Review

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Pseudomonas aeruginosa: all roads lead to resistance

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Pseudomonas aeruginosa **is often resistant to multiple antibiotics and consequently has joined the ranks of 'superbugs' due to its enormous capacity to engender resistance. It demonstrates decreased susceptibility to most antibiotics due to low outer membrane permeability coupled to adaptive mechanisms and can readily achieve clinical resistance. Newer research, using mutant library screens, microarray technologies and mutation frequency analysis, has identified very large collections of genes (the resistome) that when mutated lead to resistance as well as new forms of adaptive resistance that can be triggered by antibiotics themselves, in *in vivo* growth conditions or complex adaptations such as biofilm growth or swarming motility.**

Pseudomonas aeruginosa as a superbug

Since the introduction of antibiotics in clinical therapy, bacteria have developed increasingly more sophisticated resistance strategies. This has led to the appearance and spread of the so-called 'superbugs', resistant to practically all antimicrobial drugs available on the market. The Gram-negative bacterium *Pseudomonas aeruginosa*, which can infect a wide range of animal and plant hosts [1], has become a superbug. This opportunistic pathogen is a leading cause of nosocomial infections, as well as chronic lung infections in cystic fibrosis (CF) patients [2,3] (Box 1).

Compared with other pathogens, *P. aeruginosa* is very difficult to eradicate as it displays high intrinsic resistance to a wide variety of antibiotics, including aminoglycosides, fluoroquinolones and β-lactams. This is largely because of the low permeability of its outer membrane [4], which limits the rate of penetration of antibiotic molecules into the cells due to the inefficient porins in this bacterium. This then enables secondary adaptive resistance mechanisms to work more efficiently, including increased efflux and enzymatic antibiotic modifications (e.g. β-lactamase).

These intrinsic mechanisms are part of the genetic make-up of *P. aeruginosa*, leading to very high baseline minimal inhibitory concentrations (MIC) and rendering many common antibiotics ineffective against *P. aeruginosa*. These intrinsic mechanisms can be stabilized or enhanced by mutation. The occurrence of mutational events affecting the expression of these resistance genes or the activity of their respective products can make most other drugs clinical unusable. Other mutations can slightly increase resistance and probably contribute to the gradual decrease in baseline susceptibility of *P. aeruginosa* that has been observed for most antibiotics [5–9]. These mutations do not lead to clinically meaningful resistance although this can occur through the accumulation of several mutations. In addition, some strains acquire increased resistance via the horizontal transfer of resistance determinants.

In addition to these more traditional mechanisms, the extraordinary adaptability of *P. aeruginosa* to changing environmental conditions and stresses, including exposure to antibiotics, altered media conditions or altered growth states, can lead to the development of adaptive resistance to antimicrobials [10]. This phenomenon, termed adaptive resistance, has been known for a long time; however, its broad applicability to clinical issues has only recently been investigated.

Considering the multiple ways in which *P. aeruginosa* can become resistant, it is not surprising that resistance can be observed for all currently available anti-pseudomonal antibiotics. Although antibiotics are available and usable for most *P. aeruginosa* infections, resistance rates are on the rise and the high intrinsic resistance of *P. aeruginosa* makes it very challenging to find new drugs. For this reason, it is important to understand the mechanisms that participate in *P. aeruginosa* antibiotic resistance. The objectives of this review are thus to describe the most recent findings with regard to the mechanisms of resistance observed in *P. aeruginosa* and to discuss how they can impact antibiotic therapy failure.

Mechanisms of intrinsic resistance of *P. aeruginosa*

P. aeruginosa is intrinsically resistant to most antibiotics, and wild type strains exhibit an inherently reduced susceptibility to most antibiotics compared with most other Gram-negative bacterial species. The main mechanism underlying this characteristic feature is its low outer membrane permeability, which is 12–100 times less than that of *E. coli* [11]. The outer membrane of Gram-negative bacteria acts as a selective barrier to uptake of antibiotics [4]. It has been compared to a molecular sieve in which the uptake of most hydrophilic molecules is size dependent due to diffusion through the channels of water filled porin molecules. *P. aeruginosa* has a large exclusion limit owing to the limited number of large channels of its major porin OprF (most OprF channels being very small), and the small size of the channels of other porins that mediate the passage of other molecules the size of antibiotics, including OprD and OprB. Other antibiotic uptake pathways include uptake through specific porins (e.g. carbapenems through

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Box 1. Resistance in CF patients

