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REVIEW

Multidrug Resistance Pumps as a Keystone of Bacterial Resistance

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Abstract—Antibiotic resistance is a global problem of modern medicine. A harbinger of the onset of the postantibiotic era is the complexity and high cost of developing new antibiotics as well as their inefficiency due to the rapidly developing resistance of bacteria. Multidrug resistance (MDR) pumps, involved in the formation of resistance to xenobiotics, the export of toxins, the maintenance of cellular homeostasis, and the formation of biofilms and persistent cells, are the keystone of bacterial protection against antibiotics. MDR pumps are the basis for the nonspecific protection of bacteria, while modification of the drug target, inactivation of the drug, and switching of the target or sequestration of the target is the second specific line of their protection. Thus, the nonspecific protection of bacteria formed by MDR pumps is a barrier that prevents the penetration of antibacterial substances into the cell, which is the main factor determining the resistance of bacteria. Understanding the mechanisms of MDR pumps and a balanced assessment of their contribution to total resistance, as well as to antibiotic sensitivity, will either seriously delay the onset of the postantibiotic era or prevent its onset in the foreseeable future.

Keywords: multidrug resistance (MDR), pumps, antibiotics, xenobiotics, biofilms, persisters, bacteriophages
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1. END OF THE GOLDEN ERA OF ANTIBIOTICS

The problem of antibiotic resistance is relevant today more than ever. Antibiotics took medicine to the next level and helped save countless lives, but the "golden era of antibiotics" did not last long. Currently, antimicrobial resistance threatens the very foundations of modern medicine [1], and multidrug resistance (MDR) may become a more common cause of death than cancer in the coming decades [2].

This problem has become especially acute in connection with the SARS-CoV-2 coronavirus pandemic, when hospital treatment of diseases with different and mixed etiologies can serve as a trigger for accelerating the formation of antibiotic resistant hospital strains. The results of many studies testify in favor of the existence of a direct relationship between the increase in the consumption of antibiotics and the spread of bacterial resistance to their action [3–5], and, as a result of the globalization of the economy, antibiotic resistance is rapidly spreading around the world.

Meanwhile, the search for new antibiotics has slowed down dramatically. Despite the urgent need for antimicrobial agents, very few new compounds are currently being developed, most of which, moreover, belong to the classes of antibiotics already in use [6]. Over the past 15 years, only one new class of antibiotics against Gram-positive bacteria has been introduced into clinical practice, and the last class of broad-spectrum antibiotics was introduced into clinical practice in the 1960s [7]. Thus, humanity is on the verge of a global crisis and can be thrown back into the preantibiotic era.

However, the phenomenon of antibiotic resistance is not new. Bacteria competed with each other long before the emergence of eukaryotes, and there is evidence for the presence of genes encoding resistance to beta-lactam antibiotics, tetracycline, and glycopeptide antibiotics in DNA of bacteria that were in the 30 000-year-old ancient permafrost [8]. How quickly bacteria manage to adapt to new antibiotics is an evidence of the diversity and complexity of their defense mechanisms. Some of them may be due to genetic factors (random mutation transferred in the population), and some due to special conditions (persistence, location in the deep layers of the biofilm), where cells are insensitive to the action of antibiotics. Currently, the main mechanisms of resistance are considered to be

the limitation of drug absorption, modification of its target, inactivation of the drug and its active release, and target switching and target removal [7, 9]. In addition, bacterial defense can be specific (against a particular class of antibiotics or a particular antibiotic) and nonspecific (against a wide range of antibacterial substances), and also include the formation of metabolic shunts, bypasses that do not involve the molecule target of the antibiotic.

2. NONSPECIFIC PROTECTION

Although the concept of nonspecific protection, or immunity, is inherent in complex multicellular organisms, it is also realized in bacteria at the cellular level. In animals, for example, in mammals, the main defense systems are localized on the skin and mucous membranes and represent the secrets of the mucous, sebaceous, or salivary glands as well as gastric juice. The cells of the immune system are able to recognize and destroy pathogens, which creates a powerful barrier between the body and the external environment. The main task of such protection is to prevent the pathogenic organism from entering and, if this failed for some reason, to start the inflammatory response. At the same time, animals also have highly effective specific immunity in their arsenal, which makes them able to resist various infections of a viral, bacterial, and fungal nature [10].

