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The intrinsic resistome of *Pseudomonas aeruginosa* to β-lactams

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Pseudomonas aeruginosa **is a relevant opportunistic pathogen particularly problematic due to its low intrinsic susceptibility to antibiotics. Intrinsic resistance has been traditionally attributed to the low permeability of cellular envelopes together with the presence of chromosomally-encoded detoxification systems such as multidrug efflux pumps or antibiotic inactivating enzymes. However, some recently published articles indicate that several other elements can contribute to the phenotype of intrinsic resistance of bacterial pathogens. In a recently published article, we explored the chromosomally-encoded determinants that contribute to the phenotype of susceptibility of *P. aeruginosa* to ceftazidime, imipenem and carbapenem. Using a comprehensive library of transposon-tagged insertion mutants, we found 37 loci in the chromosome of *P. aeruginosa* that contributed to its intrinsic resistance, because mutants in these loci were more susceptible to antibiotics than their parental strain. Forty one further loci could potentially be involved in the acquisition of resistance, because mutants in these loci were less susceptible than their wild-type counterpart. These results indicate that the intrinsic resistome of *P. aeruginosa* involves several elements, belonging to different functional families and cannot be considered as a specific mechanism of adaptation to the recent usage of antibiotics as therapeutic agents. In the current article, we summarize the findings of the paper and discuss their implications for understanding the evolution of antibiotic resistance and for defining novel targets for the search of new antimicrobials. Finally, the validity of recent theories on the mechanisms of action of antibiotics is discussed taken into consideration the results of our paper and other recently published works on the mechanisms of intrinsic resistance to antibiotics of *P. aeruginosa*.**

Key words: Intrinsic resistance, *Pseudomonas aeruginosa*, β-lactam resistance, β-lactamase, resistome

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Antibiotic resistance is frequently considered as an acquired trait of bacterial populations, which has become prominent very recently (in evolutionary terms) as the consequence of the introduction of antibiotics for the treatment of infectious diseases.[1,2] Since resistance can be achieved either as the consequence of mutation[3] or due to the horizontal acquisition of resistance genes,[4] it has been largely assumed that the origin of such resistance genes are the microorganisms producing antibiotics, since they need to carry resistance elements to avoid the inhibitory action of the antibiotics they produce.[5,6] Possibly due to these views regarding the origins of resistance, and the forces that shape its evolution, intrinsic resistance has not been analyzed in full detail until recently. It is of critical importance in opportunistic pathogens that present a characteristic low natural susceptibility to antibiotics. Intrinsic resistance has traditionally been attributed to a reduced permeability of the cell envelope due to decreased uptake. This is a dependent mechanism in the sense that restricted permeability is hard-wired into the cell and slows down rather than prevents the uptake of antibiotic. The characterization of chromosomally-encoded antibiotic-inactivating enzymes (such as β-lactamases) and multidrug (MDR) efflux pumps demonstrated that bacterial

cells harbor further intrinsic mechanisms that can act in synergy with slowed uptake to reduce the activity of the antibiotics. Differing from reduced permeability, the latter are detoxification elements that resemble the classical determinants of antibiotic resistance acquired by horizontal gene transfer. However, since these elements are widespread, can be encoded in the core-genome and, in the case of MDR efflux pumps, are present in the chromosomes of all organisms, including those that do not produce antibiotics,[7,8] they might have evolved for purposes other than just avoiding the activity of a given antibiotic. Indeed, the recent analyses of comprehensive transposon-tagged mutant libraries in different organisms such as *Escherichia coli*[9,10] or *Pseudomonas aeruginosa*[11-14] demonstrated the existence of several genes that cause changes in antibiotic susceptibility when they are inactivated. Among those genes, several encode for proteins involved in bacterial metabolism, indicating that intrinsic antibiotic resistance is not just the consequence of bacterial adaptation to the presence of antibiotics, but rather a characteristic phenotype highly dependent on the metabolic networks of each bacterial species. Indeed it may reflect an adaptation to a distinct growth state such as afforded by biofilm development and swarming motility both of which are multigenic phenomena associated with major increases in antibiotic resistance and metabolic changes.[15,16]

In a recent paper, we analyzed a comprehensive library of transposon-tagged insertion mutants with the aim of finding genes that changed the susceptibility of *P. aeruginosa* to β-lactams upon inactivation.[17] This bacterial species is one of the most important opportunistic pathogens,[18] causing severe infections in hospitals and being the major pathogen associated with eventually fatal chronic infections that afflict patients with cystic fibrosis, the most prevalent inherited disease in Caucasian populations. *P. aeruginosa* is particularly problematic due to its low intrinsic antibiotic susceptibility[19] in part based on its exceptionally low outer membrane permeability.[20] The antibiotics chosen for the analysis were a cephalosporin (ceftazidime) and two carbapenems (meropenem and imipenem) that are currently used for treating *P. aeruginosa* infections. Two carbapenems were included in order to check whether intrinsic resistance to drugs belonging to the same structural family might have some degree of specificity. We studied mutants that showed a higher susceptibility, reflecting proteins that contribute to intrinsic resistance, as well as mutants with decreased susceptibility, which define the genetic reservoir of *P. aeruginosa* for evolving towards resistance without acquiring foreign DNA.