P. aeruginosa lung infections of CF patients constitute an excellent environment for the development and acquisition of antibiotic resistance. The ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) is the most common mutation leading to CF [74]. This ultimately has an impact on chloride transport and sodium absorption, which leads to a dehydrated and thickened mucus. Therefore, upon colonization with a non-mucoid and susceptible strain, the conditions of the CF lung together with long-term antibiotic therapy can lead to important phenotypic changes as well as selecting for cells with a higher degree of resistance. *P. aeruginosa* undergoes certain morphological changes while invading the lung: loss of LPS O-antigen, production of exoproducts, formation of small-colony variants, transitions to mucoidy due to alginate overproduction, formation of biofilms and evolution of mutator strains (reviewed in [75]). These adaptations and genetic switches are associated with increased resistant strains and maintenance of a chronic infection state.

OprD channels), self-promoted uptake of polycationic antibiotics (described below) and uptake of hydrophobic molecules through the outer membrane bilayer. Although low outer membrane permeability plays a decisive role in drastically reduced drug uptake, equilibration of hydrophilic molecules across the outer membrane is still managed in a matter of seconds. Therefore, the high intrinsic resistance of this pathogen is absolutely dependent on other intrinsic and adaptive secondary mechanisms such as rapid efflux [12,13] due to the intrinsic or induced expression of efflux pumps, particularly the resistance-nodulation-cell division (RND) systems MexAB–OprM and MexXY–OprM, and AmpC β-lactamase production [14], which take advantage of the reduced flow of antibiotic across the outer membrane (Table 1).

Recent studies screening comprehensive mutant libraries for mutants that lead to increased antibiotic susceptibility have identified new candidate mechanisms as being involved in the intrinsic resistome of *P. aeruginosa* to several antibiotics [5–9,15]. Results support the participation of dozens of genes from different functional classes, and the wide range of newly identified mutations form part of a very complex resistome. For example, strains with mutations in many genes affecting DNA replication, recombination and repair showed an increase in ciprofloxacin susceptibility [7,8]. Interestingly, mutations in the cell division gene *ftsK* indicated a role for this in intrinsic resistance to ciprofloxacin and β-lactams [6–8]. Likewise, mutations in genes involved in alginate production led to increased susceptibility to the β-lactam imipenem. Another study revealed that *ampG* (PA4393) is required for the induction of AmpC β-lactamase and mutation of this gene reduced susceptibility to β-lactams eightfold [16].

An overlap between genes dysregulated in microarray experiments upon treatment with subinhibitory or lethal exposure to antibiotics and genes involved in antibiotic resistance has been observed. This indicates that *P. aeruginosa* adaptively activates defence (resistance) mechanisms to combat the inhibitory effects of antibiotics [7,9,17,18].

These studies collectively demonstrate that the high intrinsic resistance of *P. aeruginosa* is ultimately a result of the combination of several mechanisms acting simultaneously. The underlying mechanisms probably play a significant role in clinical outcome.

Acquired resistance: horizontal transfer and mutational resistance

In addition to its high intrinsic resistance, *P. aeruginosa* can become even less susceptible owing to the acquisition of inheritable traits. The two types of acquired resistance involve horizontal transfer of genetic elements and mutational resistance (Table 1).

DNA elements, including plasmids, transposons, integrons, prophages and resistance islands, can harbour antibiotic resistance genes and can be acquired by conjugation, transformation or transduction. This can increase antibiotic resistance and even multidrug resistance due to plasmids that can contain multiple resistance cassettes. Such horizontal transfer mainly affects aminoglycoside and β-lactam resistance in *P. aeruginosa*, but has been observed for several other classes of antibiotics. For example, aminoglycoside modifying enzymes located on mobile genetic elements can inactivate aminoglycosides leading to various chemical modifications of the aminoglycoside, which in turn leads to reduced affinity for the 30S ribosomal subunit, the main target of aminoglycosides [19]. In addition to the inducible chromosomal AmpC β-lactamase, some *P. aeruginosa* strains acquire plasmids encoding new β-lactamases that confer resistance to penicillins and cephalosporins [20]. Of great concern is the proliferation of plasmid mediated extended-spectrum β-lactamases (ESBLs), which were originally described in the *Enterobacteriaceae*, and metallo-β-lactamases (MBLs) that inactivate carbapenems [21].

