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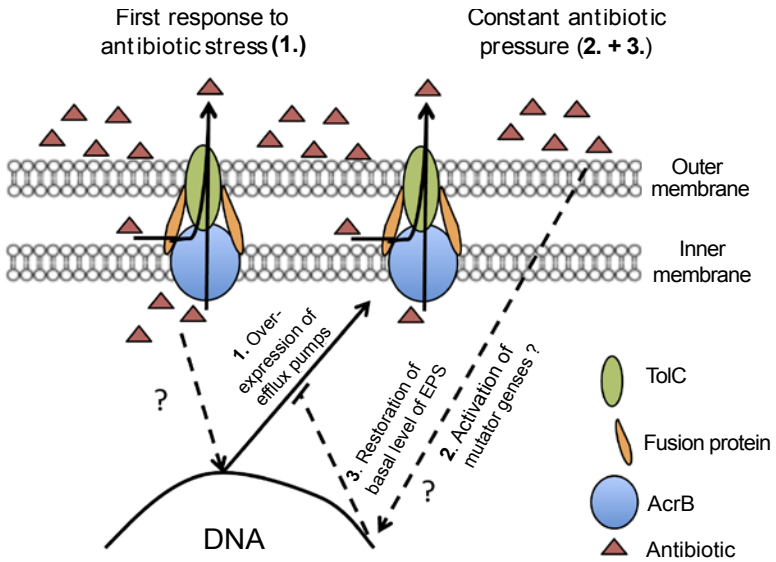
Efflux Pumps that Bestow Multi-Drug Resistance of Pathogenic Gram-negative Bacteria

Amaral L[1,2*], Spengler G[2], Martins A[2,3] and Molnar J[2]

[1]Travel Medicine of the Centre for Malaria and Other Tropical Diseases (CMDT), Institute of Hygiene and Tropical Medicine, Lisbon, Portugal
[2]Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary
[3]Unit of Parasitology and Medical Microbiology (UPMM), Institute of Hygiene and Tropical Medicine, Lisbon, Portugal

Abstract



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Efflux pumps are integral plasma membrane protein systems that recognize and bind noxious compounds present in the cytoplasm (toxic products produced by metabolism; compounds that have penetrated the cell), or periplasm of the bacterial cell and extrude it into the environment in which the bacterium resides [1]. The efflux pump machinery gives the cell additional protection to the one provided by the constituents of its cell wall (example: lipopolysaccharides), and provides an initial protection to noxious agents present in its natural environment that have penetrated into the cell (example: bile salts in the colon) [1]. The efflux pump machinery is divided into five superfamily classes; the major facilitator (MF), the ATP-binding cassette (ABC), the resistance-nodulation-division (RND), the small multi-drug resistance (SMR) and the multi-drug and toxic compound extrusion (MATE). With respect to Gram-negative bacteria, although they all play important roles in the protection of the bacterium from noxious agents present in the environment, the main efflux pump of the Gram negative bacterium is a member of the RND superfamily, and because multi-drug resistance of clinical isolates have been associated with the over-expression of this pump, it has received a great deal of attention [2].

The first *in vitro* response of bacteria to a given noxious agent, such as an antibiotic, is to over-express its main efflux pump [2]. If the bacterium is serially exposed *in vitro* to increasing concentrations of that compound, it responds by increasing the effective number of its main efflux pump, as well as others that provide redundant protection [2]. However, if that "adapted" bacterium is now maintained at a constant level of a noxious agent, the level of efflux pump activity increases up to a maximum, followed by a gradual return of efflux pump activity to its basal level. Concomitant to this process, an accumulation of mutations of essential proteins located in the plasma membrane (example penicillin binding proteins), mutations 30 S component of the ribosome and gyrase take place [3]. These events suggest that when the organism is faced with an environment that contains a constant toxic level of a compound, and the cost for maintaining an energy consuming system, such as that needed for the energy dependent efflux pump, is too great a price to pay. Therefore, in order to survive in this unchanging environment, other mechanisms are activated. For example, activation of a mutator master gene is thought to be an important step at this level, which results in the mutation of genes that code for essential proteins, reversing the over-expression of efflux-pumps, but still conferring the bacterial resistant to the environmental pressure *via* other mechanism(s), yet to be understood [4,5]. This sequence of

***Corresponding author:** Amaral L, Travel Medicine of the Centre for Malaria and Other Tropical Diseases (CMDT), Institute of Hygiene and Tropical Medicine, Lisbon, Portugal, E-mail: LAmaral@ihmt.unl.pt

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events can be an explanation for what takes place when a patient is treated with an ineffective constant dose of antibiotic(s). During such therapy, the level of resistance increases many fold higher than that of the initial infecting strain. Hence, clinical isolates from treated patients often show much higher levels of antibiotic resistance than that of their wild type counterpart (sometimes it can even present a 1000 fold increase) [6]. Moreover, at this stage, resistance is usually related with the presence of mutations, which reduces the survival of the resistant bacteria, once it is transferred to a noxious agent-free environment that contains the competing wild type counterpart [3,4]. However, depending upon when during therapy a clinical strain is isolated, its resistance to two or more antibiotic classes (multi-drug resistance (MDR)), may be due entirely to over-expressed efflux pumps; to a mixture of over-expressed efflux pumps and increasing accumulation of mutations; and only to mutations [3,4]. The degree of resistance due to any of the aforementioned possibilities can readily be determined with methods that employ compounds known for their modulation of efflux pump activity, such as phenothiazines [7] or phenyl-arginine-beta-naphthylamide (PAβN), the latter which competes with the antibiotic as substrate of the efflux pump [8]. If in presence of such compounds, the MDR bacterium is rendered fully susceptible to the antibiotic(s) to which it was initially resistant, resistance is most likely due to its over-expressed efflux pump systems. Contributions made by accumulated mutations render the organism less and less affected by the EPI. This type of information is of great value to clinicians faced with long-term therapy of a bacterial infection that progresses to an MDR phenotype. It should be understood that although the Gram-negative bacterium has essentially one main efflux pump, such as the AcrAB (*Escherichia coli*) or the MexAB (*Pseudomonas aeruginosa*), the deletion of the main efflux pump results in the over-expression of one or more other RND efflux pumps, such as is the case for deletion of the AcrAB, followed by the over-expression of the AcrEF pump [2]. Redundancy of as many as nine RND efflux pumps [2], provides additional protection to the organism.

