Cloning & Transgenesis

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Unraveling Resistance Mechanisms against New Antimicrobials Using Transposon Mutagenesis

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Abstract

Transposon mutagenesis historically has been an invaluable tool for the discovery of bacterial gene functions and for the identification of numerous cell processes, including those related to bacterial virulence and pathogenesis. This review discusses the role of transposition in the emergent research field of identifying genes involved in the mechanisms of resistance to antivirulence compounds, biofilm inhibitors, and non-redox iron analogs.

Keywords: Transposon mutagenesis; *Pseudomonas aeruginosa*; *Escherichia coli*; Quorum quenching; Biofilms; Gallium nitrate

Introduction

Bacterial infections and antibiotic resistance

A current global health challenge is to develop effective strategies to deal with the ever increasing antibiotic resistance of many bacterial pathogens, some of which are currently resistant to all known antibiotics [1]. Unfortunately, despite the more restricted use of antibiotics, there is an increasing number of reports of multi-resistant (able to resist at least three different kinds of antimicrobials) and pan-resistant (able to resist all known antimicrobials) strains of pathogens causing nosocomial infections. Among the major nosocomial pathogens, *Pseudomonas aeruginosa* is particularly able to produce refractory infections [2]. This remarkable organism has the ability to infect almost any tissue or organ of immune compromised patients, including patients suffering from diseases such as lung infections, cancer, Human Immunodeficiency Virus (HIV) infections, and severe burns. *P. aeruginosa* is responsible for approximately 10% of all intra-hospital infections, which are very difficult to treat due to several factors [2,3]. These factors include the bacterium having: i) very high genome complexity, with many genes devoted to regulatory functions, such as those encoding transcription factors and two-component systems [4]; ii) a very robust metabolism and hence minimal growth requirements, which allow it to survive in diverse environments; iii) a high biofilm formation capacity (biofilms being linked to refractory chronic infections [5] and the ability to tolerate doses of antibiotics several orders of magnitude higher than planktonic cells; moreover, biofilms are induced by stress [6], including the damage produced by clinical antibiotics such as amino glycosides [7]); iv) and an intrinsically high tolerance to antimicrobials due its low cell membrane permeability and the presence of several multidrug resistance pumps [2,3], allowing it to readily develop resistance against new antimicrobials, including those that target cell-cell communication and virulence [8,9] and non-redox iron analogs such as gallium [10]. Another common bacterium responsible for nosocomial infections is *Escherichia coli*. This bacterium, which is the most studied organism in the world, is normally harmless and in fact commensal for humans, being an important part of the healthy human intestinal tract. However, some *E. coli* strains are pathogenic and able to produce infections, such as cholecystitis, bacteremia, cholangitis, urinary tract infections, intestinal infections, neonatal meningitis, and pneumonia [11,12]. Notably, *E. coli* also has the ability to form robust antibiotic-resistant biofilms [13]. Like *P. aeruginosa,* it can develop dormant "persister" cells, which are tolerant to antibiotics; these are generated in stationary phase cultures and in biofilms, and are largely responsible for the refractory nature of chronic infections [14]. For all these reasons it is urgent to develop new therapies to better treat the infections produced by these and other important bacterial pathogens.

Transposon mutagenesis for the study of bacterial virulence

Transposon mutagenesis has been ideal for the discovery of genes implicated in several important bacterial processes, such as those related to virulence. For example, the role of Phenazines in the infective phenotype of *P. aeruginosa* was demonstrated by assaying a library of transposon-generated mutants of the PA14 laboratory strain in the nematode *Caenorhabditis elegans;* moreover, most of the genes implicated in the killing of *C. elegans* are also important for *P. aeruginosa* infections of plants and mice [15,16]. The transposition for those studies was done using the transposon TnphoA carried on the suicide plasmid pRT731 in the *E. coli* strain SM10 λ-pair, and was previously used in the identification of *P. aeruginosa* virulence genes specific for plants [17]. Later, Fred Ausubel's group developed a non-redundant library of PA14 transposon mutants in which nonessential PA14 genes are represented by a single transposon insertion. The PA14NR library, as it was named by the authors, was generated using *MAR2xT7*, which is based on the mini-transposon *mariner*. The library was first used by its creators to identify important genes relating to the attachment of the bacteria to abiotic surfaces. The library (either a single mutant or the complete set) is commercially available at the PA14 Transposon Insertion Mutant Database, http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi [18]. Several important discoveries have been made by other researchers using that library. For example, in 2009 the library was screened to determine mutants that made altered biofilms; seven uracil biosynthesis-related mutations that abolished biofilm formation were found, among them *pyrF* that encodes an enzyme that catalyses the last step in uridine monophosphate synthesis. In addition to defective biofilm formation, the *pyrF* mutant was also severely impaired in its expression of several QS-controlled virulence factors and in the production of the PQS (Pseudomonas Quinolone Signal) autoinducer. The defects in all phenotypes were reversed by the addition of uracil to

