first renamed *Burkholderia solanacearum* and its current denomination is *Ralstonia solanacearum*. Control of Bacterial Wilt is difficult given that it is a soil borne disease and the current major strategy is breeding for host resistance to disease.

In 1985 I was asked by the Australian Centre for International Agricultural Research (ACIAR) to undertake a research and training program on the genetics of *P. solanacearum,* working with developing country agricultural scientists on the application of molecular genetic techniques to this organism. Bacterial Wilt caused by this organism is a major problem in tropical and sub-tropical regions in which ACIAR has a major interest. I did not anticipate that this program would continue until 2002. I established collaborations with other research groups in Australia notably those led by at Chris Hayward at the University of Queensland and Jeremy Timmis at the University of Adelaide. The training program on molecular genetics involved selected agricultural scientists from developing countries coming to one or more of the three Australian institutions involved to obtain laboratory experience. We established procedures by which the home laboratories of those we trained were equipped with the necessary laboratory equipment for this work, and we sought to build up research groups so that the people we trained would not be working in isolation. One of the outcomes of this program was the development of a DNA probe for the specific identification of *P. solanacearum* which could be used in developing country institutions (Opina et al. 1997). I also started a program at Monash to develop the genetic analysis of *P. syringae* (Nordeen and Holloway 1990)

Pre-genomic genetic analysis of phytopathogenic pseudomonads had only limited success. Gene transfer systems, either or both transduction and conjugation were found for *P. glycinea* and *P. syringae* with all that work done since the early 1970's (Panopoulos and Peet 1985). It has been demonstrated that while single genes can decide the virulence of a bacterial strain for a particular host, in most cases disease production is the result of multigenic control. As well, there may be specific host—parasite gene interactions. Of particular interest was the demonstration that in some strains of *P. solanacearum* some virulence genes known as *hrp* genes were clustered on the DNA of a very large plasmid. It would be shown later (Salanoubat et al. 2002) by whole genome sequencing that the genome of this organism consists of a 3.7 Mbp chromosome and a megaplasmid of 2.1 Mbp, with the *hrp* genes located on the megaplasmid. Genomic techniques have subsequently created successful new opportunities for the understanding of phytopathogenic pseudomonads.

Bacterial Chromosome Mapping in the Genomic Era

In line with our long term aim to get the most detailed map of the *P. aeruginosa* chromosome, we had established circularity of the map in 1981 (Royle et al. 1981). This map had been constructed using a variety of sex factors and there was no certainty that the different plasmids mobilised chromosome at the same rate. Hence the measured distances between genes on the map were of questionable accuracy. What was needed was a circular chromosome map constructed using only one sex factor. This was achieved using two new tools. A mutant of the IncP 1 plasmid R68 temperature sensitive for replication was isolated by Carol Crowther. This was then loaded with a newly found transposon, Tn2521, which Martha Sinclair and I had isolated from clinical strains of *P. aeruginosa.* This hybrid plasmid, pMO514, was then transferred to selected PAO strains with appropriate auxotrophic markers. These were grown at 43 °C and selection made for streptomycin resistance, a marker encoded by Tn2521. It was found that pMO514 had integrated into the PAO chromosome at a range of different sites. When so integrated, it mobilized chromosome at high frequency from each site, thus creating donor strains comparable with the Hfr strains in *E. coli*. Time of entry data obtained from a range of crosses enabled the recalibration of the *P. aeruginosa* PAO1 chromosome map to 75 min, compared with the former value of 95 min (O'Hoy and Krishnapillai 1987).

Conventional genetic mapping in various species of *Pseudomonas* clearly demonstrates the frustrating labour intensive techniques involved, with limited data outputs which vary from species to species. The availability of DNA interactive techniques to create physical chromosome maps provided an approach which can be used for all bacteria. However, such maps do not eliminate the need to identify individual genes through mutation, complementation or other approaches.

The discovery and characterisation of restriction endonuclease enzymes provided the key to this novel mapping paradigm. Unlike cloning, where restriction enzymes having frequently occurring sites are required, physical mapping needs such enzymes which cut infrequently. This approach is combined with the techniques of pulsed field gel electrophoresis (PFGE) which permits the separation of DNA fragments of widely disparate sizes, even those having several megabases of double stranded DNA. Together with techniques which enable the order of the fragments to be determined, the size of an entire bacterial genome can be determined. (Smith and Condamine 1990; Fonstein and Haselkorn 1995).

The location of individual genes on such fragment maps can then be located using a combination of cloned material, DNA probing and complementation mapping (Holloway 1993). This combined approach was widely used in the 1990's for the construction of combined physical and genetic maps for a wide variety of microorganisms including *P. aeruginosa*, *P. putida*, *P. solanacearum*, *P. fluorescens* and *P. stutzeri*.

