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Mechanisms of Resistance in Bacteria: An Evolutionary Approach

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Abstract: Acquisition of resistance is one of the major causes of failure in therapy of bacterial infections. According to the World Health Organization (WHO), thousands of deaths caused by *Salmonella* sp., *Escherichia coli*, *Staphylococcus aureus* or *Mycobacteria tuberculosis* are due to failure in therapy caused by resistance to the chemotherapeutic agents. Understanding the mechanisms of resistance acquisition by the bacterial strains is therefore essential to prevent and overcome resistance. However, it is very difficult to extrapolate from *in vitro* studies, where the variables are far less and under constant control, as compared to what happens *in vivo* where the chosen chemotherapeutic, its effective dose, and the patient's immune system are variables that differ substantially case-by-case. The aim of this review is to provide a new perspective on the possible ways by which resistance is acquired by the bacterial strains within the patient, with a special emphasis on the adaptive response of the infecting bacteria to the administered antibiotic.

Keywords: Multi-drug resistance, acquisition of resistance, efflux pumps.

INTRODUCTION

Resistance to antibiotics represents a worldwide healthcare problem that affects therapy of infectious diseases caused by a large variety of organisms including Gram-negative, Gram-positive bacteria or mycobacteria. Since the discovery of penicillin, antibiotics and other antimicrobial agents have been successfully employed for treatment of infections. On the other hand, traditional medicine, which is probably as old as mankind, has been extensively used in a wide variety of applications. Within tribal communities, various plants, fungi and even animal body parts have commonly been ingested or topically applied with a therapeutic aim for various diseases including infections [1]. Traditional Chinese medicine is an excellent example of a complex therapeutic system that has a strong focus on natural products and provided valuable subjects also for chemistry-based drug discovery [2-4].

Development of antibiotic resistance by a bacterial strain is most typically due to antibiotic misuse consequentially leading to an ineffective therapy [5, 6], which, for example, in economically disadvantaged countries can also occur due to inadequate access to drugs [7]. Bacterial resistance has been detected for all classes of antibiotics [8] and with respect to a wide variety of infections including those caused by *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* or *Mycobacterium tuberculosis*, resistance to one or more antibiotics has become a commonplace issue [9]. Excellent and extensive reviews that cover different mechanisms of resistance including modulation of porins [10-12], efflux pumps (EPs) [13-15], and lipopolysaccharide components of the cell wall [16] have been published and characterization of these mechanisms will not be the main focus here. Instead of that, this review aims to discuss possible relationships between environmental conditions and the mechanisms of resistance developed by bacterial strains during infection and therapy.

ACQUISITION OF RESISTANCE

The process of resistance acquisition by the bacterial cells can apparently be divided into two major stages: 1) a first and fast response which includes the reorganization of the membrane and its permeability [17] (change in lipopolysaccharide composition [17], decrease of porin content [10-12] and/or over expression of efflux pumps [13-15]), and 2) a second, slow response that would involve genetic changes.

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Many of the possible genetic changes are not well described or completely understood, however, one may suppose that they would involve the acquisition of mutations, promoted for example by activation of "mutator genes" [18]. Other mechanisms may also determine the process of resistance acquisition, like horizontal gene transfer between organisms, or activation of cell signalling responses which are closely related to the behaviour of bacteria in the wild; bacterial communication (quorum sensing) [19-21] and biofilm formation [22]. This latter phenomenon may also play an important role in bacterial pathogenicity and host colonization [23]. Nevertheless, it is almost sure that all these events are correlated and play an important role in the acquisition of resistance by a bacterial strain in a patient treated with antibiotics.

1. Signalling Molecules

Adaptation of bacterial strains to the environment within the living organism before and/or after starting therapy has an undoubtedly close relationship with evolutionary processes [24, 25]. This is supported by many studies on the role of antibiotics and other molecules used by microorganisms as cell signalling agents, and by the responses of pathogenic bacteria to different stimuli [20, 26]. Interestingly, sub-inhibitory concentrations of commonly used antibiotics were found to induce responses in the bacterial cell significantly different to those observed at greater concentrations [27, 28]. Moreover, response of bacteria to environmental changes has been described along with successful co-operation and co-evolution among species [29, 30]. The role of natural antibiotics (firstly isolated from bacteria, fungi, plants, etc) was also studied in their original environment [31], and Linares and his co-authors suggested that antibiotics can also be interpreted as signalling agents instead of "weapons" [32]. The generally accepted point of view, that these compounds are used by bacteria to fight competitors in their natural environment [32] may completely be overwritten by the idea that antibiotics serve as signalling molecules and regulators of the homeostasis of the bacterial community [33], playing an important role in biofilm formation, symbiosis, etc. It was demonstrated that for example tobramycin, an antibiotic aminoglycoside produced by *Streptomyces tenebrarius*, can inhibit the quorum sensing (QS) system of an environmental *Pseudomonas aeruginosa* strain when applied at sub-antibiotic concentrations [28].

