Molecular mechanisms of bacterial resistance to antimicrobial agents

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Infectious diseases caused by bacterial pathogens represent a serious public health concern. Antimicrobial agents such as anti-bacterial drugs are often indicated for chemotherapy of bacterial infections in clinical medicine. Thus, it is important to study the biological mechanisms that confer bacterial pathogenesis and virulence. Under selective evolutionary pressure when in the presence of antimicrobial agents, bacterial variants evolve mechanisms to survive in the presence of these inhibitory agents. Drug resistant bacteria that are selected with a single drug are also frequently multi-drug resistant against multiple structurally different drugs, thus confounding the chemotherapeutic efficacy of infectious disease caused by such pathogenic variants. There are several major classes of mechanisms for bacterial resistance to antimicrobial agents: (a) enzymatic inactivation of the drug results from the metabolic degradation of the drug into a form that is rendered ineffective in inhibiting bacterial growth; (b) alteration of the drug target results in the inability of the drug to bind to its biological target, thus rendering the drug unable to kill the bacteria. Bacterial cellular drug targets may include the protein synthesis apparatus, nucleic acid synthesis enzymes, cell wall synthesis machinery, and metabolite pathway enzymes; (c) drug permeability reduction mechanisms prevent cellular entry of drug into the inside of the bacterial cell; and (d) active efflux of drugs from bacteria results in the intracellular dilution of drugs, making the extruded drugs unavailable for their inhibitory action. Unfortunately, these drug and multi-drug resistance mechanisms are poorly understood at the molecular level, impeding our advances towards identifying new targets for possible inhibition of clinical multi-drug resistances; this prevents chemotherapeutic usefulness. Understanding how these bacterial resistance mechanisms work from the standpoint of molecular physiology and biochemistry will identify new targets for potential inhibition of multi-drug resistance and thus restore clinical utility of chemotherapy of infectious disease caused by serious bacterial pathogens.

Keywords antimicrobial agent; drug; antibacterial drug; bacteria; antibiotic resistance; efflux; multidrug efflux

1. Introduction

Bacteria that are causative agents of infectious disease represent a serious public health concern globally. Antimicrobial agents are indicated for the treatment of bacterial infections. Bacteria may be intrinsically resistant to antibacterial agents or acquire resistance by mutation or acquisition of resistance determinants. Use of antimicrobial agents selects for bacterial variants within a population that are less susceptible, or resistant, to the antimicrobial agent used, leading to a situation where the resistant variant predominates under such selective pressure [1]. Furthermore, selection of resistance to a single antimicrobial agent often results in bacterial variants that harbor transferable multidrug resistance determinants [2]. These selective pressure phenomena are thought to occur in areas where antimicrobial agents are extensively used, such as in human clinical medicine [3, 4], agriculture [5-7], and in natural soil and aquatic environments [8-12]. Therefore, antimicrobial use fosters bacterial drug resistance and dissemination of drug resistance determinants within populations.

Multidrug resistant bacteria may be recalcitrant to clinically relevant chemotherapeutic agents, resulting in treatment failures of infectious diseases [13]. Study of these antimicrobial resistance mechanisms in infectious disease causing microorganisms is, therefore, necessary in order to find ways to circumvent conditions that foster such recalcitrant pathogens. Molecular, biochemical, physiological and structural analyses of bacterial multiple drug resistance mechanisms will foster their putative modulation and make possible the restoration of the efficacy of infectious disease chemotherapy [14-18].
Table 1 The bacterial mechanisms of antibiotic resistance are diverse.