Unlike animals, plants rely on the innate immunity of each cell and systemic signals from infection sites rather than on mobile defense cells and the somatic adaptive immune system. However, even in this case, infection with pathogenic microorganisms is not always successful for bacteria due to structural changes in the cell wall or programmed cell death [11]. The cell wall of plants consists of microfibrils of cellulose and hemicellulose; it is reinforced with lignin and contains a significant amount of proteins that perform structural and enzymatic functions [12]. The heterogeneity of the plant cell wall structure forces pathogens to use different strategies for penetrating through it.

Despite the fact that bacteria are monocellular organisms, they also have elements of nonspecific protection. Bacteria have a special state of pseudomulticellularity, biofilms, when the cells of the population are in a different metabolic, expression, and energy state, forming a complex conglomerate of cells that perform the protective and adaptive function. The state of the biofilm allows the bacterial population to effectively resist antibiotics but, at the same time, makes it extremely vulnerable to viral infections caused by bacteriophages. At the same time, the role of nonspecific protection is played by MDR pumps, which determine the ability of individual cells to resist in the war of antibiotics lasting many millions of years [13, 14]: apparently, since the emergence of the last common ancestor of all living beings (Last Universal Common Ancestor, LUCA) on Earth [15].

3. MAIN COMPONENT OF NONSPECIFIC PROTECTION OF BACTERIA—MULTIDRUG RESISTANCE PUMPS

As mentioned above, MDR pumps play an important role in nonspecific protection. They are highly potent and broadly specific, providing protection to bacteria against a wide variety of xenobiotics. At the same time, it should be taken into account that MDR pumps are present in all living cellular organisms without exception and are the most important element of not only bacterial nonspecific cellular defense.

The contribution of MDR pumps to bacterial resistance has not yet been fully appreciated. This can be illustrated by the example of the mitochondria-targeted antioxidant SkQ1. Until recently, it was believed not to be an antibiotic [16]. When its antibacterial properties were discovered, it was decided that the sensitivity of bacteria to SkQ1 is determined by its ability to permeate a complex cell wall; therefore, Gram-positive *Bacillus subtilis* were sensitive to SkQ1, while Gram-negative *Escherichia coli* were not [17]. Further studies showed [18] that deletion of AcrAB-TolC pump proteins leads to a complete loss of the resistance to SkQ1 [19].

AcrAB-TolC is the main efflux pump for many antibiotics [20], so it can easily seem that the resistance of Gram-negative bacteria is determined not by the difficulty of permeating the substance through two membranes but by the presence of a pump that effectively pumps it out. However, this was also a too simple explanation. It turned out that Gram-negative bacteria *Rhodobacter sphaeroides* and *Photobacterium phosphoreum* sensitive to SkQ1 have protein components of the AcrAB-TolC pump [18, 19], which negates the hypothesis of its presence as a necessary and sufficient condition for the resistance. It turned out that the amino acid sequences of the AcrB protein from *E. coli*, *R. sphaeroides*, and *P. phosphoreum* are 35–60% similar and are formally phylogenetic homologs (orthologs) but they are not functionally homologous and should be considered as paralogs [21, 22]. This is confirmed by the fact that, in the case of *Klebsiella pneumoniae* with the homology of 91.5%, phylogenetic and functional homology is observed, which is typical for most orthologs [19, 21].

It is clear that, in the course of research, the mechanism of SkQ1 resistance was mistaken for limiting its uptake by a complex cell wall, when in fact it was based on the active release of the drug by the MDR pump.

Problems with determining the resistance mechanisms in each case are associated with the insufficient study of antibiotics as well as the pleiotropy of the pump functioning. Many pumps can pump out the same antibiotic, and removing even a few of them has little effect on the total effect. If we look at the sensitivity profile of *E. coli* to antibiotics by their efflux [23], we can see that the "active drug release" mechanism leads to a 50–100-fold increase in the minimum