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the medium, indicating that there is a previously unknown connection between uracil and QS/biofilm formation. Furthermore, in the same study, uracil analogs were tested for their effects on QS inhibition; the commercially available chemotherapeutic agent 5-Fluoro Uracil (5-FU) was found to be an effective quorum quencher [19]. Later, the same group of researchers found that 5-FU was also effective against *E. coli* biofilm formation [20]. Interestingly, 5-FU may be the first QQ with clinical application, since it has been used successfully as a coating for catheters (to prevent bacterial biofilm formation) in large-scale human trials [21]. In addition to the PA14 Tn mutant library, another library was developed for the other main *P.aeruginosa* reference strain, PAO1. That library was created by delivering the mini-Tn5-*lux* transposon to PAO1 by conjugation with the donor strain *E. coli* S17-1 λ-pair (pUTmini-Tn5-*luxCDABE*-Tc), and is also available to the public at http://pseudomutant.pseudomonas.com/methods.html. The library was first used to identify genes important for the adaptation to magnesium and phosphate limitation, as well as genes involved in resistance to cationic antimicrobial peptides [22]. The PAO1 mini-Tn5-lux library was later used to identify genes important for swarming motility, including genes involved in the synthesis and function of flagellin and type IV pilus; genes that codified components of the type II secretion system; genes implicated in regulatory, metabolic and chemosensory functions; as well as genes with unknown functions [23]. In addition, the library was used successfully for the identification of genes important for resistance against aminoglycosides [24] and ciprofloxacin [25], and in several other studies. In addition to the virulence genes identified by transposon mutagenesis, mentioned before, there are many other not reviewed here and perhaps many more will be discovered in the future, for this purpose, transposon mutagenesis will continue to be an excellent tool in the upcoming years.

Targeting cell-cell communication as a way to attenuate bacterial virulence

Quorum Sensing (QS) is a widespread process of cell-cell communication in both Gram negative and Gram positive bacteria, which leads to the coordinated expression of hundreds of genes in response to the presence of small signaling molecules named autoinducers. Since autoinducer concentration correlates with the number of bacterial cells present in the environment, bacteria can use this concentration to estimate their population size and act accordingly. In general, QS-controlled phenotypes are energetically costly, so their expression is limited to conditions under which those phenotypes could provide a fitness advantage to the population, even when they are potentially detrimental to the individuals; this constitutes classic cooperative behavior. Such conditions are established once a sufficiently high population threshold is reached. Several QS-regulated phenotypes have been identified; these include bioluminescence, conjugation, nodulation, sporulation, production of antibiotics and other secondary metabolites, and competence. Other QS-controlled behaviors include production of certain virulence factors together with features such as swarming and biofilm formation, and the production of toxins, adhesion molecules, lytic enzymes, and siderophores [26,27]. Due to the importance of QS in controlling virulence, several compounds able to inhibit it (quorum quenchers, or QQ) have been identified. Among them, probably the best described are signal analogues (receptor inhibitors), such as brominated furanones, that are secreted by the marine algae *Delisea pulchra* to prevent biofilms from inhibiting its photosynthesis. While the natural compound (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone from *D. pulchra* inhibits both acyl-homoserine lactone-based and autoinducer 2-based QS [28], the synthetic furanone C-30 decreases acylhomoserine lactone-based

signaling as well as the virulence of *P. aeruginosa* in mouse pulmonary infection models [29]. These brominated furanones interrupt QS by interacting with transcriptional regulators that propagate the QS response, such as LuxR in Vibrio and its homolog LasR in Pseudomonas [30], and they do not affect bacterial growth in rich medium [28,29]. Hence, it was speculated that those compounds would not generate resistance, as they do not produce selective pressure by affecting individual cell survival and fitness. Current research, however, is challenging this assumption, and actually showing that resistance against anti-virulence compounds can be developed easily [8]. In addition, recently it was reported that the brominated furanone (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8) can restore the antibiotic susceptibility of *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* RP437 persister cells. This compound is effective against both planktonic and biofilm cells (at growth non-inhibitory concentrations) and promotes biofilm disruption, although the process by which it inhibits persister cell formation is currently unknown and apparently not related to QS [31,32].