In the bacterial genome the information for the biosynthesis of antibiotics and other signalling molecules compounds is usually present in clusters, which also contain genetic information for activation of the so called resistance mechanisms and which are also common to several different signalling molecules [26]. The information present in such gene clusters can than pass among bacteria either vertically (generation through generation of a bacterial species) or horizontally (between species) [25].

It was shown by Hosaka and co-authors that different bacterial strains known for producing several antibiotics contain a larger pool of silent genes encoding a higher variety of antibiotics that can be biosynthesized by that specific bacterial strain under certain conditions [34]. Presence of a similarly large pool of silent resistance genes encoding all necessary mechanisms for self-defence against all possible (evolutionary already "invented") endogenous antibiotics seems to be a logical consequence of this; however to our knowledge such studies have not been extensively connected with the specific mechanisms used by bacteria to acquire resistance.

2. Reorganization of the Membrane and Its Permeability as a Response to Antibiotic Pressure

2.1. Lipopolysaccharides (LPS)

Acquired resistance to antibiotics through a decrease in the permeability of the cell membrane requires major structural changes in the membrane [35-37]. However, due to the composition of the cell barrier, there is a general difference in the susceptibility to hydrophobic and hydrophilic compounds [38]. Gram-negative bacteria have an extra "protection" given by the outer membrane. For this reason some antibiotics that are active against Gram-positives are not active against Gram-negatives. LPS composition increases the asymmetry in the membrane architecture and the cross binding between LPS and divalent cations decrease permeability to hydrophilic agents [39].

Salmonella sp. is an example demonstrating the importance of LPS in virulence as well as intrinsic and acquired resistance to antibiotics [16, 39, 40], which shows well that LPS are more than a simple barrier: it is an essential part of the complex system responsible for the response of the bacterial strains to their environment. These systems are regulated by host-derived signals that control gene expression for optimal establishment and maintenance of infection and activate virulence-factor expression, which allows the bacteria to survive, for example, within a neutrophil [16].

2.2. Porins

The regulation of membrane permeability in Gram-negative bacteria is also a function of membrane proteins. Regulation involves the joint action of porins and efflux pumps. Porins, found in Gram-negatives and mycobacteria, are trimers of identical subunits, each consisting of an anti-parallel β-barrel forming a pore [41]. These proteins form channels that traverse the outer membrane and end in the periplasm. They serve as the main entry for different classes of antibiotics such as β-lactams or fluoroquinolones, as well as a large variety of small hydrophilic molecules [42-45]. Indeed, some β-lactam resistant strains of *E. coli* have shown a deficiency in the expression of the outer membrane protein (Omp) with alterations in its loop structure, caused by mutations. This can interfere with the interaction of the antibiotic with the surface of the channel, which determines its penetration inside the cell [45]. In fact, clinical isolates with modifications in the structure of their porins were already identified in many Gram-negative strains [12]. Moreover, porin-deficient mutants are also more resistant to quinolones, tetracyclines, chloramphenicol, nalidixic acid and trimethoprim [44]. *P. aeruginosa* has innate low susceptibility to β-lactams due to its low porin content with distinct physicochemical properties as compared to other strains [12].

It was also observed that the expression of OmpC and OmpF, controlled by the concentration of some antibiotics in the environment, regulates the permeability of the outer

membrane to glucose under nutrient deficient conditions [46]. Most of the related studies aimed to explore the mechanisms involved in this. [45-48]. It was shown that some clinical isolates, obtained from patients undergoing treatment, had their membrane permeability changed due to a switch in the expression of porins from OmpF to OmpC, which latter one has a smaller pore size. This modification in the porin balance was suggested to have occurred during the treatment [12]. OmpC-OmpF balance is strongly regulated by different genetic control systems, such as EnvZ-OmpR and RNA anti-sense regulators (MicF and MicC) [12, 49, 50].