A second form of acquired resistance is mutational resistance. The spontaneous mutation frequency varies

Table 1. Overview of the different types of resistance exhibited by *P. aeruginosa*

Class of resistance	Stable[a]	Inheritable	Dependency on environment	Mechanisms	Examples of genes involved
Intrinsic	+	+	–	Low outer membrane permeability, β-lactamase production and efflux pump overexpression	*crc, lon, psrA*
Acquired	+	+	–	Horizontal transfer, mutations leading to reduced uptake and efflux pump overexpression	*ampD, gyrA, nalA, nfxB, cbrA,* MBLs
Adaptive	–	–	+	Gene expression changes including β-lactam and efflux pump overexpression owing to factors triggering expression of regulatory genes	*ampC, mexZ, phoQ*

[a]+, property applies; –, not a property of this form of resistance.

between antibiotics with resistance frequencies ranging from 10^{-6} to 10^{-9} for individual antibiotics. The mutation rate can further increase under certain conditions such as in the presence of DNA-damaging agents or during growth in a biofilm. For example, the mutation frequency for meropenem increased tenfold when the culture was pre-incubated with subinhibitory concentrations of ciprofloxacin [22]. Similarly, a >100-fold increase in mutation frequency to ciprofloxacin resistance was observed in biofilms compared to free-living cells [23], possibly owing to the downregulation of antioxidant enzymes during growth in a biofilm leading to increased DNA damage. Mutation frequencies are increased by as much as 70-fold in hypermutator strains [24] that contain mutations in genes involved in the efficient repair of DNA replication errors. These strains can acquire resistance to several different antibiotics. Examples of strong hypermutators are *mutL* and *mutS*, which are commonly found in patients with CF [25], although a variety of weaker mutators have been found in mutant library screens [24], and could play a role during the early stages of CF lung infections [26].

'Breakthrough' mutations that make *P. aeruginosa* untreatable by given antibiotics include those leading to overexpression of efflux pumps, reduced uptake of antibiotics, hyperproduction of β-lactamases and altered antibiotic targets. For example, an important mutational mechanism is the derepression of the efflux pumps MexAB–OprM and MexCD–OprJ owing to mutations in genes encoding *mexR* and *nfxB,* respectively [27]. Furthermore, overexpression of MexXY–OprM owing to a mutation in *mexZ* leads to aminoglycoside, fluoroquinolone and cefepime resistance in clinical strains of *P. aeruginosa* [28]. Mutations in the specific porin OprD reduce the uptake of the antibiotic imipenem and therefore lead to clinical resistance [29], whereas mutations in either *mexT* or *mexS* (*nfxC*) are known to reduce the expression of OprD as well as increasing expression of the efflux pump MexEF–OprN leading to imipenem and multiple antibiotic resistance [30]. Hyperproduction of β-lactamases occurs upon mutation of an effector of the *ampC* β-lactamase, AmpD, which controls activity of the AmpR regulator. Mutations in target enzymes can also lead to clinically meaningful resistance [31], e.g. mutations in *gyrA* and *gyrB* (gyrase) as well as *parC* and *parE* (topoisomerase IV) reduce fluoroquinolone binding affinity, which leads to clinical resistance [32].

Recent screening studies employing the *P. aeruginosa* PA14 comprehensive Harvard library [33] have shown that many additional mutations can also lead to increased resistance, although in most cases, the observed increases are modest (~twofold) and might easily be missed in the clinic [5–9]. It has been proposed that low-level resistance might evolve to high-level resistance in a stepwise manner, a phenomenon termed creeping baselines in the literature [10]. The accumulation of several mutations with modest changes in MIC can, over time, result in a stepwise increase in resistance, which ultimately leads to high-level resistance. Indeed, single mutations in either *galU* (central intermediary metabolism), *nuoG* (energy metabolism), *mexZ* (transcriptional regulator) or *rplY* (adaptation) exhibited only a twofold increase in resistance to tobramycin, but a quadruple mutant had a major 16-fold increase in resistance [34].

These screening studies define the resistome, which collectively represents all mutations that can lead to resistance. A wide variety of such mutations make up the resistomes for aminoglycosides, β-lactams, fluoroquinolones, tetracycline and sulfonamide. For example, the *P. aeruginosa* aminoglycoside resistome [8,9] involves 150 different genes from many functional categories including those related to energy metabolism, DNA replication and repair and lipopolysaccharide (LPS) biosynthesis. Resistance to fluoroquinolones [7,8] can be mediated by mutations in NADH dehydrogenase genes, phage-related genes, iron transport genes and efflux regulators. β-lactam resistance genes can affect efflux, overproduction of β-lactamase and cell wall and LPS biosynthesis [6,8]. In summary, mutations in a large number of unrelated genes can give rise to acquired resistance to different antibiotics.