Inhibition of biofilm formation

Since biofilm formation, especially in its early stages, is controlled by QS, Quorum Quenchers (QQ), such as brominated furanones, are effective in decreasing it, as well as in rendering the formed biofilms more susceptible to antibiotics such as Tobramycin [29] or detergents such as Sodium Dodecyl Sulfate (SDS) [33]. However, biofilm inhibitors that are not related to QS have also been identified; among them is ursolic acid, which is able to inhibit *E. coli*, *P. aeruginosa,* and *Vibrio harveyi* biofilms without decreasing growth. Although the exact mechanism of action for this biofilm inhibitor is currently unknown, DNA microarrays showed that it interferes with chemotaxis/mobility and sulfur metabolism, including cysteine synthesis [34]. Other recently discovered biofilm inhibitors include D-amino acids, such as D-leucine, D-methionine, D-tyrosine, and D-tryptophan. These components of the cell wall (peptidoglycans) of Gram negative bacteria are able to inhibit biofilm formation and to disrupt mature biofilms of *Bacillus subtilis*, *Staphylococcus aureus* and *P. aeruginosa*. D-amino acids interact with the protein component of *B. subtilis* biofilm, breaking down the amyloid fibers that link cells together [35]. Also, the same researchers demonstrated that the polyamine norspermidine, which is produced by mature *B. subtilis* cells, prevents biofilm formation of the same organisms, but interacts directly and specifically with the exopolysaccharide biofilm matrix component. In fact, D-amino acids and norspermidine can act together to break down existing biofilms, which probably could explain the dispersal of mature biofilms in natural environments [36]. Another important development of the field of biofilm inhibition is the discovery of the anti-biofilm properties of bacterial extracellular polysaccharides. These compounds mediate many of the cell-to-cell and cell-to-surface interactions that are required for the formation, cohesion, and stabilization of bacterial biofilms. Recently, several bacterial polysaccharides that inhibit biofilm formation in a wide spectrum of bacteria and fungi (both *in vitro* and *in vivo*) have been identified; among them are the A101 polysaccharide produced by *Vibrio sp.* QY101; the Ec111p, Ec300p, and K2 polysaccharides produced by *E. coli;* and Pel and Psl produced by *P. aeruginosa* [37].

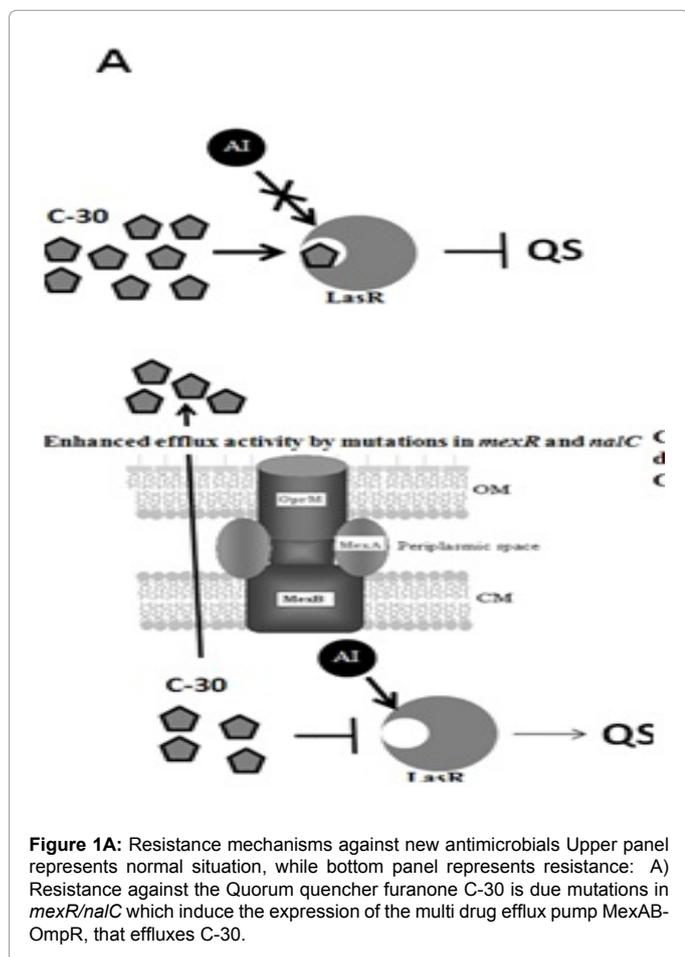## Transposon mutagenesis for the study of resistance against virulence and biofilm inhibitors