<table>
<thead>
<tr>
<th>Basis of resistance</th>
<th>Mechanism</th>
<th>Bacterial proteins/targets responsible</th>
<th>Antibiotic targets</th>
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<tr>
<td>Enzyme</td>
<td>Hydrolysis</td>
<td>β-lactamases</td>
<td>β-lactams</td>
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<td>Esterase</td>
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<td>C-P lyase complex</td>
<td>Fosfomycin</td>
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<td></td>
<td>Group transfer</td>
<td>Acetyltransferase</td>
<td>Streptogramins, aminoglycosides, chloramphenicol</td>
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<td>Phosphotransferase</td>
<td>Aminoglycosides, macrolides</td>
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<td>Nucleotidyltransferase</td>
<td>Lincomycin, clindamycin, aminoglycosides</td>
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<td>Glycosyltransferase</td>
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<td>Ribosyltransferase</td>
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<td>Thiol transferase</td>
<td>Fosfomycin</td>
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<td>Redox process</td>
<td>TetX</td>
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<td>All major antibiotics</td>
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<tr>
<th>Target modification</th>
<th>Reduced permeability</th>
<th>Reduced protein expression/defective</th>
<th>Porins</th>
<th>Antibiotic targets</th>
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<tbody>
<tr>
<td>Structural alterations/modifications</td>
<td>Penicillin binding proteins</td>
<td>Cell wall precursors</td>
<td>β-lactam antibiotics</td>
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<td>Ribosomal subunits</td>
<td>RNA polymerase</td>
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<td>Amino acid substitutions</td>
<td>DNA gyrase/topoisomerase</td>
<td>16S rRNA</td>
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<td>Methylations</td>
<td>23S rRNA</td>
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<td>Mutations</td>
<td>23S rRNA</td>
<td>23S rRNA</td>
<td>Oxazolidinones</td>
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| Efflux              | Active extrusion     | Membrane proteins                    | All major antibiotics            |

1.2. Bacterial drug and multidrug resistance mechanisms

Bacteria have developed diverse means to circumvent the growth-inhibitory properties of antimicrobial agents (see Table 1). Major mechanisms of bacterial resistance to antimicrobial agents include the following: (a) enzymatic drug inactivation; (b) drug target modification; (c) drug permeability reduction; and (d) active efflux of drugs. These drug resistance mechanisms allow bacteria that harbor these mechanisms to survive, or even to actively grow, in the presence of a given antimicrobial agent. Furthermore, certain bacterial variants have evolved mechanisms to resist multiple drugs, making such variants recalcitrant to chemotherapy against such bacterial strains that are the causative agents of infection in patients.