Adaptive resistance

Adaptive resistance is inducible and dependent on the continuing presence of either an antibiotic or another environmental stimulus. Although first observed in 1966 [35], no important connection to a clinical outcome was established for adaptive resistance in contrast to intrinsic and acquired resistance and therefore this phenomenon did not receive much attention. Subsequently, it was discovered that plasmid-mediated tetracycline resistance was often induced by tetracycline, that biofilms demonstrated broad spectrum non-mutational resistance, and that in *P. aeruginosa,* inducible chromosomal β-lactamase limits the efficacy of several β-lactams that are effective in other bacterial species.

The advent of the genomic era has enabled a broader understanding of the complex phenomenon of adaptive resistance. A number of triggering factors are now recognized to induce this type of resistance including antibiotics, biocides, polyamines, pH, anaerobiosis, cations and carbon sources, as well as social activities such as biofilm formation and swarming (reviewed in [10]). These triggering factors modulate the expression of many genes leading to effects on efflux pumps, the cell envelope and enzymes. The importance of adaptive resistance in *P. aeruginosa* is consistent with the large repertoire of regulatory genes (9.4% of all genes) in its genome [36].

Environmental cues and subinhibitory concentrations of antibiotics lead to defined changes in the gene expression pattern of *P. aeruginosa* and allow the bacterium to withstand subsequent exposures to lethal concentrations of the inducing and related antibiotics. An important feature of adaptive resistance is that once the inducing factor or condition is removed, the organism reverts to wild type susceptibility. This might explain the observation that *in vitro* efficacy does not predict *in vivo* success in *P. aeruginosa* therapy [37], which probably reflects adaptive resistance. This is of particular concern in clinical settings where *P. aeruginosa* grows as a biofilm, for example in CF, catheter-associated infections or ventilator-associated pneumonia.

Polycationic antimicrobials such as aminoglycosides, polymyxins and cationic antimicrobial peptides pass across