Despite all the benefits exhibited by the new antimicrobials described above, the assertion that antivirulence compounds are impervious to resistance [38,39] was recently challenged, at least for
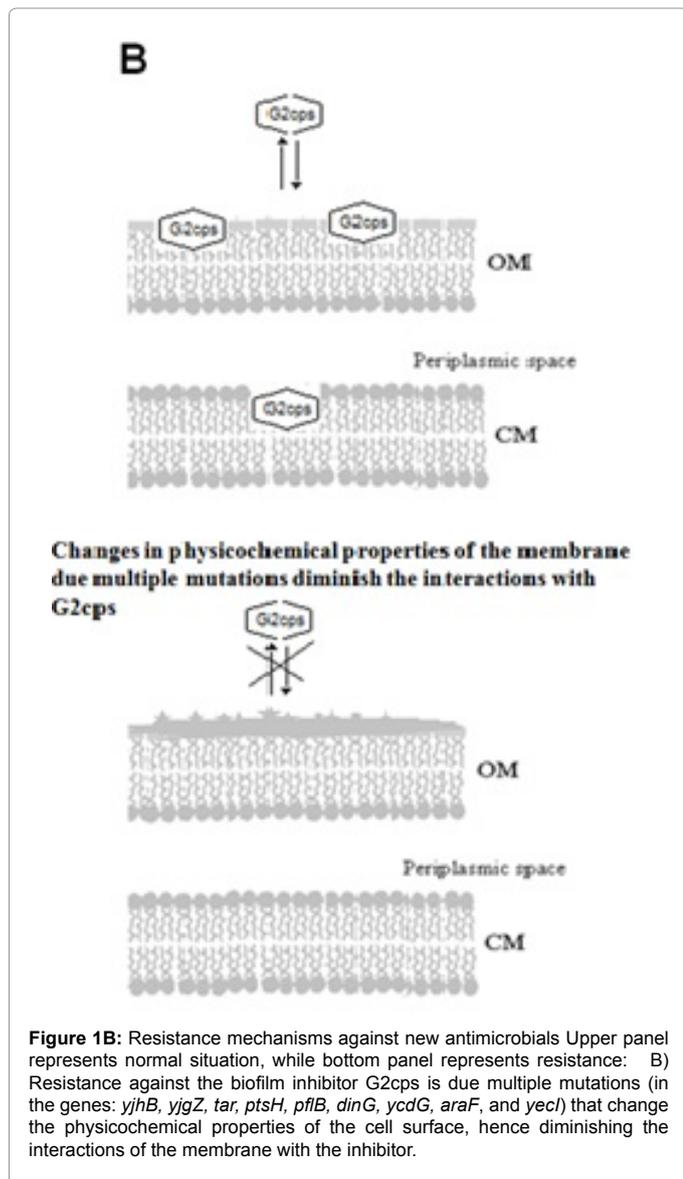
the brominated furanone C-30. In a 2010 opinion paper by Defoirdt et al. [1] it was first suggested that bacteria may evolve resistance to QQ compounds. These authors formulate their hypothesis based on several studies showing that the expression of core QS genes involved in the production/detection of autoinducers, as well as QS signal transduction, is highly variable, and since this variability is heritable and may confer an increase in fitness under QS disruption, natural selection would favor the spread of QQ resistance. A year later, the first experimental demonstration of the resistance against a QQ, the brominated furanone C-30, was published [8]. In this work the *P. aeruginosa* PA14 strain was used, and in order to create the selective pressure necessary for the isolation of C-30 resistant mutants, a novel screening procedure was developed, using adenosine as the sole available carbon source in the medium. Growth on adenosine requires an active LasI/LasR *N*-3-oxododecanoyl homoserine lactone QS system, due to the tight QS control of the expression of the nucleoside hydrolase (*nuh*) gene, which encodes an enzyme essential for adenosine catabolism. Under those conditions, C-30 was very toxic to the cells, and indeed the clones able to cope with the effects of the QQ had a higher fitness and growth rate than the sensitive wild-type strain. For the identification of genes related to C-30 resistance, a transposon mutant library was assayed. To construct this random library, conjugation with *E. coli* S17-1 (λpair)/pUT-miniTn5 *luxAB*-TcR was used, in order to deliver the miniTn5 *luxAB* transposon to PA14. Sequencing of the transposon adjacent zone in the isolated resistant mutants led the authors to identify two interrupted genes, *mexR* and *nalC*. Both genes encoded transcriptional repressors of the MexAB-OprM multi-drug resistance pump, and

