Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*

Martina Valentini*, Diego Gonzalez§, Despoina A.I. Mavridou#, Alain Filloux**

#: MRC Centre for Molecular Microbiology and Infection, Department of Life Sciences, Imperial College London, SW7 2AZ London, United Kingdom;

§: Département de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland.

*: corresponding authors. E-mail: m.valentini@imperial.ac.uk; a.filloux@imperial.ac.uk

Abstract

*Pseudomonas aeruginosa* acute and chronic infections are of great concern to human health, especially in hospital settings. It is currently assumed that *P. aeruginosa* has two antagonistic pathogenic strategies that parallel two different lifestyles; free-living cells are predominantly cytotoxic and induce an acute inflammatory reaction, while biofilm-forming communities cause refractory chronic infections. Recent findings suggest that the planktonic-to-sessile transition is a complex, reversible and overall dynamic differentiation process. Here, we examine how the Gac/Rsm regulatory cascade, a key player in this lifestyle switch, endows *P. aeruginosa* with both a permissive lifecycle in nature and flexible virulence strategy during infection.

Highlights

- *Pseudomonas aeruginosa* causes acute and chronic infections in humans.
- Virulence factors are co-regulated with lifestyles.
- The Gac/Rsm cascade regulates the lifestyle and infection strategy switch.
- The Gac/Rsm action is modulated during biofilm formation.
- The Gac/Rsm cascade has a social nature and responds to bacterial competition.

Introduction

*Pseudomonas aeruginosa* is an opportunistic human pathogen associated with a wide range of infections affecting, among others, skin, ear, eye, urinary tract, heart, airway and lung tissues [1]. The high frequency of *P. aeruginosa* strains causing nosocomial infections, the increasing occurrence of multi-drug resistant strains, and the adaptive antimicrobial resistance displayed by the bacterium during chronic infections, cause a severe threat to human health worldwide [2]. *P. aeruginosa* pathogenesis has been shown to be multifactorial and combinatorial [3]. Some virulence factors have been known for decades, while others have only been identified recently, thanks to whole genome sequencing of environmental and clinical isolates [4]. *P. aeruginosa* infections can be acute or chronic.
Interestingly, the type of infection is independent of the pathogen's genotype, but possibly linked to the host health status and the lifestyle adopted by the bacteria when colonising the host [6]. Acute infections are predominantly associated with bacteria adopting a planktonic lifestyle, while biofilm-related functions (attachment, capsule components) play a major role in persistent infections. It has been proposed that a binary regulatory switch controls the transition between the two lifestyles and infection modes of \textit{P. aeruginosa} [6]. This switch model predicts that biofilm-associated functions and virulence factors of acute infection cannot physiologically co-exist in the bacterium. However, recent studies on the formation of biofilms indicate that the lifestyle switch model might be a simplified and static view of a plastic cell differentiation process, where acute and chronic infectious traits can co-exist within a bacterial population. Here, we review the lifestyle/infection model with an emphasis on the role of the Gac/Rsm global gene regulatory network in ensuring behavioural plasticity in \textit{P. aeruginosa}. We propose that it is indeed the permissive lifecycle of the bacterium which is key for its success in the environment as well as for its adaptive pathogenesis during host infections.