2. Drug inactivation mechanisms

Bacteria have evolved several mechanisms of rendering antimicrobials inactive such as the enzymatic hydrolysis of antibiotics, group transfer and the redox process [19]. The classical example of this mechanism is the production of β-lactamases that hydrolyze the β-lactam ring of penicillins. The discovery of β-lactamase precedes the discovery of the first antibiotic penicillin itself, and the enzyme is thought to have some important role in cell wall peptidoglycan assembly. The genes encoding β-lactamases (bla) are either on the chromosome (e.g. AmpC β-lactamase) or on the plasmids, the TEM-1 β-lactamase being the first one to be discovered on the plasmid in a strain of *Escherichia coli* [20], followed by a second plasmid-mediated β-lactamase SHV-1 (sulphydril variable active site) [21]. TEM and SHV enzymes, in due course of time, evolved to hydrolyze a broad range of extended spectrum cephalosporins, and these are collectively called extended spectrum β-lactamases, or ESBLs [22]. TEM and SHV were the major ESBLs until the discovery third unique cefotaxime degrading enzyme CTX-M type in 1990 in *E. coli*, and now there are more than 40 types of CTX-M ESBLs [23, 24]. ESBLs hydrolyze a wide range of cephalosporins including the oxyimino group of cephalosporins such as ceftriaxone, cefazidime, cefotaxime and the monobactam drugs such as aztreonam, but do not hydrolyze cephamycins and carbapenems [25]. In addition, ESBLs derived from OXA-type β-lactamase confer resistance to clavulanic and oxacillin antibiotics and are referred to as OXA-type ESBLs. Unlike TEM and SHV ESBLs, OXA types are not inhibited by clavulanes or tazobactam. Currently, more than 200 ESBL types have been discovered worldwide, and these have evolved from TEM, SHV and CTX-M types by point mutations [26]. Though Enterobacteriaceae such as *E. coli* and *Klebsiella pneumoniae* are the predominant producers of ESBLs, many Gram-negative bacteria are now known to produce them. Pathogenic bacteria that are causative agents of urinary tract infections and other infectious diseases are capable of producing multiple ESBLs and, thus, offer a great therapeutic challenge leaving the physicians with very few treatment options. Like β-lactamases, bacteria produce antibiotic hydrolyzing enzymes such as the macrolide esterases and fosfofycin epoxidases. Macrolide esterases produced by many members of Enterobacteriaceae inactivate erythromycin A and oleandomycin by hydrolyzing the lactone ring.
The second mechanism of antibiotic inactivation involves enzyme-mediated structural alteration of the drug via transfer of a functional group such as an acyl, ribosyl, phosphoryl or thiol group [29]. The reaction is irreversible and the modified antibiotic is unable to bind to the target due to the resultant change in the structure. The antibiotics susceptible to this bacterial mechanism include aminoglycosides, fosfomycin, macrolides, lincomycin and chloramphenicol [19]. For instance, bacteria have evolved acetyltransferases which inactivate chloramphenicol [30], tetracycline-metabolizing enzymes that are largely uncharacterized [31, 32], and beta-lactamases that inactivate beta-lactams such as penicillin [33]. Highly active variants of these enzyme inactivation mechanisms for drugs are ubiquitous in the environment and have yet to be found within clinically-relevant bacterial pathogens [29, 34]. The enzymatic O-acetylation of chloramphenicol by chloramphenicol acetyltransferase (CATs) is responsible for the inactivation of this drug [30]. Similarity, the modification of aminoglycoside antibiotics into their inactive forms leading to bacterial resistance is achieved by aminoglycoside acetyltransferases or AACs [29]. The enzymes of this group vary in their choice of groups (hydroxyl or amino) as well as their positions on aminoglycoside antibiotics for acetyl group transfer, but their actions invariably lead to drastically reduced affinity of the antibiotics to their ribosomal targets [35]. The other enzyme-mediated inactivation of antibiotics include acetylation of streptogramins by streptogramin acetyltransferases (VATs), for virginiamycin acetyl transferases), aminoglycoside modification by aminoglycoside phosphotransferases (APHs), phosphorylation of macrolides by macrolide kinases (MPHs), glutathione induced fosfomycin inactivation by FosA (or FosB), ADP-ribosylation of rifampin by ADP-ribosyltransferases (ARRs), nucleotidylation of aminoglycosides and lincomycin by nucleotidyl transferases (ANTS and Lin), glycosylation of macrolide antibiotics by glycosyltransferases [29]. A less common mechanism is the inactivation of an antibiotic by a redox process which involves flavin-dependent monoxygenase enzyme TetX. This enzyme transfers a single hydroxyl group to tetracycline at position 11a resulting in a structure that is less able to sequester Mg²⁺ ions which are critical for binding of tetracycline to its bacterial target [36, 37]. TetX is present on a transposon, and this mechanism has been recently found to be responsible for bacterial resistance to a third generation tetracycline, tigecycline [32].

3. Ribosome protection

Certain bacteria have developed resistance mechanisms that protect the antimicrobial target. For example, in the case of bacterial protein synthesis inhibitors, such as tetracycline, the bacteria have the ability to produce ribosome protection proteins that bind to the ribosomal target thus preventing the binding of tetracycline to the ribosome [38]. Such ribosome-protected bacteria will be able to grow in the presence of tetracycline as protein synthesis will be possible. Disease-causing bacteria harboring such ribosome protection mechanisms have been demonstrated to be clinically important, and these resistance determinants have been discussed extensively elsewhere [39, 40].