Understanding the physical chemistry, physiology and regulation of efflux pumps of Gram-negative bacteria is, therefore, important for the development of drugs that may be used as adjuvants, in combination with antibiotics for therapy of an efflux pump dependent MDR Gram-negative infection. The main efflux pumps of Gram-negatives are members of the resistance-nodulation-division (RND) superfamily. The pumps belonging to the RND family form a tripartite complex together with the periplasmic proteins belonging to the membrane-fusion-protein (MFP) family and the outer membrane channels. RND transporters consist of a transporter protein that recognises and binds the noxious agent in the cytoplasm or periplasm and transports it to the contiguous channel (TolC), ending at the surface of the outer membrane. The transporter is attached to the plasma membrane by two or three fusion proteins, which are believed to assist the extrusion of the substrate by peristaltic actions [9]. Although the actual structure of RND efflux pumps in the cell envelop is not completely understood, the structure of the transporter, TolC and fusion proteins are well established for major Gram-negative bacteria [10].

RND efflux pumps obtain their energy for activity from the proton motive force (PMF). The PMF results from metabolic activity of the cell. However, whereas at pH above 6.5, the efflux pump is totally dependent upon metabolic activity controlled by the ATP synthase, at lower pH efflux activity is independent of metabolism [11]. Because the dissociation constant of the bound substrate from the transporter is pH dependent (at pH of 7 dissociation is very slow compromising the activity of the pump), we have from many of our studies concluded that the PMF energy dependent efflux pump most likely needs the passage of hydronium ions through its internal cavity, for the release of the substrate that is in turn ejected into the TolC channel *via* the peristaltic action of the fusion proteins [11]. Because at low pH, the concentration of hydronium ions at the surface of the cell results in a pH difference of 2 or 3 pH units, as opposed to that of the milieu, the surface concentration of hydronium ions provides the force for the mobility of hydronium ions through porins leading to the acidification of the periplasm, and thereby, providing the low pH needed by the transporter for the release of the substrate. At high pH, these hydronium ions come from hydrolysis of ATP by ATP synthase, and are therefore, passed into the transporter, thereby reducing its internal pH, so that the release of the substrates can take place [11,12]. EPIs, such as the phenothiazines chlorpromazine or thioridazine, exert their inhibition at pH above 6, and are believed to affect hydrolysis of ATP, hence, denying the efflux pump transporter hydronium ions needed for release of the bound substrate [11,12]. The search for EPIs that are clinically useful continues, although with respect to thioridazine, this old neuroleptic has been shown to inhibit efflux pumps of pathogenic mycobacteria [13], and has been successfully used to treat extensively drug resistant tuberculosis infections [14].

Moreover, the regulation of the main efflux pump of *Escherichia coli* may take place *via* distinct pathways. The induced synthesis of the transporter component of the AcrAB efflux pump, when the organism is exposed *in vitro* to a noxious agent, involves the activation of the stress gene *soxS,* followed by the activation of the local regulator *marA*, and then by the activation of the transporter gene *acrB* [8]. On other hand, in case of *Salmonella spp.* two component resistance mechanisms, such as the PmrA/PmrB system, directly activate the master efflux pump regulator *ram A* g ene [15]. The activation of the PmrA/PmrB system takes place readily when *Salmonella spp.* is phagocytosed due to the acidic nature of the phagolysosome [15], as follows: PmrB is a sensor that self-phosphorylates, and then transfers the phosphate to PmrA. PmrA activates a nine gene operon, which eventually codes for Lipid A that is introduced into the nascent lipopolysaccharide layer of the outer membrane. The increased presence of Lipid A renders the phagocytosed bacterium practically immune to everything, including the hydrolases of the phagolysososome [15]. It is for this reason that when *Salmonella spp.* that invades the peritoneum from a resected colon produces high mortality, even though the neutrophil has in essence done its job.

The role of efflux pumps in the therapy of MDR bacterial infections is still evolving, and the need for adjuvants that are safe and effective for rendering the MDR bacterium susceptible to antibiotics is obvious. Moreover, because the EPI may render MDR bacteria susceptible to old antibiotics that have fallen out of use, their economic potential benefit is huge. Although some EPIs are in clinical trials, none have yet to reach the marketplace, mainly due to their common toxicity against healthy mammalian cells, affecting intrinsic mammalian efflux pumps, as for example those of the blood brain barrier. Lastly, it should be noted that compounds that inhibit the efflux pump of bacteria also have the capacity to promote the removal of plasmids that carry antibiotic resistant genes [16,17]. This alone has huge implications for the animal husbandry industry, and is worth considering.

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