**The planktonic-to-sessile switch model**

The main elements and differences of the two \textit{P. aeruginosa} lifestyles/infection modes are depicted in Figure 1, and are briefly described below. \textit{P. aeruginosa} acute lung infection (pneumonia), occurring mainly in immunocompromised or intensive-care patients, is initiated by bacteria binding at the mucosal barrier through two major adhesins, flagella and retractile type IV pili, which trigger a host inflammatory response [1]. Once in contact with the epithelial cells, bacteria cause significant tissue damage by translocating cytotoxic effectors directly into the eukaryotic cells via their type III secretion system (T3SS), and by secreting another set of virulence factors in the extracellular milieu [7]. The T3SS effectors are also involved in the inhibition of phagocytosis and, together with the LasB protease, eventually cause loss of the endothelial barrier integrity. The disruption of this barrier allows the pathogen to grow and spread rapidly within the host, sometimes resulting in sepsicaemia [7]. Overall, the process of acute infection leads to a massive mobilization of the immune system, starting with the recruitment of neutrophils and macrophages to the lungs. By contrast, bacteria involved in chronic infections are slow growing, less cytotoxic and immunogenic, and can persist in the host for decades without reaching the bloodstream. Chronic infections are common in the lungs of patients with cystic fibrosis (CF), primary ciliary dyskinesia and bronchiectasis [8]. In chronically-infected CF lungs, clusters of \textit{P. aeruginosa} are found encased in a polysaccharide matrix within a thick layer of mucus [9]. Common adaptation routes of \textit{P. aeruginosa} in the CF lungs include the loss of major determinants of the planktonic cells, like motility or a functional T3SS, and the conversion to a mucoid colony phenotype. This has as a result that CF-adapted lineages are usually avirulent in mouse model of acute infection, but unhampered in their ability to establish chronic infections [10]. Altogether, these observations on the lifestyle of the bacterium led to the suggestion of a binary model
of P. aeruginosa pathogenesis, in which cells in the planktonic state are equipped for the aggressive host-invasion strategy seen in acute infections, while biofilm-forming cells are pre-adapted for long-term persistence and immune evasion, as observed in the airways of chronically-infected CF patients [6].

The identification and initial mapping of the Gac/Rsm global regulatory network (Box 1) substantiated this model, grounding it into the fundamental decision-making processes of P. aeruginosa [11]. Briefly, it was found that RsmA positively regulates the expression of virulence factors which are important during the planktonic state and acute infection, while it negatively regulates factors associated with biofilm formation and chronic infection. RsmA-induced virulence factors include flagella, rhamnolipids, type IV pili, the T3SS and its effectors, the Xcp type II secretion system (T2SS), exotoxin A and lipase. On the other hand, genes repressed by RsmA encode biofilm matrix components (pel, pel), Quorum Sensing (AHL), the type VI secretion system (T6SS) and secondary metabolites (siderophores, HCN, phenazines) [12]. Moreover, the rsmA mutant showed reduced cytotoxicity for epithelial cells and decreased dissemination efficiency in a mouse model of acute infection, but displayed increased persistence and lung inflammation in a mouse model of chronic infection [13]. Although the molecular cues which determine the choice between the two infection strategies remain elusive, the detection of kin-cell lysis, via RetS and GacS, and the sensing of high calcium levels, via the LadS kinase, have recently been identified as major players in the initiation of P. aeruginosa biofilm formation via the Gac/Rsm cascade [14,15].

The Gac/Rsm cascade as lifestyle modulator

Generally, planktonic and sessile bacteria differ in their physiology and behaviour; they have different growth rates, forms of motility, degrees of virulence, propensities for social interactions and antibiotic resistance or tolerance. However, planktonic and biofilm-forming populations of P. aeruginosa are not as significantly distinct as, for example, the infective and dissemination forms of primary pathogens like Legionella pneumophila or Salmonella enterica [16,17]. Several studies have measured the difference in gene expression profiles between P. aeruginosa planktonic and biofilm cultures, but surprisingly little consensus is found across the studies. Estimates of the difference in gene expression between the two lifestyles vary from 1% to 13% of the total genes, and a consensus regarding a putative biofilm-specific regulon has, so far, not been reached [18-21]. To account for this, it is proposed that although changes in cells morphology and physiology always occur during the lifestyle transition, the precise experimental condition or environment in which the transition takes place has a major impact on the process. The transition to a sessile state in P. aeruginosa has recently been described as a progressive differentiation program, which involves several, possibly not clearly delimited stages, generally found during biofilm formation, starting by surface attachment and going on to microcolony formation, biofilm maturation and eventually dispersal or detachment [22]. The bacterial physiology and behaviour continuously change throughout this process. For example, cells
undergoing surface-attachment or detaching from biofilms have different gene expression profiles and show increased virulence compared to their planktonic counterparts [23,24]. Bacterial functionalities within a lifestyle are therefore continuously modulated as part of a differentiation program and/or as a response to environmental conditions. Some studies have already offered evidence that *P. aeruginosa* lifestyles and infection strategies are more flexible than previously thought, as some functions traditionally associated with planktonic cells were found to occur within biofilms and vice-versa. Individual-cell chemotaxis, for example, is not exclusive to flagellated cells, as previously thought, but also plays a role in pili-mediated motility [25]. In addition, Turner and colleagues observed that planktonic/acute- and sessile/chronic-related virulence genes are not mutually exclusive in acute and chronic wound infections in a mouse model. For example, an up-regulation of T3SS, rhamnolipids and pyochelin (siderophore) expression was observed in both chronic and acute infections [26]. On the same lines, *P. aeruginosa* biofilm components were shown to contribute to acute infections of burn wounds in mice [27], and the T3SS and T6SS genes, which were thought to be antagonistically regulated, were found to be both upregulated during acute murine respiratory infection, along with iron uptake genes, usually associated with chronic infection [28].