4. Biofilm formation

Biofilm production occurs in many loci, including teeth plaque, water environments, medical catheters, trauma wounds, etc. [41-43]. As such, microorganisms that are found in biofilms are protected from the entry of multiple antimicrobial agents [44]. Thus, biofilms are increasingly becoming a challenge in the human clinical medicine arena when considering potential chemotherapies with antibacterial agents; and this recently recognized new mode of resistance has been reviewed previously [45-47].

5. Target modification

Bacteria have found ways to alter the molecular targets of antimicrobial agents. Altered targets may include, for example, DNA gyrase, a target of quinolone antimicrobials [48], RNA polymerase, a target of rifampin [49, 50], the prokaryotic ribosome, a target of tetracycline and other protein synthesis inhibitors [51-53], and targets of antimetabolite drugs, such as the sulfonamides and related drugs [54]. One classical example of drug target modification is the staphylococcal mechanism of variously altering the penicillin binding protein (PBP) which is the target of β-lactam antibiotics. Staphylococcus aureus, the causative agent of serious infectious disease, becomes resistant to these antibiotics by any one of the several mechanisms such as mutation in PBP or acquisition of new PBP with reduced affinity to penicillins, over expression of PBP, etc [55]. Another example of an altered target mechanism includes substitution of amino acids in the quinolone-resistance determining region (QRDR) of DNA gyrase and topoisomerase IV resulting in less efficient binding of quinolone antibiotics [56]. This mechanism has been responsible for widespread quinolone resistance among the Enterobacteriaceae.

Methylation of drug binding targets on 16S rRNA by rRNA methyl transferases is responsible for aminoglycoside resistance in several bacterial species [35]. On the other hand, mutations in genes (rrs) encoding ribosomal subunits
lead to altered ribosomal protein targets which resist aminoglycoside binding, a mechanism responsible for streptomycin resistance in *Mycobacterium tuberculosis*, the causative agent of tuberculosis and other infections [57]. Methylation of the 23S rRNA component of 50S ribosomal subunit by adenine-specific N-methyltransferases is a common mechanism of macrolide resistance in many Gram-positive and -negative bacteria [58]. Also, mutations around the methylated sites have also been responsible for additional macrolide resistance. Modification of the drug target site which involves a G to A substitution at position 2,032 in the peptidyl transferase center of 23S rRNA results in reduced affinity of linezolid to the 50S subunit [59].

The vancomycin resistant enterococci (VRE) have evolved a unique mechanism of synthesizing peptidoglycan using alternate pathway thereby producing the peptidoglycan precursors ending with acyl-D-Ala$_5$-D-Lac$_5$ instead of the vancomycin target acyl D-Ala$_5$-D-Ala$_5$ [60].

6. Reduced permeability

A drug resistant phenotype of a bacterium may be due to the inability of the antimicrobial agent to gain entry into the cell where the drug targets are located [61]. One mechanism that results in reduced drug permeability in bacteria is the cell wall’s lipopolysaccharide (LPS), which consists of lipid A, a core consisting of polysaccharide and O-antigen [62-65]. Bacteria that harbor LPS moieties show resistance to erythromycin, roxithromycin, clarithromycin and azithromycin in Gram-negative bacteria such as strains of *Pseudomonas aeruginosa*, *V. cholerae* and *S. enterica*, all of which are serious pathogens, especially in immune-compromised patients [66-68]. Another mechanism that confers reduced permeability involves the porin channels that reside in the outer membrane and allow small molecular weight molecules, such as antimicrobial agents, to gain cellular entry [62, 69-71]. Drug resistant bacteria alter the expression of these outer membrane proteins such that they fail to integrate into the outer membrane or are functionally defective, thus preventing the entrance of growth-inhibitory molecules [61, 69, 72, 73]. Clinically important bacterial pathogens like *Serratia marcescens*, *E. cloacae*, *S. enterica*, *E. aerogenes*, *Klebsiella pneumoniae*, and *P. aeruginosa*, have utilized this reduced drug uptake system to resist important antimicrobial agents, such as the beta-lactams, fluoroquinolones, aminoglycosides, as well as chloramphenicol [62, 74].