One of our main objectives was to look for small changes in antibiotic susceptibility. Antibiotic resistance can be defined using operational criteria, which take into account the pharmacokinetics and pharmacodynamics of the antibiotics to establish those values above which a therapeutically useful concentration is difficult to achieve. If the MIC for a bacterium is above these values, a risk exists that the infection cannot be successfully treated. Because of this, it is usually assumed that the microorganisms should be categorized as resistant when their MICs are above a pre-defined threshold. This definition, which has a clear relevance in the clinical world, does not take into consideration low-level resistance mechanisms. In our work we took into consideration this type of mutants because low-level resistance is relevant to the development of high level resistance[21,22] and is also likely the cause of MIC-creep, defined as the constant rise over time in the basal intrinsic resistance of an average isolate of a given bacterial species.[23] Low-level resistance is difficult to track using conventional double-dilution tests of antibiotic susceptibility. Because of this, we confirmed our screen results by determining MICs using the Epsilon-Test, which allows for the accurate discrimination between small changes in MIC values. Applying a threshold on MIC change of two-fold, we found that 37 loci in the chromosome of *P. aeruginosa* contributed to its intrinsic resistance to antibiotics (mutants in these loci were more susceptible than their wild-type parental strain), whereas 41 could potentially be involved in the acquisition of resistance upon their inactivation (mutants in these loci were less susceptible than the wild-type). As these studies were restricted to transposon mutants it is likely that there are other genes in the resistome that cannot be knocked out due to their essentiality for growth on common lab media. Additionally other proteins expressed in infection of the host may not be expressed in vitro and such proteins contributing to the resistome would not have been found in our study.

The antibiotics used in our screen affect cell wall synthesis by interacting with penicillin-binding proteins and murein hydrolases, therefore we expected to detect a core set of loci involved in the susceptibility to this family of antibiotics. To our surprise, the overlap among the different phenotypes was very low. Only one mutant (in PA0908) presented reduced susceptibility to all three antibiotics and two (in *glnK* and *ftsK*) showed an increased susceptibility to all three antibiotics. These last two mutants revealed genes that are potential good targets in looking for drugs that, like the β-lactamase inhibitors, increase the efficacy of antibiotics against resistant organisms.

Those genes that when inactivated resulted in changes in susceptibility to β-lactams encode for proteins that belong to a variety of functional groups, including metabolic enzymes such as phosphoenolpyruvate carboxiquinase, elements involved in cell attachment and motility such as fimbrial proteins or chemotaxis proteins, elements involved in the biosynthesis of LPS and in alginate production, and transcriptional regulators. More classical resistance elements such as the transporter of carbapenems, OprD2, regulators of efflux pumps like NalC, elements of these efflux pumps, like OprM or elements involved in the regulation of the expression of the *P. aeruginosa* chromosomally-encoded β-lactamases, like those encoded by *dacB*, *mpl*, *ampR* and *ampD* emerged as well in our screening further validating our experimental approach. Altogether our results indicate that the intrinsic resistome of *P. aeruginosa* involves several different elements and might be considered as an emergent property of the system more than a specific mechanism of adaptation to the presence of antibiotics. A recently

published theory on the mechanism of action of bactericidal antibiotics suggests that they share a common pathway in bacterial killing involving the generation of oxygen radicals, through the interference of such antibiotics with the bacterial metabolism.[24,25] From this model, it can be predicted that mutations in genes coding for proteins involved in the bacterial metabolism might be relevant in the development of resistance or supersusceptibility. Unfortunately, our results did not support a general role of oxygen radicals in killing. Indeed although some of the mutations analyzed in our work were previously found to be involved in the intrinsic resistance of *P. aeruginosa* to other drugs, most of the mutants were specific, indicating that the mechanisms of activity of the antibiotics and thus the mechanisms of intrinsic resistance are not as general as might be expected based on the common pathway concept. Furthermore, the percentage of mutants presenting the same phenotype (increased or decreased susceptibility) for imipenem and meropenem was not high, despite both antibiotics being carbapenems. Another interesting issue, raised as well in other studies on intrinsic resistomes[26] is the finding of some degree of strain-specificity. Whereas some elements contributed to resistance in both *P. aeruginosa* strains PAO1 and PA14, others are strain-specific. This might be due to different expression levels of these elements in either of the strains or to the existence of changes in their respective metabolic and/or regulatory networks.

As a conclusion of our work, and consistent with other published studies, it can be stated that the intrinsic resistome of *P. aeruginosa* involves a large array of elements. Furthermore, the analysis of mutants causing a reduced susceptibility to β-lactams indicates that this bacterial species has a high potential to evolve towards resistance. Given that mutation is the main mechanism whereby *P. aeruginosa* develops resistance during chronic infections,[27,28] the results presented in our article and others dealing with the intrinsic resistome of this bacterial pathogen might help to define novel elements involved in the acquisition of resistance during such infections.

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