A more detailed genetic analysis of the Gac/Rsm cascade has also suggested that the initial binary switch model was not fully capturing the complexity of the regulatory system. The core components of the cascade (Box 1), being post-transcriptional regulators, *i.e.* RNA-binding protein(s) and small RNAs, have an intrinsic modulatory potential. It is known that post-transcriptional regulation allows both an ON/OFF switch and fine-tuned control of gene expression [29]. RsmA (and its homologue RsmN) shows a wide range of binding affinities for its mRNA targets and high affinity for its titrating small RNAs (RsmY/Z) [30,31]. Therefore, even a subtle variation in small RNA levels could drastically affect the RsmA regulatory output. The regulation of the small RNAs levels can occur either via activation/repression of the Gac/Rsm accessory sensors (Box 1) or via other cross-regulatory pathways [12,32,33]. The latter can be global gene regulatory networks, like cyclic di-GMP signalling, but also regulatory modules, like sensor kinases (*e.g.* RetS, LadS and PA1611), two-component systems (*e.g.* BfiSR) and small RNAs (*e.g.* RsmW) [34-36]. The Gac/Rsm flexibility is particularly important during the planktonic-to-sessile lifestyle transition. The increase in RsmY/Z levels, occurring after the activation of the Gac/Rsm cascade, was initially considered to be the key step initiating biofilm formation by increasing surface attachment [11]. However, RsmY and/or RsmZ levels, and consequently RsmA activity, have more recently been shown to be continuously modulated during the process of biofilm development (Figure 2) [19,23,34,37]. A decrease in RsmZ levels, through activation of the BfiSR two-component-system, ensures the progression of the biofilm development after surface attachment [37]. Moreover, in mature biofilms, the levels of RsmY/Z and RsmW, a newly discovered RsmA-binding small RNA, increase sharply compared to the planktonic state [19,34]. Finally, in dispersed cells, the expression of *rsmY* and *rsmZ* is down-regulated compared to both biofilm and planktonic cells [23].
In addition to its modulatory action, it is an open question whether the Gac/Rsm cascade is involved in the generation of physiological heterogeneity in bacterial populations. Phenotypic heterogeneity occurs in biofilm structures, in part because of oxygen and nutrient gradients, and in part because of inter-bacterial communication through secretion of chemical signals in their immediate surroundings [38]. Localised gene expression in *P. aeruginosa* biofilms has been shown for virulence-factor genes like *aprA* and *phzA1* [39]. The RsmA regulon includes both cooperative (e.g. exopolysaccharide and siderophore production) and competitive (e.g. T6SS, phenazine, pyocyanin) traits [40-42], which could benefit from being expressed heterogenously by specialised subpopulations [41].