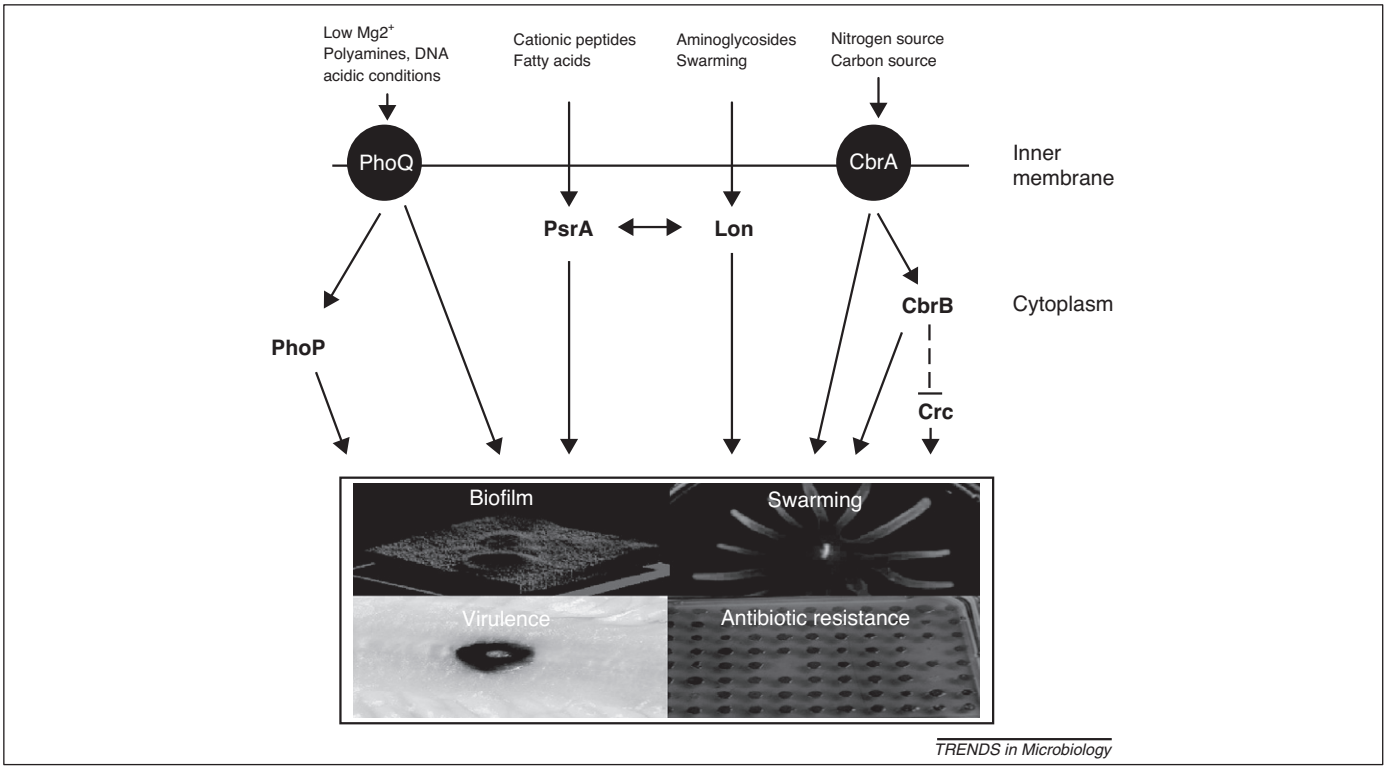

Figure 1. Antibiotic resistance and virulence in *Pseudomonas aeruginosa*. Overview of the complexity underlying the regulation of antibiotic resistance and virulence-related processes. The interaction among regulatory networks dictates the contribution of each gene to both antibiotic resistance and virulence. The figure represents different stimuli that induce inner membrane and cytoplasmic proteins, which then positively (thin arrow) or negatively (dotted line) regulate other proteins. All of them are involved in antibiotic resistance and virulence (thick arrow). A possible crosstalk between CbrA and PhoPQ has been recently suggested [53]. The *P. aeruginosa* biofilm picture is reproduced, with permission, from [73].

the outer membrane by self-promoted uptake, which involves the interaction of the polycations with divalent cation binding sites on LPS to competitively displace these cations, causing localized disruption that enables the passage of the polycation across the membrane [11]. The *arnBCADTEF* operon mediates the addition of 4-aminoarabinose to Lipid A of LPS, which blocks self-promoted uptake leading to resistance. Adaptive resistance to polymyxins and host cationic antimicrobial peptides can be mediated by low concentrations of divalent cations (Mg^{2+} and Ca^{2+}), leading to the activation of two two-component regulatory systems, PhoPQ and PmrAB, and consequent induction of the *arn* operon (Figure 1); however, antimicrobial peptides or polymyxins are more likely to be the physiological triggers [38]. The *arn* operon can also be induced in biofilms by extracellular DNA, which creates a cation-limited environment [39]. Recently, a new two-component regulatory system, ParRS, was identified and demonstrated to mediate the upregulation of the *arn* operon and adaptive resistance in the presence of the anti-pseudomonal drug colistin and certain antimicrobial peptides [40].

A major adaptive mechanism in *P. aeruginosa* is the induction, by pre-exposure to β-lactam antibiotics, of a chromosomally-encoded β-lactamase (encoded by the *ampC* gene) that can cause enzymatic inactivation of many β-lactams. Clinical failure of ceftazidime, anti-pseudomonal penicillin or cefotaxime treatment is strongly associated with dysregulation of AmpC because these drugs all lead to a strong upregulation of the *ampC* gene. Conversely, some β-lactams, including the most recent fourth generation of cephalosporins, cefepime and cefpirome, show weaker or no upregulation of *ampC* [41].

Recently, Lee *et al*. [42] demonstrated that the two-component regulator AmgRS, which is involved in the adaptive membrane stress response, is activated after tobramycin exposure. Similarly, Kindrachuk *et al*. [18] demonstrated that the heat shock stress response, which leads to low-level resistance to aminoglycosides, is controlled through the heat shock sigma factor RpoH and the ATP-dependent protease AsrA. The heat shock response allows the cell to repair protein damage that is caused by aminoglycosides. Although the Lon protease, which controls the DNA stress response and fluoroquinolone susceptibility, is upregulated by aminoglycosides [43], no connection to adaptive aminoglycoside resistance was observed.

Another mechanism resulting from the exposure to subinhibitory concentrations of antibiotics is an overexpression of genes encoding efflux pumps. For example, aminoglycosides induce the MexXY efflux pump [44]. Through these regulatory changes in pump expression, the antibiotic is more rapidly effluxed and the bacterium becomes adaptively more resistant. Moreover, efflux pumps often mediate multidrug resistance.