For more precise linkage analysis, we needed a vector that while retaining the wide host range of the P1 plasmids, transferred a smaller fragment of chromosome. While there were an increasing number of available cloning vehicles, for our work

we needed one with wide host range and low copy number. Other laboratories had similar needs and we were lucky that Ron Hanson's laboratory at the University of Minnesota developed a series of cosmids (a plasmid vector containing the *cos* site of lambda phage) that were derived from a P1 group plasmid. They generously let us have some of their strains and we found that one, pLA2917, was ideal for our purposes. It was to become a major tool for our genomic analysis of a number of bacteria including methylotrophs. We constructed combined physical and genetic maps of *P. aeruginosa*. For the genetic mapping we had the advantage of a lot of data from conjugation and transduction, so we knew the relative location of a lot of genes. In addition, we had a large collection of mutants for *P. aeruginosa*, some of them characterised for the metabolic function involved. While we had obtained some of this data ourselves, most of the gene function data had been obtained by making our mutants available to anyone who wanted them. We had sent mutants to many laboratories and in turn they had done the biochemical investigations on our strains. For the gene bank we adopted a new approach in that individual cosmids were selected for storage rather than the mixtures of cosmids which was the standard approach of the time. We stored fragments of *P. aeruginosa* chromosome in cosmids carried in individual *E. coli* cultures. A library of about 1000 individual cosmids was needed to be sure of covering the whole chromosome with some degree of duplication. By using the individual cosmid approach, we could identify and retain fragments that contained particular chromosomal regions identified by genes or restriction fragments and in this way, identify the genes carried by individual cosmids.

In summary, the essential components that we used to construct combined physical and genetic maps of the bacterial species that interested us were an individual cosmid library, data obtained by PFGE and restriction enzyme site mapping, complementation of known mutants, DNA probes used in Southern hybridisation and eventually DNA and protein sequence data bases. Genetic data obtained by pre-genomic approaches were an added bonus. A combined genetic and physical map of *P. aeruginosa* PAO1 was constructed (Ratnaningsih et al. 1990). Burkhardt Tümmler and his colleagues in Hanover had also constructed a physical map of *P. aeruginosa* PAO1 (Romling et al. 1989) and we collaborated to produce a more comprehensive physical and genetic map (Holloway et al. 1994).

In 1993, mandatory retirement at age 65 changed the nature of my relationship with Monash University. In 1994 I was appointed an Emeritus Professor for life and I continued as an Honorary Professorial Fellow until 2002 when my research funding ended.

During my time at Monash we were fortunate to attract a range of distinguished visitors from overseas. Given the geographical isolation of Australia it is more difficult and more expensive to do this than other regions. Some have been mentioned in the text above, others included Pat Clarke (England), Jack Leary (USA), Larry Bryan (Canada), Pieter van der Putte (Holland), Kyoshi Tagawa (Japan), Alexander Boronin (the then USSR), Norberto Palleroni (USA), Hideki Matsumoto (Japan), and Hans Schlagel (Germany). For our graduate students these visits were very stimulating and provided linkages that were important for their subsequent career

paths. For me they established and reinforced friendships which have continued over the years.

The success of the physical and genetic approach to mapping encouraged other workers to construct similar maps for other species of *Pseudomonas* using more sophisticated probing techniques. In 1996 Rainey and Bailey at Oxford University constructed a physical and genetic map of the *P. fluorescens* SBW25 chromosome (Rainey and Bailey 1996). This was followed 2 years later by Ramos-Diaz and Ramos in Granada, who constructed the same sort of map for *P. putida* (Ramos-Diaz and Ramos 1998). These maps enabled more detailed comparisons of the three species. There are many similarities, such as the lack of clustering of genes in biosynthetic pathways and similarities in the grouping of other genes. However the overall chromosome arrangement of all three species was different, reflecting the importance of chromosomal rearrangements and plasmid insertions to acquire genes. Over the years, a lot of our effort was put into mapping *P. aeruginosa* and establishing systems by which mapping could be achieved in other pseudomonads. This approach became largely redundant with the development of more efficient techniques for sequencing entire genomes. In 1995, the complete genomic sequence of a free living bacterium was first achieved with *Haemophilus influenza* Rd (Fleischmann et al. 1995). Sequences of other bacteria have followed with increasing frequency.