To conclude, the architectural complexity of the Gac/Rsm cascade can not easily be reduced to an ON/OFF switch. Elements of this complexity include the multiple, possibly conflicting, inputs which feed into it, the apparent redundancy in the transducing small RNAs (RsmWYZ), and the generalised cross-regulation which links the Gac/Rsm cascade with other networks acting during biofilm development. The impact of these regulatory intricacies on the dynamics of *P. aeruginosa* pathogenesis and infection strategies remains to be fully evaluated.

**Conclusions and perspectives**

*P. aeruginosa* strains, including lineages which are infectious in humans, are widely distributed in the environment and can be found both in association with non-human eukaryotic hosts and in host-free ecosystems [43]. Environmental strains express typical virulence factors and multidrug resistance determinants, while clinical strains can use oil hydrocarbons as carbon source [44]. Therefore, many of the *P. aeruginosa* traits and behaviors as a pathogen might have been shaped by selective pressures unrelated to human pathogenesis [45]. It is likely that the continuous cycling between a planktonic and a sessile state, encountered by *P. aeruginosa* during its evolution, is particularly relevant in changing environments outside the host. Many environmental bacteria, like *Caulobacter crescentus*, *Rhodopseudomonas palustris*, and other opportunists like *Vibrio cholerae* present similar cycles [45-47]. Plasticity in lifestyles has been recently shown to contribute to the evolutionary success of *V. cholerae* under fluctuating conditions [48]. This could also be the case for *P. aeruginosa*. In most environmental niches, but also in acute and early chronic infections, the aptitude for lifestyle transitions and the behavioural modulation associated with it might be favored [26-28]. By contrast, in relatively stable environments, this flexibility could be lost through drift or counter-selected in favour of niche specialisation. In long-lasting infections, like in CF-lung chronic infections, ecological stability and constant selection against immunogenicity are two important selective forces. Accordingly, over time, *P. aeruginosa* often looses its behavioural/virulence flexibility and traits exclusively associated with the biofilm-forming lifestyle are fixed [49]. Mutations in global regulators (such as *lasR*, *vfr*, *rpoN*, *mucA* and *retS*) are frequently observed in CF isolates, because they affect multiple cell functions or phenotypes simultaneously [50]. However, these mutations are not selected
in natural environments, presumably because they are detrimental (evolutionary dead-end) [51]. Further work will be necessary to fully characterize the features and functions of *P. aeruginosa* phenotypic and virulence-related plasticity during infection and to understand how the Gac/Rsm cascade and other regulatory networks contribute to it.

**Acknowledgements**

Work in the Filloux laboratory is supported by Biotechnology and Biological Sciences Research Council (BBSRC) Grant numbers BB/N002539/1 and BB/L007959/1 (A.F. and M.V.). D.A.I.M. is supported by the Medical Research Council (MRC) Career Development Award (MR/M009505/1) and D.G. by a Swiss National Science Foundation Postdoc Mobility Fellowship (P300PA_167703).

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest


11. Mulcahy H, O’Callaghan J, O’Grady EP, Adams C, O’Gara F: The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human...


This study shows how P. aeruginosa detects ecological competition by sensing danger cues released by lysed kin cells. These cues are detected by a dedicated by the Gac/Rsm cascade which regulon can be read as a danger response program.


By combining genetics and biochemical data, this study demonstrates that calcium induces in P. aeruginosa the activation of the Gac/Rsm regulatory cascade via the LadS sensor. The authors show that the capacity to sense calcium is retained in some P. aeruginosa clinical isolates and they propose calcium serves as a signal in host–pathogen interaction.


This study describes a new RsmA-binding sRNA, named RsmW. RsmW levels increase in *P. aeruginosa* in nutrient-limiting conditions, biofilms, and at higher temperatures, but is not transcriptionally activated by GacA. RsmW indicates the presence of a new uncharacterized pathway that regulates RsmA activity under specific conditions.


By performing competition experiment with *Vibrio cholerae* strains having flexible or rigid biofilm production strategies, the authors show that lifestyle flexibility is, as evolutionary strategy, incompatible with a temporally stable environment. This study provides a direct experimental validation that fluctuating environmental conditions favour lifestyle flexibility in bacteria.