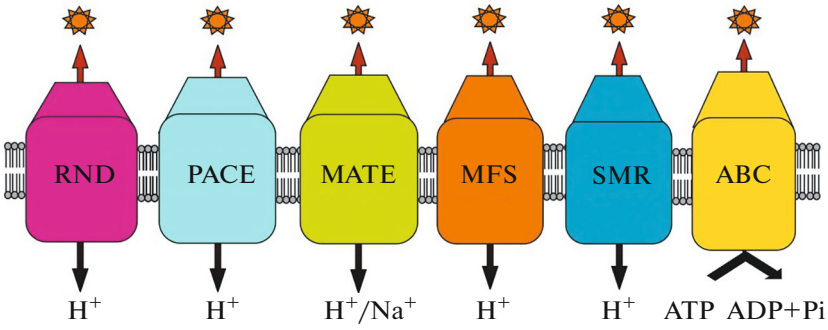

Fig. 1. Schematic representation of the functioning principles for known families of MDR pumps. To date, six families of MDR pumps have been identified. Among them, only the ABC (ATP-binding cassette) family of transporters directly uses ATP energy for transport; the remaining five families are secondary active transporters that use the electrochemical energy of the transmembrane potential: these include the superfamily MFS (major facilitator superfamily), the MATE (multidrug and toxin extrusion) family, the SMR (small multidrug resistance) family, the RND (resistance-nodulation-cell division) superfamily, and the PACE (proteobacterial antimicrobial compound efflux) family.

inhibitory concentration for antibiotics, and we observe the same order of magnitude in the case of SkQ1 [18]. Even when the permeability across the outer membrane appears to be hindered, as in the case of BACE (chlorin e6 13(1)-N-{2-[N-(1-carba-*closo*-dodecarboran-1-yl)methyl]aminoethyl}amide-15(2), 17), a conjugate of chlorin e6 and carborane [24], the effect of the pumps can still be seen [25]. Thus, MDR pumps appear to play a key but underestimated role in antibiotic resistance.

4. STRUCTURE OF MULTIDRUG RESISTANCE PUMPS

To date, six families of MDR pumps have been identified [26]. One of them is the ABC (ATP-binding cassette) family of transporters, which directly use ATP energy for transport. The remaining five families are secondary active transporters using the electrochemical energy of the transmembrane potential; they include MFS (major facilitator superfamily), MATE (multidrug and toxin extrusion), SMR (small multidrug resistance), RND (resistance-nodulation-cell division), and PACE (proteobacterial antimicrobial compound efflux). Figure 1 shows the scheme of the functioning of the main families of bacterial transporters.

Apparently, two different mechanisms of the transporter functioning are designed to maintain cell viability in different physiological states. In the absence of the potential on the membrane (for example, in the state of persistence), only ABC transporters will function, and all transporters will work in the presence of the transmembrane potential. In prokaryotes, voltage-dependent pumps predominate, which is explained by the high potential on the membrane (~140–220 mV for *E. coli* [27]) and the absence of the need to convert the potential into ATP, which provides an advantage in terms of the speed of operation.

One of the most studied MDR pumps is the AcrAB-TolC (or AcrABZ-TolC) pump of *E. coli* bacteria [28]. This pump consists of three main proteins: the outer membrane channel TolC, the AcrB transporter, and the AcrA adapter protein connecting them [29] as well as one small membrane AcrZ (YbhT) protein consisting of 49 amino acid residues and, apparently, modulating the work of the AcrB protein in the AcrAB-TolC pump [30–32]. Interestingly, the TolC channel plays the same role for another seven MDR pumps of the families RND, ABC, and MFS [15], while the AcrA adapter protein plays the same role for another AcrAD-TolC pump [33]. The AcrAB-TolC pump has rather high substrate specificity and pumps out substrates from the inner membrane of the bacterium and the periplasmatic space [23, 29, 34]. The AcrB transporter itself is located on the inner membrane and pumps substances due to the transmembrane potential; however, the mechanism of the AcrAB-TolC pump has not been fully established, although there are several works in the literature suggesting separate stages of this process [35–40].

5. EVOLUTION OF MULTIDRUG RESISTANCE PUMPS AND HORIZONTAL GENE TRANSFER

The evolutionary conservatism of pumps is evidenced by the ubiquitous distribution of pumps of the MFS and RND families both among prokaryotes (including bacteria and archaea) and among eukaryotes [41, 42], so the conserved structure of pumps within the same species is not surprising [43]. This is also supported by the fact that pump structures were chosen by bacteriophages as targets for their binding [44, 45]. It seems that the ability to efflux drugs appeared only a few times in the course of evolution and was stably preserved, but the modulation of the substrate specificity of these systems occurred repeatedly [46].