Figure 1A: Resistance mechanisms against new antimicrobials Upper panel represents normal situation, while bottom panel represents resistance: A) Resistance against the Quorum quencher furanone C-30 is due mutations in *mexR/nalC* which induce the expression of the multi drug efflux pump MexAB-OmpR, that effluxes C-30.

as a result of the disruption of the repressors' genes, the pump was overexpressed and the QQ compound was effluxed (3 ± 2 nmol h^{-1} mg protein^{-1} of efflux for *mexR* after 15 h versus no efflux for the wild-type strain) (Figure 1A). In addition to the resistance of the *mexR* mutant during growth on adenosine, C-30 had a diminished ability to reduce several QS controlled virulence factors and phenotypes in the *mexR* mutant. More importantly, the pathogenicity of the *mexR* mutant against the nematode *C. elegans* was not attenuated by the addition of C-30, as it was for the parental PA14 strain. In addition, clinical strains isolated from cystic fibrosis patients (Liverpool epidemic strain 12142) with *mexR/nalC* mutations were also resistant to C-30 [8]. The fact that the mutations arose in a clinical setting shows that bacteria can evolve resistance to QQ compounds even in the absence of previous exposure to them, likely by pre-exposure to antibiotics. Additional clinical evidence of the ability of strains to evolve resistance to QQ compounds was recently provided by studying the QQ resistance of clinical isolates originally found in urine, blood, and catheter tips from Mexican pediatric patients. The quenchers assayed were C-30 and 5-fluorouracil, and as in the case of the Liverpool strains, some resistant isolates were found. Interestingly some C-30 resistant strains were not resistant to antibiotics, indicating that efflux of the quencher is not the only way to develop resistance [9]. In addition, very recently the basis of resistance against a QS-independent biofilm inhibitor was explored for the first time: Travier et al. [40] studied resistance against G2cps (the 2-capsule polysaccharide). This molecule is able to impair bacterial surface interactions in *E. coli*. In order to identify the genes related to anti-biofilm resistance, once again a transposon mutant library was screened (the library consisted in 11,000 transposon insertion mutants in *E. coli* K-12 TG1-c background). This time the strain selection was based on an increased ability to produce biofilms in the presence of G2cps, and the library was created using the *mariner* transposon carried by Tn-psc189Km. Using this approach, several mutants displaying partial resistance to the anti-biofilm polysaccharide were identified. The identified mutations (*yjhB*, *yjgZ*, *tar*, *ptsH*, *pflB*, *dinG*, *ycdG*, *araF*, and *yecI*) ultimately lead to modifications in bacterial surface physicochemical properties, such as changes in the surface charge and in Lewis base properties, which counteracted those produced by the interaction with G2cps (Figure 1B). Interestingly, some of the identified mutants showing robust biofilm formation in the presence of G2cps (10% to 45% more biofilm than the wild-type strain) were also partially resistant to other antibiofilm molecules such as surfactin, the detergent Tween 80, and other *E. coli* produced antibiofilm polysaccharides [40]. Overall, although a limited amount of research on resistance against virulence/biofilm inhibitors has been reported, it is expected that in the future much more information may become available, and that transposon mutagenesis will continue as one of the main research methodologies.