This is a comprehensive review describing recent findings on *P. aeruginosa* genetic adaptation to cystic fibrosis lungs.

The Gac/Rsm global regulatory network comprises several regulatory components and signal transduction pathways, converging on the post-transcriptional regulator RsmA (associated figure). RsmA belongs to the CsrA/Rsm family of RNA-binding proteins which compete with ribosomes for binding to the 5’ untranslated regions of mRNA targets, and possibly affect their stability [32]. Therefore, the main action of RsmA is to repress the translation of target genes, although it has been reported that sometimes it also exerts a positive control on gene expression, either directly or indirectly. In addition to RsmA, the backbone of the Gac/Rsm signalling pathway is composed of the GacS/GacA two-component system, which in *P. aeruginosa* induces the transcription of the genes *rsmY* and *rsmZ* encoding small RNAs. The latter, are regulatory RNAs which have multiple RsmA-binding motifs (GGA motifs), exposed in stem-loops. Upon GacA activation, the small RNAs, abundantly transcribed, sequester RsmA and relieve the target mRNAs from its control. The type of interaction of RsmA with the mRNAs and the resulting levels of RsmY/Z determine the output of the regulatory pathway. The regulatory action of proteins like the sensor kinases RetS, LadS, PA1611 and SagS, the BfiS/R two-component system and the HptB phosphotransfer protein also feed into the core of the Gac/Rsm pathway (grey shaded area) [36]. Finally, additional regulators, like the third RsmA-binding small RNA, RsmW, or the RsmN/F RNA-binding protein, have a supportive role in the absence of backbone components [30]. In the box associated figure, the main Gac/Rsm regulatory components are illustrated together with their regulatory action (→ activation, –| repression). Blue is used to indicate the components which, when absent, cause an impairment in biofilm formation, while deletion of genes encoding elements in red lead to hyperbiofilm forming strains.
Figure 1: Schematic representation of the lifestyle switch model in *P. aeruginosa*. The levels of the small regulatory RNAs, RsmY/Z, are a major determinant for this transition. When the levels of these regulators are low (blue, left), *P. aeruginosa* cells have a planktonic lifestyle, causing acute infection. Motility, through the expression of type IV pili and flagella, along with the presence of LPS and the release of diffusible virulence factors or T3SS effectors, are crucial in this lifestyle. When the levels of RsmY/Z are high (red/right), the bacterium adopts a sessile lifestyle, causing chronic infection. The extracellular matrix, which encapsulates large numbers of cells (biofilm) and allows productive access to extracellular products and “common goods” (like siderophores, proteases, elastase, rhamnolipids and HCN), and the presence of the T6SS feature prominently in this lifestyle. During the switch from planktonic to biofilm lifestyle, the LPS is known to undergo reversible structure modifications; this represented by the presence of the classical form of the LPS on the left side of the figure (planktonic lifestyle) which is absent on the right side of the figure (biofilm lifestyle).
**Figure 2**: Schematic representation of biofilm development and of the role of RsmY/Z small regulatory RNAs in modulating this process. The stages of biofilm formation are (i) planktonic, (ii) attachment, (iii) microcolony formation, (iv) macrocolony formation and (v) dispersal. The “reversible attachment” phase indicates weak attachment to the surface while the “irreversible attachment” phase involves mechanisms which allow the bacterium to acquire a stable association with the surface (e.g. use of pili/flagella). Increase of RsmY/Z sRNAs in planktonic cells is cell-density dependent and results in increased surface attachment and sessility [11]. The reduction of RsmZ abundance (through BfiSR-CafA) is necessary for biofilm development progression during the transition from reversible to irreversible attachment [35]. In mature biofilms RsmY/Z levels are elevated compared to planktonic cells [19]. Finally, when cells disperse from the biofilm, the expression of RsmY/Z is down-regulated compared to both biofilm and exponentially-growing planktonic cells [23]. The proposed schematic model provides a reference framework for understanding the phenotype plasticity within a lifestyle, and its validity is currently under investigation.