Although some pump families are widely distributed, there are problems that make horizontal transfer of pump genes difficult, even between closely related taxa. Differences in the protein structure of the outer membrane of bacteria impose certain limitations, but this phenomenon can make a very important contribution to the spread of antibiotic resistance. Despite this fact, various components of pumps or even pumps themselves can be transferred as a result of horizontal gene transfer. Thus, genes of the OqxAB pump are usually localized on the chromosome and/or plasmids surrounded by IS26-like mobile genetic elements in clinical isolates of Enterobacteriaceae and confer resistance to quinoxalines, quinolones, tigecycline, nitrofurantoin, several detergents, and disinfectants [47, 48]. It was shown [49] that the OqxAB pump confers antimicrobial resistance or reduces the susceptibility of transformed bacteria (plasmid transfer from *E. coli* was carried out to *Salmonella typhimurium*, *K. pneumoniae*, *Kluyvera sp.*, and *Enterobacter aerogenes*) to various substrates. OqxAB is one of the few pumps encoded on conjugative plasmids. Another similar example is the MexCD pump [50]. Thus, MDR pumps and their components can be transferred horizontally between close groups of bacteria, but their ability to transfer pump function can be severely limited and require the presence of suitable protein components, such as, for example, the TolC protein for the OqxAB pump [51].

6. ROLE OF MULTIDRUG RESISTANCE PUMPS IN ANTIBIOTIC RESISTANCE

MDR pumps are a universal tool that protects the bacterial cell itself and its microenvironment from the negative effects of xenobiotics, including a wide variety of antibiotics [15]. Moreover, bacteria use various approaches to increase its effectiveness. Resistant phenotypes can arise as a result of an increase in the pump activity due to their overexpression, as is observed when antibiotics are added at sublethal concentrations, when an increase in expression of MDR pump genes that pump out these antibiotics is induced due to a cascade of interactions [52].

Another interesting aspect is the asymmetric arrangement of MDR pumps during cell division. In the process of fission, previously synthesized pumps are mainly located at the old poles, and new poles are newly created, and MDR pumps are synthesized de novo [53]. This creates a variable resistance profile during the cell cycle, which allows the population to retain bacteria with different expression status of MDR pumps. When faced with an antibiotic, the least-resistant cells die and use two strategies: adsorption of antimicrobial drugs on the surface of dead cells, which protects the remaining bacteria [54, 55], and release of a "necrosignal" by dead cells, causing activation of protective pathways in surviving bacteria [56]. Surprisingly, the AcrA adapter protein, a component of the AcrAB-TolC pump, acts as a "necrosignal," as a result of which the bacterial population acquires increased resistance to antibiotics. In addition, under the influence of antibiotics, the mutation rate of certain genes can increase [57, 58]. Changes in the number of gene copies occur quite frequently in genomes [59], and duplication of MDR pump genes leads to an increase in the chances of bacterial survival when antibiotics are added. Thus, gene duplication leads to an increase in the resistance and may be an alternative to changing the expression level.

Summarizing the above, we can conclude that bacteria take full advantage provided by MDR pumps to increase resistance, and this protects them even at high concentrations of antibiotics.

6. ROLE OF MULTIDRUG RESISTANCE PUMPS IN THE FORMATION OF BIOFILMS AND PERSISTER CELLS

The bacterial population usually exists as two subpopulations: planktonic and attached. Between them, there is an equilibrium maintained by various factors, such as quorum sensing signals [60] or electrical signals [61]. The stability of bacteria in biofilms can exceed the stability of planktonic forms by two orders of magnitude [62]. Biofilms can be formed by bacteria of different species, which has its own advantages both in terms of metabolism and protection [63, 64].

At the same time, the role of MDR pumps in the formation of biofilms is quite significant. These are the transport of extracellular matrix components outside the cell to form protection, the export of quorum sensing signals, the prevention or promotion of adhesion to other cells and substrates, and protection from toxins, antibiotics, and metabolites resulting from combined presence in a limited biofilm volume [15].