Sequencing the *Pseudomonas Aeruginosa* Genome

The first pseudomonad genome to be entirely sequenced was *P. aeruginosa* PAO1 in 2000 by a consortium led by the Pathogenesis Corporation in Seattle, Washington (Stover et al. 2000). The method used for sequencing was the same as that used for *H. influenza* by Venter and his group and details of the collaborative project and the strategy used are elegantly described by Pete Greenberg (Greenberg 2000). At 6.3 Mbp, more than the previous physical mapping had indicated, the genome of *P. aeruginosa* is large compared to all other bacteria. The sequence agrees in most aspects with the previously constructed physical map with the exception of an inversion comprising about 25 % of the entire genome. Subsequent studies have shown that such inversions occur in the propagation of *P. aeruginosa* on laboratory media and artificial storage. There is little gene duplication in the sequence from which it can be concluded genetic and functional diversity are a result of the genome being larger than usual and it is the basis for this organism's established ability to inhabit a wide variety of ecological niches. Knowledge of the whole sequence has given valuable clues as to the ability of *P. aeruginosa* to develop resistance to antibiotics and successfully and persistently inhabit such specific environments as the human lung. With easier and less expensive access to sequencing techniques, the knowledge of pseudomonad sequences will be a rich source of information for the development of strategies to combat current intransigent infections by this organism.

The Twenty-First Century

The last half of the twentieth century saw an amazing expansion of knowledge about *Pseudomonas*. This was driven by the intellectual curiosity of a cohort of top rank scientists in different countries and the need to seek a solution to infections associated with cystic fibrosis. Two driving forces that sustained that drive were the major increases in funding for research and tertiary education in most of the developed countries of the world and molecular genetics which enabled a greater understanding of how biological information is coded, stored and used.

Increased funding was at the heart of the enormous expansion of the biological knowledge base. For example, in 1950, the National Science Foundation was established in the United States. As well, there was a nine hundred fold increase in research funding for that nation's National Institutes of Health between 1950 and 2000 (Manton et al. 2009). Along with all other aspects of microbiology, *Pseudomonas* has benefited from this bonanza. Science funding in Europe has increased markedly since the formation of the European Economic Union.

The biochemical versatility of *Pseudomonas* which provided such an attractant for so many distinguished scientists to work on this organism in the 1950's should have resulted in major commercial biotechnological achievements by the turn of the century. However, it has taken another 10 years or more for that this expectation to be realised. Another goal is the control of Bacterial Wilt caused by *Ralstonia solanacearum* in tropical and sub-tropical developing countries where global food security has become a more urgent issue.

As with almost all current and future aspects of biological and medical research, it is apparent that genetics, combined with bioinformatics, has been a major factor in the development of knowledge and outcomes. In 1955 it was possible and commonplace for anyone working on bacterial genetics to have read the entire literature relating to conjugation, transduction, and transformation in all the bacterial species being studied. This probably amounted to about 200 publications. By comparison, the flood of articles on *Pseudomonas* in the present century is daunting and awesome. Personal computers, on line data bases, search engines and electronic publishing have entirely changed the way information is available and how it be found and used in science. For example, information about *Pseudomonas* genomes is available at http://www.pseudomonas.com/.

For *Pseudomonas* and other areas of biology, the availability of quicker and less expensive genome sequencing, combined with more sophisticated means of analysis of the data is leading to experimental approaches unforeseen even at the beginning of the current century. More species and strains of *Pseudomonas* are being sequenced (Silby et al. 2011) and it is estimated that the cost of sequencing an isolate of *Pseudomonas* will soon be less than US$10 and be accomplished in a day. In 1994 when a single chromosome of *Saccharomyces cerevesiae* was completely sequenced for the first time, it cost US$10 per base.

In 1954, the first information on the structure of the *Pseudomonas* genome was obtained by demonstrating the linkage in conjugation in *P. aeruginosa* PAO1 of a

streptomycin resistance gene to several but not all tryptophan biosynthesis genes. In the following years, the Monash *Pseudomonas* group and other *Pseudomonas* geneticists using pre-genomic techniques identified the location of more genes, insertion sequences, prophages, pyocin determinants, transposons and plasmids on the *P. aeruginosa* chromosome map. The genomic complexity of the pseudomonad genome determined by the sequencing of a number of *Pseudomonas* species could not have been predicted. There is a core genome comprising genes which are found in all isolates of *Pseudomonas* sequenced to date. In addition there is a pan or accessory genome which comprises the range of all the other genes and genetic elements found in all Pseudomonas isolates. There is a variety of different types of genetic elements which can be differentiated in the pan genome. These include integrative and conjugative elements, genomic islands, prophages, integrons, transposons and insertions sequences as well as other sequences which do not fit neatly into any of these elements. Genomic islands are rich in genes and prophages which are associated with virulence. Genomic analysis combined with other data gathering techniques such as proteomics will surely reveal why *Pseudomonas* has the environmental habitat range, substrate versatility, metabolic diversity and disease producing attributes which continue to mystify and excite. As the noted physicist Neils Bohr said "Prediction is very difficult, especially about the future".

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