7. Active drug efflux

One of the most common drug resistance mechanisms is active efflux of drugs from the inside of bacterial cells [75]. Such drug resistant bacteria harbor energy-driven drug efflux pumps which extrude antimicrobial agents thus reducing their intracellular concentrations to sub- or non-inhibitory levels. There are two main types of active efflux pumps. The first type, called primary active transport, uses the hydrolysis of ATP to actively efflux drugs from cells, while the second type, called secondary active transport, uses an ion gradient for active drug efflux from cells [76-79]. The ATP-driven transporters are also known as ABC (for ATP-binding cassette) or P-glycoprotein transporters [80, 81]. Both active transport systems are used by bacteria to resist the inhibitory effects of antimicrobial agents and are often referred to as efflux pumps [16, 82, 83].

In addition to single-drug efflux pump systems [83-86], bacteria may also express efflux pumps that are able to extrude multiple structurally-different antimicrobial agents and are referred to as multidrug efflux pumps [16, 84, 87-93]. These efflux pumps function by using the energy of the cation gradient generated by cellular respiration to catalyze the “uphill” transport of solute (e.g., drug substrate) across the membrane by translocation of the cation (e.g., H$^+$ or Na$^+$) down its concentration gradient in a process called antiport, where cation moves in one direction across the membrane and drug (substrate) moves in the opposite direction [77, 94, 95]. Since the secondary active drug and multidrug efflux pumps are considered to be predominant virulence factors in bacterial pathogens, we focus our discussion on these types of resistance determinants [57].

7.1. The tetracycline efflux pumps

The first antimicrobial efflux pump was discovered by Stuart Levy and co-workers in which the bacterium *E. coli* harbored an integral membrane protein specific for the efflux of the tetracyclines [96-98]. The tetracycline efflux pump is a secondary active transporter as it is energized by a membrane proton gradient [99]. The tetracycline efflux pumps are referred to as TetA and fall into several classes, such as Tet(A), Tet(B), Tet(C), Tet(D), etc., sometimes referred to simply as Tet(A), Tet(B), Tet(C) and Tet(D), respectively [85, 100-103]. Both Gram-negative and -positive bacteria harbor the TetA pumps, which are encoded on their genomes or on extra-chromosomal molecules such as transposons or on plasmid molecules [84, 104]. The deduced amino acid sequences of the TetA pumps are highly related, share predicted protein secondary structures in the biological membrane, and posses a common evolutionary origin with seemingly unrelated transporters that have diverse substrates, such as structurally-unrelated drugs, sugars, amino acids, and Kreb’s cycle intermediates [105-109]. These similarities predict that these transporters share a common molecular mechanism for the transport of structurally dissimilar substrates across the membrane [76, 85, 86, 106]. These transporters constitute individual members of a very large superfamily of homologous and related transport
proteins, called the Major Facilitator Superfamily (MFS) which are cleverly organized into a Transporter Classification (TC) Database http://www.tcdb.org/ [110-113].