The upregulation of efflux pumps and other resistance mechanisms has also been observed in cells growing in biofilms. Biofilms represent a form of social behaviour involving the formation of microbial aggregates on surfaces including the epithelia and medical devices. Cells growing

in such communities show a distinct transcriptome compared to planktonic cells and are much more resistant to many different antimicrobials [45–47]. Resistance probably occurs in several ways. The broad dysregulation of many genes (by several mechanisms including quorum sensing) leads to the upregulation of efflux pumps, enzymes, various regulators and products identified through genomic resistome studies. Also, differential access to nutrients within the biofilm leads to differential metabolic activity, whereby cells in the outer layer are metabolically active and cells in the inner part of the biofilm grow more slowly. Some antibiotics only work on growing cells (e.g. most β-lactams and aminoglycosides), whereas a few (e.g. polymyxins) preferably kill more poorly growing bacteria and these would affect different regions of the biofilm. Although the extracellular matrix has been suggested to act as a barrier to antibiotic penetration, it might also act to concentrate extracellular enzymes, such as secreted β-lactamases, near the bacterial surface. Biofilms are also known to have a greater fraction of so-called persisters than planktonic cells. Persisters represent slowly growing or non-dividing cells that can easily withstand stress conditions, such as antibiotic pressure. Little is known about the mechanisms underlying the development of persister cells in *P. aeruginosa*; however, it is known that *spoT, relA, dksA, rpoS, dinG, spuC, algR* and *pilH* mutations can affect persistence [48]. These genes might be good targets for drugs to overcome the issue of *P. aeruginosa* persistence and reduce resistance development.

Swarming is a special form of motility, unlike flagella-mediated swimming on liquid and semi-solid media or pilus-mediated twitching on solid surfaces. It is a social behaviour involving dysregulation of many genes [49] and is thought to be relevant to the movement of *P. aeruginosa* through mucosal layers because the conditions that trigger swarming (intermediate viscosity and amino acids as a poor nitrogen source) exist in the lung [50]. Swarming colonies exhibit a greater resistance to multiple antibiotics [49,51] and have a higher expression level of virulence-related factors compared to planktonic cultures. The mechanisms leading to adaptive resistance in swarming cells, a type of social behaviour distinct from biofilm formation, are only beginning to be understood [52] and appear to involve at least Lon and CbrA mediated dysregulation of PhoPQ [53].

Adaptive resistance can also have long term consequences. If cells are not completely eradicated, regrowth can be observed once the treatment is stopped [54]. This is particularly relevant in the clinic where biofilm formation has been observed, e.g. in the lungs of CF patients [55]. A related problem is the high rate of persister cells in CF and biofilms [56,57].

Relationship between antibiotic resistance and virulence

Bacterial virulence is an important contributor to infectious diseases and complicates treatments with the currently available antibiotics. Interestingly, a number of bacterial regulatory genes that participate in complex intricate regulatory networks have been identified to influence both virulence and antibiotic resistance. The products of many genes influence the virulence of *P. aeruginosa* and these genes are often controlled by conditions found in the host environment (e.g. low iron, anaerobicity, specific nutrients and stresses) [53,58–60]. Virulence genes can influence the production of virulence factors (toxins, proteases and lipases), cytotoxicity and swarming motility. Furthermore, biofilms have been shown to compromise infections *in vivo* [55]. For example, biofilm formation and swarming are relevant to *in vivo* growth and lead to a broad increase in antibiotic resistance [49,61]. Indeed, antibiotic resistance and virulence properties are often coregulated [49,58,60,62], and specific examples are described here.

Box 2. Important regulators in *P. aeruginosa*

Crc: is a global regulator that controls the metabolism of carbon sources and catabolite repression in *P. aeruginosa* [53,76]. Crc is necessary for biofilm formation and swarming and plays a role in intrinsic antibiotic resistance.

PsrA: is a type III transcriptional regulator and positively regulates the transcription of the alternative sigma factor RpoS [63,65]. PsrA is involved in biofilm formation and swarming and takes part in adaptive and intrinsic antibiotic resistance.

Lon: the ATP-dependent protease Lon is involved in the degradation and refolding of abnormal proteins and in protein quality control [77]. Lon is necessary for biofilm formation and swarming motility and plays a role in both intrinsic and adaptive resistance.

CbrA: the sensor kinase CbrA is part of a two-component system along with its cognate response regulator CbrB. The two proteins have been previously identified to be involved in the metabolic regulation of carbon and nitrogen utilization in *P. aeruginosa.* CbrA has been proposed to repress Crc through a regulatory cascade that involves CbrB and a small RNA (CrcZ) [53]. CbrA regulates swarming and biofilm development and plays a role in acquired resistance.

