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Review

Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*

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ABSTRACT

Multidrug efflux is an obstacle to the successful treatment of infectious diseases, and it is mediated by multidrug efflux pumps that recognize and export a broad spectrum of chemically dissimilar toxic compounds. Many bacterial genome sequences have been determined, allowing us to identify drug efflux genes encoded in the bacterial genome. Here, we present an approach to identifying drug efflux genes and their regulatory networks in *Escherichia coli* and *Salmonella*. Multidrug efflux pumps are often regulated by environmental signals and they are required for bacterial virulence in addition to multidrug resistance. It is now understood that these efflux pumps also have physiological roles. In this article, we investigate the physiological roles of drug efflux pumps in virulence. Because multidrug efflux pumps have roles in bacterial drug resistance and virulence, we propose that drug efflux pumps have greater clinical relevance than previously considered.

1. Introduction

Multidrug resistance plays a crucial role in the failure of cancer chemotherapy and the treatment of infectious diseases. Antibiotics have been highly effective in the treatment of disease; however, excessive use for many years have selected for drug-resistant strains. Now, drug-resistant microorganisms are a major worldwide health issue. A number of important human pathogens have acquired mechanisms that make them largely resistant to currently available treatments.

Bacteria have developed various ways to resist the toxic effects of antibiotics and other drugs [1,2]. One of these mechanisms involves the production of enzymes that inactivate antibiotics by hydrolysis or lead to the formation of inactive derivatives [3]. Well-known examples are β-lactamases [4,5] and enzymes that phosphorylate, adenylate, or acetylate the aminoglycosides class of antibiotics [6]. A second mechanism of resistance is target alteration. Cellular targets can be altered by mutation or enzymatic modification in such a way that the affinity of the antibiotic for the target is reduced [7–10]. For example, virtually all clinically important fluoroquinolone resistance can be attributed to mutations within the drug's targets, DNA gyrase and topoisomerase IV [11]. These mechanisms are all specific for a single drug or a single class of drugs. However, there are more general mechanisms of resistance in which access of the unaltered agent to the target is prevented by the barrier and active transport functions of biological membranes. The barrier cannot prevent the drugs from exerting their toxic action once they have entered the cell, and the active efflux of drugs is essential to ensure significant levels of drug resistance [12,13].

Multidrug efflux pumps are integral membrane proteins that utilize cellular energy to extrude antibiotics or biocides actively out of

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the cell [14–18]. Most bacteria possess several genes encoding such proteins. Multidrug efflux pumps are found among all the major categories of bacterial membrane transporters that have been characterized by sequence homology [19–21] and include the ATP binding cassette (ABC) family, multidrug and toxic compound exporters (MATE), the small multidrug resistance (SMR) family (a member of the much larger drug/metabolite transporter superfamily), resistance-nodulation-division proteins (RND), and the major facilitator superfamily (MFS).

Recently, many bacterial genome sequences have been determined, making it possible to identify the genetic sources of bacterial multidrug resistance. It was hypothesized that there are numerous efflux pump genes in bacterial genomes [22,23]. Moreover, recent studies have shown that not only does the multidrug efflux pump confer antimicrobial resistance on bacteria but it also contributes to virulence [24,25]. These studies underline the importance of understanding the physiological function of drug efflux pumps in bacterial cells. In this review article, we introduce the post-genomic approach to analyze putative drug efflux genes and their regulation responding to environmental signals in *Escherichia coli* and *Salmonella*. We also introduce the physiological roles of drug efflux pumps in virulence, which is an ongoing research area.

2. Drug efflux pumps in the bacterial genome

Genomic analysis has revealed a number of genes encoding putative drug efflux pumps on the chromosomes of most bacteria [22,23]. The entire genome sequence of *Escherichia coli* was determined in 1997 [26]. Among nearly 4300 open reading frames (ORFs) identified on the 4.6 M bp chromosomes of *E. coli*, 354 ORFs (approx. 77 transporters per Mb of genome) are predicted to be transporter genes [27]. Of these genes, 37 are putative drug efflux genes. Among these efflux pump genes, several are reported to actually contribute to drug resistance. The conventional method of screening new antibiotics is based on testing antibacterial activity against existing clinical bacterial strains; thus, unpredicted drug resistant bacteria always emerge after these antibiotics are used in the clinical field. If latent drug resistant genes could be screened ahead of time, we might be able to predict the evolution of future drug-resistant bacteria during new drug development. Based on this idea, we used *E. coli* as a model to clone all the putative drug efflux genes to investigate whether they contribute to drug resistance [22,28].