The class B tetracycline efflux pump, TetA(B) from transposon Tn10, is the most well-studied antimicrobial efflux pump of the MFS [114]. TetB was studied by cysteine-scanning mutagenesis in which all amino acids were systematically replaced by cystiene and analyzed for their accessibilities to N-ethyl maleimide (NEM), which binds sulfhydryl-containing Cys residues [114]. Over 40 NEM-assessable TetA(B) residues were found and thought to line an aqueous-filled channel through which the tetracycline molecules are thought to be transported across the membrane [114]. These residues lie in six of the 12 helices that constitute the TetA(B) pump. The majority of the MFS transporters share a highly conserved amino acid motif, G-62 x x x D-66 R x G R-70 R, also known as Motif A, which lies in the loop structure between helices 2 and 3 [115]. The functional roles of these residues were examined in Tn10 TetA(B) and in the model sugar transporter lactose permease, LacY, of E. coli [116-118], which showed that residues corresponding to Gly-62, Asp-66, and Arg-70 of TetA(B) are required for function [119-122]. Another study found that Asp-120 and Arg-70 form a salt-bridge [123, 124]. Taken together, these findings suggest that the loop between helices 2 and 3 acts as a gate during tetracycline transport [114]. The functional role of a highly conserved arginine in TetB was established in another study, further suggesting that residues of motif A play a gating role during antimicrobial efflux [125]. The tetracycline efflux pumps represent a well-studied and important model system for analysis of drug resistance [126-128].

7.2. Secondary active multidrug efflux pumps from bacteria

To date, several major groups of secondary active multidrug efflux pumps have been discovered in prokaryotes and eukaryotes [57, 129, 130]. One group is the Multidrug and Toxic Compound Extrusion (MATE) efflux pump family, which has recently been elegantly reviewed [131]. Another efflux pump system is comprised within the Resistance-Nodulation-Division (RND) superfamily [132, 133]. The last group is the very large MFS that was mentioned above and will be discussed below [91, 108, 134].

7.2.1. Multidrug efflux pumps and the major facilitator superfamily

The MFS was discovered by Prof. Peter Henderson and colleagues [105, 107, 135-137]. They noticed that members of the MFS had structurally diverse substrates, similar deduced amino acid sequences, similar predicted secondary membrane structures, and shared a common evolutionary origin [106, 111, 112]. Taken together these similarities suggest that these seemingly diverse transporters share a common transport mechanism. A model MFS transporter is the lactose permease of E. coli, a component of the well known lac operon, in which mutations with altered sugar-binding specificities, energy-coupling, expression, salt-bridging between charged amino acids and loss of the proton translocation have been discovered [138-141]. Elucidation of LacY crystal structures and molecular simulation dynamics have confirmed previously discovered biochemical, physiological and mechanistic properties of solute transport across the membrane [142-163]. Therefore, LacY is a good model system for comparative studies with newer MFS transporters. Using the well established LacY sugar-cation symport mechanism formulated by key work from many laboratories, some seminal studies of which originate back to the 1950s [78, 105, 138-140, 153, 164-170], a drug/cation antiport mechanism was elucidated [76, 91, 114, 171] in which the proton motive force drives the proton transport inwardly across the membrane down its concentration gradient to drive drug efflux outwardly against its drug concentration gradient [77]. The proton motive force is produced by cellular respiration resulting in the outside proton concentration being greater than that inside, producing a proton gradient across the membrane that can be used for biological work such as solute transport [77, 172]. In the initial state, the drug efflux pump is empty of substrate and cation; and the substrate binding site faces inward while the proton binding site faces outward. The proposed drug efflux transport mechanism [129, 171] is as follows: (a) the H+ binds the outside of empty pump (b) the drug binding affinity inside increases (c) the drug binds the inside of the pump (d) a conformational change occurs where drug and proton binding sites switch orientations so that the bound drug faces outside, and the bound H+ faces inside (e) the drug is released outwardly (f) the H+ is released inwardly, and (g) the efflux pump then reorients drug binding site back to the inside and the H+ binding site back to the outside. The empty efflux pump is thus ready to start another drug transport cycle.

