Chapter 8
Antimicrobial Drug Efflux Pumps in Other Gram-Positive Bacteria

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Abstract Gram-positive bacteria have a more ancient and primitive membrane structure than their Gram-negative counterparts which generally results in higher levels of intrinsic susceptibility to various lipophilic and amphiphilic antimicrobial drugs. Nonetheless, these bacteria encode similar numbers of efflux pumps in their respective genomes. In this chapter, we provide a historical overview of the identification and current understanding of such systems in Gram-positive genera of practical and industrial significance – including some clinically relevant organisms not covered elsewhere in this book. In general, these systems have been less thoroughly investigated than their Gram-negative counterparts with respect to transporter and substrate identification and their associated regulation. However, some key findings in the progression of the bacterial drug efflux field were first identified in less clinically relevant organisms such as *Bacillus subtilis* and *Lactococcus lactis*. Given this framework, the physiological relevance of efflux has become increasingly significant with concepts involving the innate immune response, metabolites, and bactericidal host-derived resistance and "natural" substrates.

Keywords Gram-positive bacteria • *Bacillus* • *Clostridium* • *Enterococcus* • *Lactococcus* • *Listeria* • *Streptococcus* • Antimicrobial resistance • Multidrug transporters • P-glycoprotein • ABC superfamily • Major facilitator superfamily

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8.1 Introduction

Model Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* encode a number of membrane efflux pumps that are responsible for significant levels of resistance to a variety of noxious compounds. In the *E. coli* genome alone, there are approximately 37 efflux pumps that belong to five different phylogenetic families and represent approximately 9 % of the encoded transporters [1–3]. The main constitutive pump, AcrB, covered in-depth elsewhere in this book, is a member of the resistance-nodulation-cell division (RND) superfamily and is a part of a system of three proteins that spans the inner membrane, periplasm, and outer membrane to coordinate efflux of amphiphiles simultaneously across both membranes into the extracellular milieu. This archetype complex alone is responsible for significant levels of efflux of several classes of antibiotics (β-lactams, macrolides, fluoroquinolones, etc.), dyes, natural and synthetic detergents (including bile acids), organic solvents, and even steroid hormones [4–6]. Furthermore, *E. coli* also encodes five other systems in this phylogenetic family that are poorly expressed except under specialized conditions, usually as a result of upregulation via two-component sensor kinases. Such systems have been extensively characterized in *P. aeruginosa*, a more clinically significant microbe, but are slightly more complex. In fact, several Gram-negative bacteria of various genera have been shown to chromosomally encode AcrB homologs [7].

On the contrary, Gram-positive efflux-based resistance is less well reported in the literature. Nevertheless, genomic analysis suggests that Gram-positive bacteria encode as many putative multidrug transporters as Gram-negative. Thus, the *Enterococcus* genome reveals 34 potential drug efflux-related genes [8]. Likewise, further comparative genomics of 11 Gram-positive bacteria of importance to the health and food industry is generally similar to *E. coli* in genomic prevalence ranging around 10 %, the exception being *Bacillus subtilis* in which 17 % of its transporter cadre is putatively dedicated to drug and toxic compound extrusion [1]. Gram-positive bacteria have a cell envelope that consists of a single phospholipid bilayer, surrounded by a thick layer of peptidoglycan. Consequently, unlike multidrug transporters of Gram-negative bacteria which often assemble into multicomponent complexes designed to span both membranes, multidrug transporters of Gram-positive bacteria have only a single transmembrane component. Since the archetypal RND superfamily transporter, the major clinically relevant superfamily of multidrug transporters in Gram-negative bacteria, consists of three components spanning both inner and outer membrane, it was believed for a long time that there are no RND-type multidrug transporters in Gram-positive bacteria. In 2001, this view was modified when YerP, a transporter of the RND superfamily, was identified in *B. subtilis* [9]. However, the number of multidrug efflux pumps belonging to RND superfamily in Gram-positive bacteria (except mycobacteria) is generally very limited [10]. Four other efflux families or superfamilies found in Gram-negative bacteria are well represented in Gram-positive bacteria: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and

toxic compound extrusion (MATE) family, and the ATP-binding cassette (ABC) superfamily [11]. Considering the vast difference in membrane structure between Gram-negatives and Gram-positives [12, 13], it is important to determine whether such proteins can contribute to similar levels of intrinsic resistance in this, a more ancient division of bacteria.

Identification of multidrug transporters remains quite a challenge even today, in a post-genomic era. Multidrug transporters lack definitive signatures for substrate specificity, so while it is possible to identify putative multidrug transporters by analysis of the genomic DNA sequence, their ability to transport multiple unrelated compounds has to be confirmed experimentally. Thus, putative SMR family proteins PsmrAB were cloned in *E. coli* from metagenomic DNA from a halophilic environment and were found to function as a two-component Na^+/H^+ antiporter, rather than involved in resistance to drugs [14]. Also, often, multidrug transporters are not expressed under physiological conditions, and whereas some of them are activated by their substrates, for many, the activator(s) are still not known. These transporters need to be overexpressed in order to confirm their identity, but for many bacteria, the difficulties in culturing or genetic manipulation and the availability of genetic tools make overexpression or disruption of the gene very difficult. In this chapter, we review the presently characterized multidrug efflux pumps in Gram-positive bacteria.