Some *in vitro* studies also showed that loss of OmpC is followed by the expression of another subfamily of porins. OmpN type of porin is structurally related to OmpC and OmpF. OmpN pore is a selective filter for charged molecules due to its structural organization. It allows the maintenance of bacterial fitness with the entrance of nutrients but not antibiotics. This increases the resistance to the β-lactams [12]. OmpX is another important outer membrane protein: it is small, and, together with OmpF, it is involved in the response to external stress via different regulation cascades [48].

2.3. Efflux Pumps (EP)

While porins represent entrances for compounds exogenous to the bacterium, cellular efflux systems are responsible for the extrusion of both endogenous (e.g. toxic metabolites) and exogenous (e.g. bile salts) toxic compounds [13, 51, 52], playing an important role in the physiology and homeostasis of the cell [53]. Some EPs have also been shown to have a role in colonization and persistence of bacteria in the host, as well as in bacterial pathogenicity [53-56]. Moreover, EPs were shown to play an essential role in quorum sensing signalling between bacteria and in biofilm formation [13, 57]. EPs are also useful tools for the cell to remove antibiotics conferring resistance to a given drug or class of drugs [25, 55, 58-61], as well as heavy metals, dyes or detergents [62]. Extensive reviews on the structure, classification, specificities and efflux kinetics of different EPs can be found in the literature, which will not be detailed here.

Acquisition of resistance: overexpression of EP plus acquisition of mutations?

Antibiotic therapy commonly results in the appearance of resistance of the infecting bacterium to the drug. Although a variety of mechanisms account for distinct forms of resistance, the over-expression of efflux pumps extruding the antibiotics is a major mechanism in the resistance of clinical isolates [15, 58]. The tri-partite efflux pumps of Gram-negative bacteria, for reasons yet to be completely understood, have the capacity to recognize and extrude a wide variety of unrelated compounds such as antibiotics from different classes, biocides and other noxious agents like bile salts [13]. Based on this, over-expression of these EPs results in a multi-drug resistant (MDR) phenotype leading to serious difficulties in the therapy of an infection [63].

The mechanism by which these MDR efflux pumps are over-expressed has been studied in the laboratory; gradual and prolonged exposure of the bacteria to increasing antibiotic concentrations of the antibiotic that are just below its MIC promote the over-expression of individual efflux pumps consequentially leading to the increase of resistance to the actual antibiotic as well as to other non-related ones [46, 64, 65]. Transfer of the now MDR phenotypic bacterium to drug free medium restores, over time, the initial susceptibility to the inducing antibiotic, as well as eliminates its MDR phenotypic status [46, 64]. Nevertheless, these studies do not entirely explain how MDR phenotypes develop in a clinical setting, since therapy does not involve progressive increases of dose levels: a clinical isolate has never been exposed to the drug over the therapeutic dose. Furthermore, considering that the level of resistance of the MDR clinical isolate to a given antibiotic may be hundreds of times greater than that of its wild-type reference strain, it is difficult to reconcile simple laboratory studies that would induce high levels of resistance with continuous exposure to increasing concentrations of an antibiotic.

It was shown that exposure of *E. coli* to stepwise increasing amounts of tetracycline (TET) concentrations increases resistance to TET [64] that is accompanied by an increase of resistance to many other antibiotics and non-antibiotic agents, producing an MDR phenotype. This MDR phenotype is accompanied by significantly increased activity of genes encoding transporter proteins [64]. Similarly, exposure of isoniazid (INH)-sensitive *M. tuberculosis* to increasing concentrations of INH also increased the resistance of the organism to this antibiotic [66] but resistance to INH was not accompanied by resistance to any other drug employed for the therapy of pulmonary tuberculosis. These studies, however, did not completely mimic the conditions of exposure of a given bacterium to an antibiotic, which would occur when a patient, infected with this organism, is treated for a prolonged period of time with a constant dose of the antibiotic.

Other experiments, aiming a better simulation of the clinical conditions, showed that serial culture of an *E. coli* strain whose efflux pump had been over-expressed, in medium containing a constant amount of the antibiotic to which the strain had been induced to high level resistance, results in the restoration of the activity of genes that regulate and code for the efflux pump transporters, as compared to those of the antibiotic susceptible *E. coli* strain [67]. Accompanying the restoration in these gene activities is the continuous increase of resistance to the antibiotic even though the bacterium had not been exposed to higher concentrations of the antibiotic to which it had been made resistant. This *E. coli* strain also displayed progressively increased resistance to compounds that target cell envelope constituents, gyrase and ribosomes, supporting the assumption that a large number of mutated targets emerged due to the continuous sub-culturing in the medium containing a constant concentration of an antibiotic. The inability of the obtained resistant *E. coli* strain to revert to the initial susceptibility to tetracycline or to the antibiotics that contributed to its MDR status when cultured further in a drug-free medium, as happened for its resistant *E. coli* parental strain [46], together with results from phenotypic array studies suggest that indeed mutations had taken place.