7.2.2. Key bacterial MFS multidrug efflux pump systems

The efflux pump EmrB (also called Emr) from the Gram-negative bacterium E. coli confers resistance to structurally-distinct antimicrobials [173] and shares similarity with QacA from S. aureus, a well-known member of the MFS [174, 175]. EmrD transports detergents and uncouplers of oxidative phosphorylation [176, 177]. EmrD shares homology with the multidrug efflux pumps NorA of S. aureus, LmrP of L. lactis, Flor of S. enterica, Bmr of B. subtilis, and MdfA and Bcr of E. coli [91]. Thus, EmrD is a member of the MFS [91]. The crystal structure of EmrD was elucidated and represents the first MFS efflux pump for which a detailed molecular structure was determined to high resolution [178]. Key structural features include 12 transmembrane α-helices, a largely hydrophobic interior that accommodates its
diverse substrates, and two long intra-helical loops that protrude into the membrane’s inner leaflet [178], the latter structure of which predicts that substrates are taken up from the membrane and transported to the cell’s exterior. The MdfA transporter of *E. coli* [179] was originally called CmlA [180-182] and Cmr [183], all of which confer resistance to chloramphenicol [184]. The MdfA multidrug efflux pump has been intensively studied and represents a good model system for antimicrobial efflux [185-188]. QepA, a plasmid-encoded pump was isolated from a clinical *E. coli* strain [189]. The QepA determinant was predicted to contain 14 transmembrane domains and found to transport norfloxacin, a fluoroquinolone antimicrobial agent [189]. Our laboratory cloned the gene encoding the EmrD-3 multidrug efflux pump from the genome of a toxigenic strain of the Gram-negative bacterium *Vibrio cholerae*, the causative agent of cholera [217, 218]. We found that EmrD-3 conferred resistance to a variety of structurally distinct antimicrobials and catalyzed drug/H*\(^+\)* efflux activity [57, 217, 218].

LmrP, from the Gram-positive bacterium *Lactococcus lactis*, confers resistance to ethidium bromide, daunomycin, and tetracyphenolphosphonium ion [190]. This transporter apparently binds lipophilic drugs that line the inner leaflets (cytoplasmic side) of the membrane and transports them outside, similar to that proposed for LmrA of *L. lactis* [191, 192]. The gene encoding Mdt(A) was cloned from a milk-isolate of *Lactococcus lactis* and shown to confer resistance to macrodiles, lincosamides, streptogramins, and tetracyclines [193]. A plasmid-encoded pathogen, *L. garvieae*, susceptible to erythromycin and tetracycline, contained a variant of Mdt(A) where amino acids of Motif C, also known as the antiporter motif, Val-154 and Ile-296, were altered to Phe and Val, respectively [85, 86, 194]. MdtA is important because of its origins in agriculture.

The plasmid encoded QacA determinant from *S. aureus* confers resistance to quaternary ammonium compounds [174, 195]. The *qacA* gene was cloned [196] and demonstrated to be homologous to TetA [175]. QacA-harboring pathogens are widely distributed in clinical patients and in the community [197-202]. Surprisingly, QacA has 14 transmembrane spanning domains and is a well-studied transporter [203]. In our laboratory, we cloned the lmrS gene from the genome of a methicillin-resistant *S. aureus* (MRSA) clinical isolate [204]. The LmrS efflux pump confers resistance to linezolid, trimethoprim, florfenicol, chloramphenicol, erythromycin, streptomycin, fusidic acid, and kanamycin [204], has14 transmembrane domains, is a member of the MFS, and harbors elements of the highly conserved amino acid sequence Motif C [86, 175, 204]. The gene encoding the MdrE efflux pump from the *S. aureus* genome was cloned and the pump activity characterized [205, 206]. MdrE has a predicted to have 12 transmembrane domains [205, 206].