8.2 *Bacillus subtilis*

B. subtilis, while of little clinical importance, is an excellent model organism, easily cultured and with a lot of genetic tools available. In addition, *B. subtilis* has significant genomic abundance of multidrug transporters relative to other Gram-positives [1]. For these reasons, these *B. subtilis* transporters have been extensively studied. In fact, the phenomenon of bacterial multidrug resistance was first discovered in *B. subtilis* [15]. Authors hypothesized the presence of a mechanism analogous to the mammalian multidrug transporter, P-glycoprotein. Indeed, multidrug-resistant cells were obtained after selection with increasing concentrations of one of the substrates of P-glycoprotein, rhodamine 6G. These cells exhibited resistance to some other known substrates of P-glycoprotein, such as ethidium bromide, chloramphenicol, and puromycin, as well as to tetraphenylphosphonium and cetyltrimethylammonium bromide, which are not transported by P-glycoprotein [15]. The mechanism of resistance was shown to be efflux based and was sensitive to the same inhibitors, reserpine and verapamil, as mammalian P-glycoprotein. These cells were used to clone the first bacterial multidrug transporter, Bmr, whose gene was found to be amplified in resistant cells. Analysis of the Bmr sequence, however, showed little similarity with P-glycoprotein. Indeed, Bmr is a multidrug transporter of the MFS and is very different from an ABC transporter P-glycoprotein. It was shown to use a different energy source – secondary-active transport with the transmembrane pH gradient – whereas P-glycoprotein couples the transport of substrates with

primary-active ATP hydrolysis [16, 17]. Later, a second multidrug transporter of the MFS, Blt, was identified in *B. subtilis* [18].

Blt is 51 % identical to Bmr and transports a similar set of compounds; however, the pattern of their expression is quite different. Bmr is expressed under standard cultivating conditions and is further regulated by BmrR, a member of the family of MerR-like transcriptional activators [19]. BmrR activates the expression of Bmr after binding its substrates. In contrast, Blt expression is normally not detectable. It is regulated by BltR [18], which is related to BmrR, but has a different inducer-binding domain, and its substrates are not yet known. Blt is cotranscribed with a downstream gene encoding spermine-spermidine acetyltransferase, indicating physiological function(s) apart from synthetic drug resistance per se – a theme central to efflux systems in Gram-positive and Gram-negative systems alike [11, 20]. Another layer of regulation was reported later for Bmr and Blt [21]. Their expression was found to be further controlled by a MerR-type regulator Mta. Apo-Mta acted as a repressor of the *bmr* and *blt* gene transcription. Although Mta inducer was not identified in this report, Mta was converted into transcriptional activator by the removal of the C-terminal inducer-binding domain. The authors proposed that this removal mimics the binding of inducer to Mta [21].

Several more multidrug transporters were identified in *B. subtilis*. In 1996, a stunning discovery was made of a first bacterial multidrug transporter of the ABC family, LmrA from *Lactococcus lactis* [22]. LmrA was homologous to both halves of the mammalian P-glycoprotein which is arranged in a 6 + 6 transmembrane motif [23] common to many multidrug transporters of different families [24]. Subsequently, two multidrug transporters of ABC family, BmrA and BmrC/BmrD, which functions as heterodimer, were identified in *B. subtilis*. BmrA was first identified from genome sequencing of *B. subtilis* [25] and demonstrated to transport Hoechst33342 (a fluorescent dye used to stain DNA), doxorubicin, and 7-aminoactinomycin D in highly enriched inverted membrane vesicles from *E. coli* [26]. BmrC/BmrD was likewise shown in the same system to transport Hoechst 33342, doxorubicin, BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, fluorescent compound) and mitoxantrone [27]. BmrC/BmrD expression is regulated, first, by the main transcription phase regulator AbrB, and second, via a dedicated ribosome-mediated transcriptional attenuation mechanism that requires the *bmrB*-encoded leader peptide [28]. Another substrate-specific ABC transporter deserves mentioning here because of its clinical relevance. BceAB is positively regulated by BceRS two-component regulatory system and contributes to intrinsic resistance to bacitracin [29, 30].