Disruption of iron homeostasis by the non-redox iron analog gallium

In addition to compounds designed to attenuate the virulence and biofilm formation capacity of bacterial pathogens, efforts of researchers have focused on creating new antibiotics that exploit stresses already imposed on invading organisms by the *in vivo* environment. Among such stresses are the low iron concentrations prevailing inside the host. Iron is an essential metal for the growth and survival of virtually all known organisms, and iron as a cofactor in basic metabolic pathways is essential to both the hosts and their pathogens. Iron is also an important component of the innate immune response, through its role in the generation of toxic oxygen and nitrogen intermediates [41]. Therefore, disrupting iron metabolism of pathogens is a promising

Figure 1B: Resistance mechanisms against new antimicrobials Upper panel represents normal situation, while bottom panel represents resistance: B) Resistance against the biofilm inhibitor G2cps is due multiple mutations (in the genes: *yjhB, yjgZ, tar, ptsH, pflB, dinG, ycdG, araF*, and *yecI*) that change the physicochemical properties of the cell surface, hence diminishing the interactions of the membrane with the inhibitor.

of malignancy. In addition, Ga coupled with desferrioxamine is effective for the treatment of bacterial keratitis in rabbit produced by *P. aeruginosa* [44], and has antibacterial effects on other important human pathogens, such as *Mycobacterium tuberculosis* [45] and multi-drug resistant strains of *Acinetobacter baumanii* [46]. As there is a severe dearth of new antibiotics in development, Ga appears to be a promising new therapeutic for the treatment of some bacterial infections.

Transposon mutagenesis for the study of resistance mechanisms against gallium

Despite the fact that Ga acts as a classical antimicrobial affecting bacterial growth and survival, the possibility that resistance against this novel antimicrobial could arise was assumed to be low. This assumption is based on the hypothesis that Ga has multiple potential targets (the many processes related to iron Redox cycling), so several mutations would be needed in order to confer significant resistance against it. This hypothesis is supported by the fact that the simultaneous disruption of the pyoverdine and pyochelin siderophore systems in the *P. aeruginosa* strain PAO1 does not make the cells Ga resistant, and also by the fact that Ga resistance in clinical isolates is actually rare [47]. Unfortunately, again this assumption was challenged, since recently using the same transposition systems as in [8], we have identified that disruption of the *hitAB* system, which encodes an iron active transport system, makes the PA14 strain resistant to Ga nitrate. In planktonic cells of *hitA* and *hitB* transposon-mutants, the MIC50 against Ga nitrate is 3 to 4-fold higher than in the wild-type strain, while for the eradication of biofilms, *hitA* mutants require a Ga nitrate concentration at least 8-fold higher than the parental strain. Also, since a *hitA* mutant exhibits very low levels of intracellular Ga, it is likely that in *P. aeruginosa* this system is involved in the internalization of Ga, HitAB being the first Ga
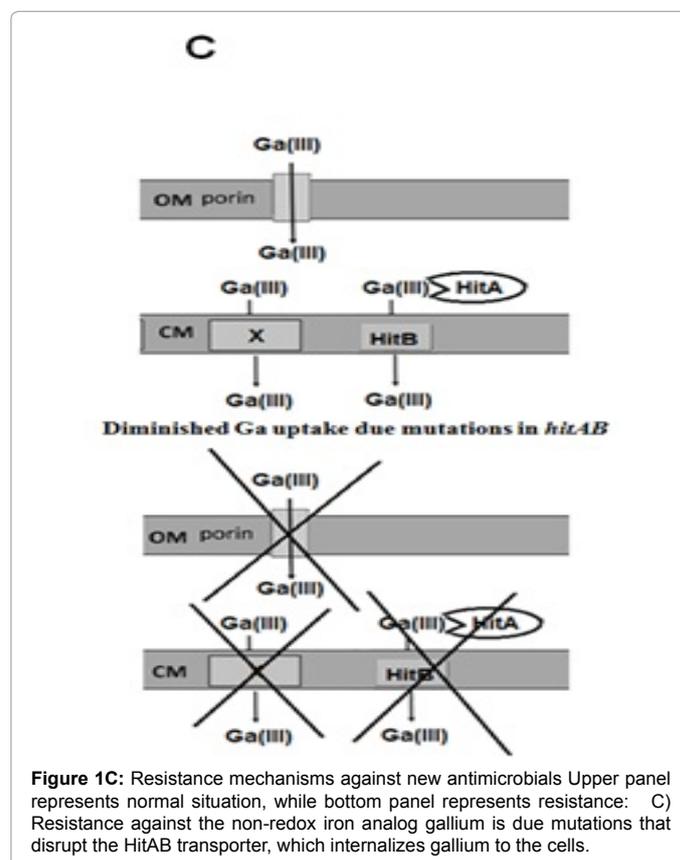
new antibacterial strategy that could be very helpful in the clinic for the treatment of infections. In fact, the utilization of siderophores and iron chelators that remove free iron from the environment has long been proposed as an alternative therapy for infections. This strategy has produced moderate success in animal models and in the clinic, though the use of chelators occasionally exacerbates infections rather than alleviating them [42,43]. An alternative approach to the use of chelators for iron metabolism disruption therapy is the recently proposed use of non-redox Fe analogs such as gallium. This semimetal acts as a "Trojan horse" that is internalized by the cell due to its chemical similarity to Fe (III). It is hypothesized, though not fully demonstrated, that it can substitute for Fe (III) in many biological systems and inhibit Fe-redox dependent processes, such as respiration, Reactive Oxygen Species (ROS) protection by antioxidant enzymes like catalase and superoxide dismutase, and DNA replication. Ga inhibits *P. aeruginosa* growth and biofilm formation, kills planktonic and biofilm bacteria *in vitro,* and is effective in murine lung infection models. Moreover, gallium nitrate is a drug approved by the Food and Drug Administration (FDA), which is administered intravenously at high doses to treat hypercalcemia