3. Post-genomic approach to identifying multidrug efflux pumps in *E. coli*

In the *E. coli* genome, 37 ORFs are predicted to be drug efflux genes [28]. We cloned these genes into the expression vector using PCR after which the constructed plasmids were transformed into the *acrB* mutant to examine whether they contribute to drug resistance. The AcrB efflux pump is an RND pump, which functions with AcrA and TolC, and is constitutively expressed in *E. coli* [29–31]. The *acrB* mutant strain is susceptible to many antibiotics and toxic compounds. This mutant was used as a parental strain for overproducing each of the putative drug efflux pumps, and minimum inhibitory concentrations (MICs) of approximately 30 kinds of chemical compounds including antibiotics, dyes, detergents, and other toxic compounds were measured. The drug efflux pumps that contribute to resistance are listed in Table 1. Among the 37 ORFs, 20 efflux genes were found to contribute to drug resistance to *E. coli*. This result indicates that a genomic information may be useful to detect drug efflux genes [22,28]. Among five families, drug efflux pumps belonging to the RND family were shown to particularly contribute to higher resistance to a wide variety of compounds (Table 1). All of the five RND efflux pumps contributed to multidrug resistance in *E. coli*. MdfA, Bcr, EmrAB of MFS, EmrE of SMR, and MdtK of MATE also conferred multidrug resistance. Moreover, we discovered for the first time the ABC type drug efflux pump, MacAB, in gram-negative bacteria. MacAB contributes to a macrolide-specific resistance [32,33]. Ten drug efflux pumps gave deoxycholate resistance to *E. coli* cells. This chemical compound is a component of bile acid, present in large amounts in the growth environment of *E. coli*, suggesting that the drug efflux pumps also play an important role in biological defense against the environment. Indeed, Thanassi et al. reported that the AcrAB system especially appeared to play a significant role in bile acid efflux, however, another efflux system(s) also plays an important role, since the accumulation level of bile increased strongly upon deenergization of *acrA emrB* double mutant cells [34]. The drug efflux pumps identified were also found to recognize novel antibiotics still under development by pharmaceutical companies, including tigecycline, a novel glycylcycline antibiotic. AcrAB and AcrEF confer tigecycline to *E. coli* cells [35]. This proved that it is possible to use genomic information in order to predict resistant strains and screen effective antibiotics at the stage of new drug development.

Most drug efflux pumps are weakly expressed under normal conditions, except for the AcrAB-TolC drug efflux system. Indeed, the gene deletions of most drug efflux pumps did not affect the drug susceptibilities of the wild-type *E. coli* strain [36]. Thus, it is important to apply the method of over-expression to identify potential drug efflux pump genes hidden in the bacterial genome. It is possible that some bacteria acquire high levels of drug resistance when drug efflux pumps are induced. Therefore, it is important to identify the regulatory networks of drug efflux pumps. Next, we discuss the regulation of drug efflux pumps through the signal transduction system.

4. Regulatory network of drug efflux pumps in *E. coli*

Bacteria can adapt to a wide range of environmental conditions. These adaptive responses are generally mediated by two-component signal transduction systems, which consist of a sensor histidine kinase and its cognate response regulator. Each sensor detects a specific environmental signal, and the histidine residue self-phosphorylates. This phosphate group is then transferred to the specific aspartic acid in the response regulator. The regulator mostly acts as a transcriptional factor to control the expression of genes that have various activities in biological reactions [37,38]. The entire genomic sequence of *E. coli* allowed us to systematically compile a complete list of genes encoding such two-component signal transduction proteins. It is thought that *E. coli* has a total of 30 sensors and 34 response regulators [39].

If we consider the role of drug efflux pumps in bacterial self-defense, it would be reasonable to assume that their expression might be regulated by a two-component signal transduction system. We investigated the relationship between drug efflux pumps and a two-component signal transduction system and found that 17 response regulators are involved in drug resistance of *E. coli* (Table 2). Among them, three two-component signal transduction systems, EvgSA [40,41], BaeSR [42–44], and CpxAR [45], are capable of inducing efflux pump expression, resulting in increased multidrug resistance. The BaeSR signal transduction system is activated by indole, a bacterial metabolite, which induces the expression of the two RND pumps, MdtABC and AcrD, conferring multidrug resistance [44,46] (Table 3). The CpxAR system is a two-component signal transduction system that responds to membrane stress [47], and also controls MdtABC and AcrD [45]. The EvgSA system induces the expression of the MdtEF and EmrKY pump, increasing the level of multidrug resistance [40,41].

In addition to the EvgSA two-component signal transduction system, several regulators control the MdtEF multidrug efflux pump (Table 3). For example, the expression of *mdtEF* is induced by *N*-acetyl-D-glucosamine (GlcNAc) and this induction is mediated by the NagE phosphotransferase (PTS) system for GlcNAc. Other PTS sugars, glucose and D-glucosamine, also induced *mdtEF* gene expression. These results suggest that *mdtEF* expression is stimulated through catabolite control