Other mechanisms of defense against antibiotics include absorption of antibiotics by bacterial cells at the biofilm boundary, whose death is both a defense [54, 55] and a source of nutrients for the population inside the biofilm due to the so-called necrotrophic growth [65]. Thus, MDR pumps not only play an important role by pumping antibiotics out of cells but also create a certain microenvironment that affects the entire population around [66].

The conditions created in biofilms protect bacteria from environmental influences but, at the same time, this limits access to nutrients, due to which the stationary phase of growth occurs faster. The proportion of metabolically inactive persister cells in the population increases and the cells begin to slow down their metabolism, so the lack of nutrients in the deep layers of the biofilm contributes to the formation of persistence [67, 68].

Since one of the mechanisms of persistence is membrane depolarization under the action of the TisB toxin [69–71], it can be assumed that it is in this case

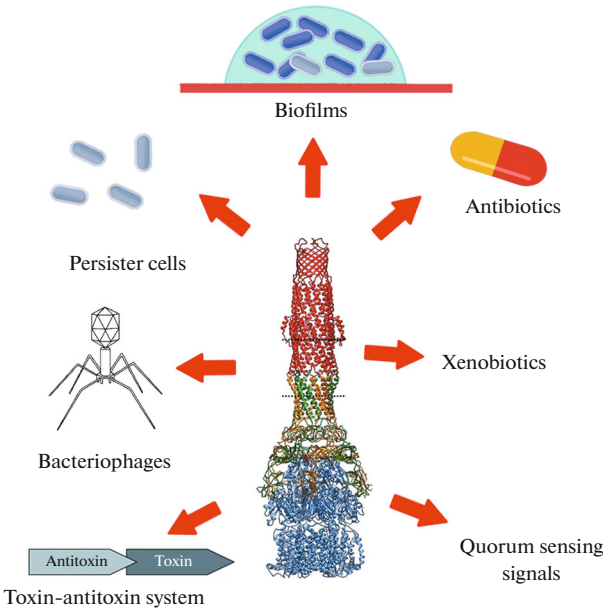

Fig. 2. Role of MDR pumps in bacterial cell processes. Pumps can perform both protective function and active attack function, maintain cell homeostasis, and even be receptors that mediate the penetration of bacteriophages into the cell.

that a state arises when the cell is deprived of the potential on the membrane and, having received energy due to membrane-independent energy processes (for example, glycolysis), can use ATP-dependent pumps to pump out harmful substances when the rest of the pumps are disabled due to a lack of the potential on the membrane.

Thus, MDR pumps play an important role not only in protecting cells from antibiotics but also in the formation of complex structures, such as biofilms, and in the implementation of a special cellular state of persistence that allows bacteria to increase resistance to antibiotics by several orders of magnitude and even avoid their exposure without having genetic determinants of specific protection.

7. CONCLUSIONS

It can be assumed that MDR pumps are the keystone of the resistance and are essential for many cellular processes in bacteria. Pumps play a key role in the processes of defense and attack due to their participation in the export of toxins, are involved in the formation of biofilms, and make an important contribution to bacterial persistence and maintenance of cellular homeostasis, and even are receptors that determine the penetration of bacteriophages into the cell (Fig. 2). Even in the absence of specialized systems for protecting against antibiotics, the very presence of these complexly regulated systems provides a high level of protection, allowing bacteria to survive at antibiotic concentrations several orders of magnitude higher than potentially lethal ones. This makes it possible to conclude that the MDR pump system of bacteria is a nonspecific protection against xenobiotics, which determines the basic, primary, resistance, "immunity" of bacteria to toxins, antibiotics, and other substances that negatively affect the bacterial cell. Other resistance mechanisms (drug target modification, drug inactivation, target switching, and target sequestration) are secondary, specific mechanisms that, when combined with the primary system, determine the existence of the phenomenon of "super-resistant" bacteria. A long-term strategy for combating such bacteria cannot be achieved solely by circumventing specific defenses without giving due attention to overcoming nonspecific defenses. The study and understanding of these processes will allow us to either seriously delay the onset of the postantibiotic era or even prevent its onset in the future.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

Statement on the Welfare of Animals. This article does not contain any studies involving animals performed by any of the authors.

Statement of Compliance with Standards of Research Involving Humans as Subjects. This article does not contain any studies involving humans as subjects.

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