PhoQ: PhoQ is a sensor kinase that phosphorylates and activates the transcription factor PhoP, comprising a two-component regulatory system. It participates in resistance to antimicrobial peptides under low Mg^{2+} concentrations and swarming and biofilm formation [60].

The regulators Crc, PsrA and Lon (Box 2) all play a role in both intrinsic antibiotic resistance and virulence. A *crc* mutant strain was more susceptible to β-lactams, aminoglycosides, fosfomycin and rifampin [58]. Mutants in Lon, an ATP-dependent protease that controls DNA damage stress, were supersusceptible to fluoroquinolones [7,15], indicating that they play a fundamental role in intrinsic resistance. Similarly, a *psrA* mutant exhibited supersusceptibility to polymyxin B and the cationic antimicrobial peptide indolicidin owing to altered outer membrane permeability [63]. Remarkably, although these regulatory mutants display different supersusceptibilities, they have certain common virulence defects, exhibiting swarming and biofilm deficiencies.

Lon also regulates the RhlR–RhlI and LasR–LasI quorum sensing (QS) systems [64] that control virulence. Interestingly, Lon expression is induced during swarming and by subinhibitory concentrations of aminoglycosides [43] that also induce biofilm formation [59], which indicates that Lon is also involved in adaptive resistance.

The swarming and biofilm deficient phenotype of a *psrA* mutant can be partly explained by the downregulation of genes involved in adhesion and motility [63,65,66], although the involvement of PsrA in type III secretion systems (TTSS) is controversial [63,67,68]. PsrA is also

Box 3. Biofilm formation and swarming motility

Biofilm and swarming are complex adaptations (i.e. social behaviours) that occur due to multi-factorial regulation (Figure I). Biofilm formation is initiated by flagella-mediated motility that permits planktonic cells to approach a surface where it can attach using flagella and type IV pili as adhesins. Subsequently, the bacteria attach more strongly and grow in an ordered (mushroom-shaped) structure held together by structural polysaccharides (Pel and Psl). Similar to biofilms, swarming motility depends on flagella and type IV pili but involves the ordered movement of bacteria across semi-solid (viscous) surfaces creating dendritic or solar-flare type colonial appearances. This process depends on the expression of more than 200 genes including transcriptional regulators, virulence genes and metabolism-related genes. The establishment of biofilms has been correlated with chronic infections on epithelial or medical device surfaces [78], whereas swarming has been proposed to occur in a virulent lifestyle where the bacterium moves through the mucous layer of the epithelium [49]. Genes involved in the genetic switch between biofilm and swarming lifestyles have been identified [72,79].