The maintenance of an over-expressed efflux pump system must consume a large, yet undefined amount of energy: EPs driven by the proton motive force depend on metabolic energy as the main source of protons in order to assure the

pH gradient [68]. However, it is expected that bacterial systems tend to a low energy level (following the second law of thermodynamics). One may suppose that after exposure to an environment that remains noxiously constant, the genetic system of the organism responds by activation of a mutator system [18] resulting in the accumulation of mutations that render the organism multi-drug resistant. Although as long as the organism remains in that environment its survival is assured, if the environment returns to that initially present, the organism cannot compete with its wild type counterpart which has all relevant biochemical targets fully functional.

The above study therefore demonstrates "evolution of a bacterial strain" within a laboratory environment, and may model the situation that takes place within a patient who is infected with a bacterium and is treated for a prolonged period of time with a constant dose of a given antibiotic. Moreover, this study also demonstrated that, by ensuring relatively short-term survival, an over-expressed efflux pump provides the opportunity by which other, less energy-consuming mechanisms of resistance may ensue, which eventually makes efflux pump overexpression itself unnecessary.

In the first phase, the induced MDR phenotype is subject to reversal by common efflux pump inhibitors (EPIs). However, after further serial passages in presence of high concentrations of the antibiotic, the strain remains resistant and cannot revert to the wild phenotype anymore. These observations are possibly related to those demonstrating that whereas some MDR clinical isolates may yield a reduced resistance to a given antibiotic when an EPI is added to the medium, the EPI does not affect, or affect less, other clinical strains [69, 70]. It can be assumed that the efflux pump overexpression represents the early response to prolonged therapy with the same antibiotic either at the same or greater dose levels whereas the latter case represents a much later adaptive response.

3. Future Perspectives

Multi-drug resistance in bacteria remains a problem yet to be fully understood. With this short review on fundamental ideas and research work we aimed to give a contribution to the overall solution to understand and overcome resistance of clinical strains to therapeutics in the patient. In fact, the solution is, most likely, not only one, and in no case uniform. It may vary strain by strain, since different bacterial species can show marginally different responses to antibiotic pressure. Some develop resistance to that antibiotic, others resistance to that and other antibiotics, others form biofilms and have increased QS signalling in presence of certain antibiotic concentration or the opposite, inhibition of QS signals in presence of an antibiotic can also occur. The overall signalling role of antibiotics between strains is a very exciting theory: in such a bacterial communication system, certain porins could be interpreted as "ears" (i.e. signal receivers), while efflux pumps would be the "mouth" (i.e. signal senders by extruding endogenous, biosynthesized "antibiotics"). Existence of such fine systems, supported by many studies, certainly has several other aspects of potential interest in view of fighting resistance, different to the approach of targeting porins or efflux pumps themselves.

In the clinical point of view many other factors, depending exclusively on the patient, also have to be taken into account, such as amount of drug delivered to the site of infection, metabolism, etc.

An approach that raised great hopes and has been studied for many years is that of finding compounds that inhibit the efflux pumps of bacterial cells leading to the increase in the intracellular concentration of the antibiotic so that it can reach the desired inhibitory concentration. However there is still much to do in this approach. Some of the compounds that were very promising *in vitro* showed high toxicity *in vivo*, due to their effect in the efflux pumps of human cells, such as those of the blood brain barrier. On the other hand, some promising compounds, such as certain phenothiazines (some of which are commonly used antipsychotic drugs), were able to cure patients with extremely drug resistant *Mycobacterium tuberculosis* when applied at much lower concentrations than those considered toxic or psychoactive in humans [71-73]. Although these compounds were first described as efflux pump inhibitors, their overall mechanism of action is probably other than a direct inhibition of the pump and is yet to be fully understood. It was however recently suggested, that phenothiazines actually target the host cell (macrophage), and enhance its killing capacity on the intracellular pathogen [74, 75]. This group of compounds may hence provide valuable tools for exploring pathways that should be given priority for a more successful fighting against resistance with a new approach.

Many publications have visualized the end of the antibiotics' era. Although, unfortunately, this point of view might indeed have a chance to be right, we do believe that, despite the great challenge, resistance can be overcome by utilizing the growing knowledge on novel possible target mechanisms.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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