Figure 1C: Resistance mechanisms against new antimicrobials Upper panel represents normal situation, while bottom panel represents resistance: C) Resistance against the non-redox iron analog gallium is due mutations that disrupt the HitAB transporter, which internalizes gallium to the cells.

transporter described for *P. aeruginosa* [48] (Figure 1C). Since Ga is also effective as an antibiotic for some other bacterial pathogens, it would be interesting to determine if those Ga-sensitive microorganisms also have the HitAB transporters and if disruption of this system also renders those organisms resistant to Ga. Performing transposon mutagenesis would be useful for identifying potential Ga resistance mechanisms in those pathogens.

Perspectives

The development of new therapies and strategies to treat refractory bacterial infections is essential, but it is equally important to study the possibility that resistance to novel antimicrobials will arise, elucidating potential resistance mechanisms even before the new compounds are used in the clinic. The utilization of transposon mutagenesis has been an ideal tool to begin to unravel those mechanisms. However, the same studies that first reported resistance against C-30 and Ga in transposon mutants also identified spontaneous mutants with much higher resistance profiles than those found by transposition, and with mutations not located in the genes interrupted by the transposons, indicating that there are other yet unknown mechanisms that can confer resistance against the novel antimicrobials [8,48]. For the study of those mechanisms, transposon mutagenesis could again be the ideal tool, since it could be used to generate mutants with lower resistance levels than the original spontaneous hyper-resistant strains. Ideally it is expected that the mutations involved in the reversion of the resistant phenotype would be located in genes participating in the unknown processes that confer resistance. Nevertheless, resistance against the new antimicrobials may not be necessarily linked to the disruption of gene functions (which in most cases will have the effect of interrupting a gene with a transposon). Therefore, genetic or genomic methods, such as the complete sequencing of the resistant strains, may be necessary to reveal those resistance mechanisms associated with an increase in, or change of, gene functions. In addition to several Quorum Quenchers (QQ), anti-biofilm and novel antimicrobials have been identified without studying whether resistance against them could evolve. Therefore, although valuable as a first approach, the results discussed here only represent the tip of the iceberg of an emergent field of research. It is expected that the identification of the resistance mechanisms against some novel antimicrobials will eventually aid in the design of new therapies and strategies with higher chances of success for the treatment of bacterial infections. Towards this end, transposition will continue to be an excellent experimental tool for identifying new resistance mechanisms in many bacterial pathogens.

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