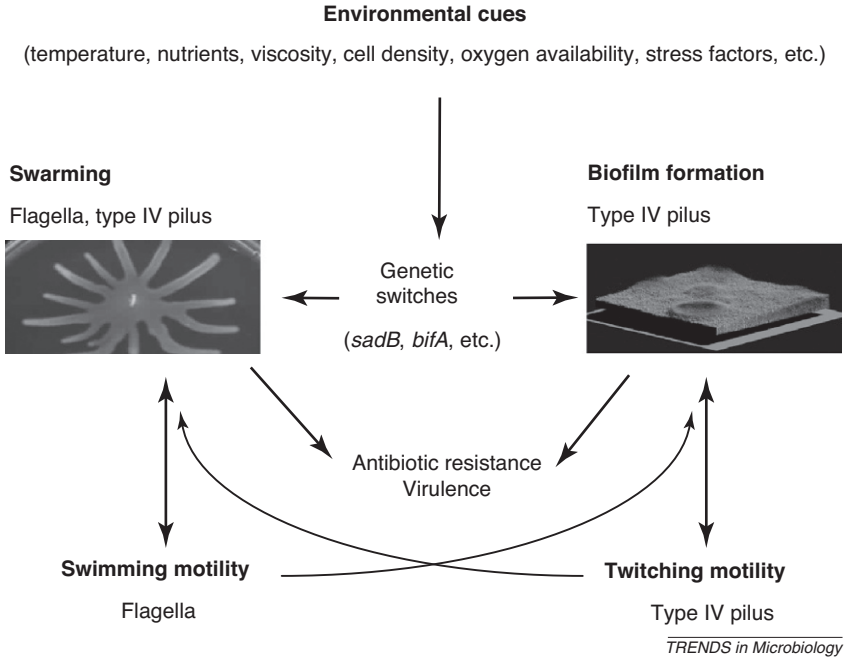

Figure I. A variety of environmental signals contribute to the occurrence of either biofilms or swarming by influencing the expression of different genetic switches (such as the ones represented by the genes *sadB* and *bifA*). *sadB* is required for an early step in the development of biofilms, and it also regulates swarming motility. *bifA* plays a role in biofilm formation and is essential for swarming. At the same time, biofilms and swarming are distinctly influenced by the expression of both swimming motility-dependent flagella and twitching motility-dependent type IV pili. The combination of these and other factors regulate biofilm formation and swarming, complex adaptations that have an influential effect on the pathogenesis-related events of resistance to antibiotics and virulence. The arrows indicate the correlation between the different virulence determinants. The *P. aeruginosa* biofilm picture is reproduced, with permission, from [73].

involved in adaptive resistance because it is induced by antimicrobial peptides [63] and fatty acids [68].

The sensor kinase CbrA, similar to Crc (mentioned above), controls central carbon and nitrogen metabolism (Figure 1) and is involved in acquired resistance. Increased resistance towards polymyxin B, ciprofloxacin, tobramycin and colistin as well as a deficiency in swarming and increased biofilm formation were observed in a *cbrA* mutant [53]. Furthermore, a *cbrA* mutant displayed increased cytotoxicity towards human bronchial epithelial cells (HBEs) when compared to wild type. Therefore, in CbrA an increase in antibiotic resistance is associated with a modulation of virulence, and these phenotypes can be explained by the dysregulation of discrete subsets of genes [53].

A number of other genes play a role in antibiotic resistance in response to triggering factors. Mutation of *phoQ* leads to a constitutive expression of the *arn* operon and thus constitutive resistance to aminoglycosides, polymyxin B and antimicrobial peptides [60,62] (Figure 1 and Box 2). PhoQ is also important in virulence and a *phoQ* mutant was deficient in swarming, twitching, biofilm formation, production of pyocyanin and pyoverdine as well as cytotoxicity, compared to the wild type. Furthermore, the deletion of *phoQ* drastically reduced virulence in lettuce leaves and competitiveness in a rat model of chronic lung infection [60].

In addition to the above mentioned genes, many others can also affect both antibiotic resistance and virulence. These include *mexS* (*nfxC*), *mexGHI–ompD*, *gacAS*, *retS*, *ladS*, *algR* and *rsmA* and have been extensively reviewed [69]. Importantly, MDR efflux pumps are relevant for virulence and resistance and it was shown that mutations in *mexI* and *ompG* reduce the production of quorum-sensing signals, therefore being essential for cell-to-cell communication. Furthermore, the loss of the pump leads to the accumulation of the toxic PQS precursor (2-heptyl-3-hydroxy-4(1H)-quinolone), which has been shown to be important for an attenuated virulence phenotype [70].

In summary, many regulators affect both antibiotic resistance and virulence. Biofilm formation and swarming are conspicuous among the virulence-related processes and appear to have a role in *P. aeruginosa* pathogenesis. Converging evidence suggests that these two complex

adaptations are interrelated and the existence of genetic switches between them have been proposed [71,72] (Box 3).

Concluding remarks

In certain types of uncomplicated infections, *P. aeruginosa* can be eradicated in a quite efficient way. However, in chronic CF infections for example, *Pseudomonas* undergoes a switch to a biofilm and swarming lifestyle and becomes highly resistant to a broad spectrum of antibiotics, which makes it extremely difficult to treat. Resistance of *P. aeruginosa* to currently available antibiotics rises every year, even when combination therapies are administered to the patient. As a consequence of this, there is an urgent need to discover more effective drugs and new therapeutic strategies to combat the infections caused by this superbug. The optimal antibiotic dosages administered in clinical settings clearly need to be defined in the future because the inappropriate use of antibiotics contributes to the rise of adaptive, stepwise and breakthrough resistance. Once breakthrough resistance arises, the chance of finding an effective treatment decreases considerably. Indeed, there are multidrug resistant (MDR) *P. aeruginosa* strains for which no effective antibiotic is available for therapy and these will probably become more common over time. Further research will start to unravel the complexities of antibiotic resistance in this recalcitrant organism, hopefully guiding the development of new and more efficacious antimicrobial agents or new strategies that can minimize resistance issues. Furthermore, differences between *in vitro* and *in vivo* efficiency of antibiotics could exist and more *in vivo* studies need to be undertaken to re-evaluate the *in vivo* efficiency of certain antibiotics. This would help to improve the use of antibiotics in human infections.

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