NorA from *S. aureus* confers resistance to fluoroquinolones, such as norfloxacin, enoxacin and sparflaxacin [207, 208]. NorA was later demonstrated to be a homologue of Bmr, a multidrug efflux pumps from *B. subtilis* [209]. Thus, NorA was suspected to also be a multidrug efflux pump and later shown to transport non-fluoroquinolone drugs [210]. NorA is relevant because of its ties to *S. aureus* pathogens and because of the discoveries of efflux pump inhibitors, representing a promising avenue for the restoration chemotherapeutic efficacy against MRSA [211]. Several pathogenic strains of *S. aureus* bacteria harbor the plasmid-encoded tetracycline efflux pump Tet(K) [212]. Tet(K) has 14 predicted transmembrane domains [213] and is closely related to Tet38 from *S. aureus* [214] and Tet(L) from *B. subtilis* [215]. Tet(K) and Tet(L) catalyze transport of Na*⁺* and K*⁺* in an Na*/K*⁻* antiporter mechanism with H*⁺*, plus transport of tetracycline and H*⁻*, demonstrating that they function in other physiological processes in bacteria that are independent of resistance to antimicrobials [216].

In summary, investigations of these and other bacterial multidrug efflux pumps from the MFS will enhance our insights into their molecular mechanisms for drug resistance and transport across the membrane. Such knowledge can be exploited in order to modulate the transport activities of these drug and multidrug resistances for the ultimate purpose of restoring the efficacy of clinically important antimicrobial agents and reducing conditions that foster infectious disease.

8. Future directions

Prudent use of antimicrobial agents is highly recommended for clinicians, veterinarians, ranchers, and farmers [217-219]. Appropriate sanitation and hand-washing practices are extremely helpful for reducing the conditions that foster transfer of bacterial resistance determinants within populations, especially in the clinical settings [220]. There will always be, however, a tremendous need for the development of new antimicrobials, especially those drugs with novel molecular and cellular targets. Until new drugs with novel targets become realized, one promising avenue lies in the study of extant multidrug efflux pump systems for the purpose of developing inhibitors [17, 211, 221-225]. Phase therapy for the treatment of infectious disease is making a long awaited comeback [226]. Genomic analysis will help identify new targets for antimicrobial agents [227]. Another area where a tremendous amount of effort is being expended is the chemical modification of extant antimicrobials to develop semi-synthetic antimicrobial agents [225]. Inhibitors of enzymatic inactivation systems have shown clinical utility [228, 229]. In short, investigators have much work to accomplish if multidrug resistant bacterial pathogens are to be effectively controlled and eradicated.

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References

[34] Park BH, Levy SB. The cryptic tetracycline resistance determinant on Tn4400 mediates tetracycline degradation as well as tetracycline efflux. Antimicrobial agents and chemotherapy 1988;32:1797-800.

528 © FORMATEX 2013


Levy SB. Active efflux, a common mechanism for biocide and antibiotic resistance. Journal of applied microbiology 2002;92 Suppl:65S-71S.


Yamaguchi A, Nakatani M, Sawai T. Aspartic acid-66 is the only essential negatively charged residue in the putative
Someya Y, Yamaguchi-Someya T, Yamaguchi A. Role of the charge interaction between Arg(70) and Asp(120) in the Tn10-encoded metal-tetracycline/H(+) antiporter of Escherichia coli. The Journal of biological chemistry 2000;275:210-4.
Someya Y, Yamaguchi A. Mercaptide formed between the residue Cys70 and Hg2+ or Co2+ behaves as a functional positively charged side chain in the metal-tetracycline/H+ antiporter of Escherichia coli. Biochemistry 1996;35:9385-91.


Mayer S, Boos M, Beyer A, Fluit AC, Schmitz FJ. Distribution of the antiseptic resistance genes

Walther C, Rossano A, Thomann A, Perreten V. Antibiotic resistance in

Tennent JM, Lyon BR, Gillespie MT, May JW, Skurray RA. Cloning and expression of

Brown MH, Skurray RA. Staphylococcal multidrug efflux protein QacA. Journal of molecular microbiology and biotechnology


Floyd JL, Smith KP, Kumar SH, Floyd JT, Varela MF. LmrS is a multidrug efflux pump of the major facilitator superfamily from Staphylococcus aureus. Antimicrobial agents and chemotherapy 2010;54:5406-12.