Additional work identified Bmr3 [31], a member of the MFS. Bmr3 was shown to transport puromycin, norfloxacin, and tosufloxacin which are also substrates of the Bmr and Blt. Bmr3 expression is growth phase dependent and is drastically reduced as the cells enter late log phase. A spontaneous multidrug-resistant mutant selected by puromycin exhibited an increased stability of *bmr3* transcripts [32]. In addition, MdtP is a multidrug transporter of the MFS that contributes to resistance to actinomycin, fusidic acid, novobiocin, and streptomycin [33]. MdtP expression is induced by its substrate fusidic acid, which binds to repressor MdtR (whose

encoding gene is cotranscribed with *mdtP*) and causes its dissociation from the *mdtP* promoter. YerP is the sole RND-type multidrug transporter characterized in Gram-positive bacteria (except mycobacteria) and has been shown to be involved in resistance to acriflavine, ethidium bromide, and surfactin, a cyclic lipopeptide biosurfactant synthesized by some species of *B. subtilis* [9]. Finally, EbrAB is a paired multidrug transporter which belongs to the SMR family and functions as heterooligomer. Its overexpression in *B. subtilis* confers resistance against acriflavine, ethidium bromide, pyronine Y, and safranin O [34].

8.3 *Clostridium difficile*

C. difficile is a major cause of nosocomial diarrhea. It is also implicated in 95 % of pseudomembranous colitis cases [35]. This species is intrinsically less susceptible to antibiotics, in particular β-lactams, fluoroquinolones, chloramphenicol, and lincosamides, than the other clostridia. Active efflux was long thought to be responsible for this resistance; however, the first description of multidrug transporter from *C. difficile* dates to 2004, when Dridi et al. [36] characterized CdeA, an MATE family transporter. When overexpressed in hypersensitive strain of *E. coli*, CdeA caused resistance to acriflavine, ethidium bromide, ciprofloxacin, and norfloxacin. CdeA was shown to cause energy-dependent efflux of ethidium bromide in *E. coli* cells, and quantitative reverse transcription-PCR assay showed that *cdeA* expression in *C. difficile* was significantly increased by exposure to ethidium bromide, but not to ciprofloxacin. The authors could not test the effect of CdeA inactivation in *C. difficile*, due to inability to genetically transform this species. The same year, Lebel et al. [37] identified four genes in *C. difficile* encoding putative proteins homologous to NorA from *Staphylococcus aureus*, a multidrug transporter of the MFS. Of these sequences, only one, designated *cme*, conferred resistance to ethidium bromide, safranin O, and erythromycin when expressed in *Enterococcus faecalis*. It was not known whether the lack of effect of the other three open reading frames was due to inefficient expression in a different species, to inappropriate substrates, or to inactivity of these open reading frames as multidrug transporters.

8.4 *Listeria monocytogenes*

L. monocytogenes is an important food-borne pathogen that can cause such severe diseases as septicemia, meningitis, stillbirth, and abortion. High-risk groups include immunocompromised patients, neonates, and pregnant women [38]. Infection with *L. monocytogenes* causes significant mortality and morbidity in these groups. *L. monocytogenes* invades host cells and replicates within their cytoplasm [39]. Acquired antimicrobial resistance in *L. monocytogenes* is a very rare event. However, this pathogen is intrinsically resistant to several antimicrobial agents and also to the

bile which has bactericidal properties and to which it is exposed during several stages of its lifecycle in the human gastrointestinal tract. In addition, multidrug transporters play a fascinating role in interaction of *Listeria* with mammalian innate immunity during its infection cycle. For these reasons, information about multidrug transporters in *Listeria* is highly clinically significant.

The first multidrug transporter in *Listeria* spp., MdrL, was identified serendipitously while sequencing genomic region around a gene encoding a putative histone-like protein, *flaR*, in an effort to find genes implicated in its regulation [40]. The authors identified a gene similar to a number of multidrug transporters of the MFS. Disruption of the allele in the wild-type strain of *L. monocytogenes* resulted in a small but reproducible increase in susceptibility to erythromycin, josamycin, clindamycin, and heavy metals and about a tenfold increase in susceptibility to cefotaxime. Functional characterization of MdrL as an efflux pump was confirmed by observing reserpine-dependent inhibition of ethidium bromide efflux, which was virtually eliminated in the MdrL disruption mutant.

Two other multidrug transporters of MFS family present in *L. monocytogenes*, MdrM and MdrT, were identified based on predicted protein sequence similarity [41]. To date, no experimental work addressing directly and conclusively their function as multidrug transporters has been published, but there is a significant indirect supporting evidence. The expression of these genes, as well as of *mdrL*, is induced by common multidrug transporter substrates. MdrL, MdrM, and MdrT were shown to be regulated by repressors LadR, MarR, and BrtA (previously TetR), respectively [42–44]. These repressors are encoded adjacently to the corresponding multidrug transporter genes. The expression of MdrL is induced by rhodamine 6G in the LadR-dependent fashion [43]. The transcription of *mdrM* and *mdrT* is also upregulated in response to rhodamine 6G and tetraphenylphosphonium, although the involvement of the aforementioned repressors in their activation by these compounds has not been demonstrated [42]. Cholic acid, another common multidrug transporter substrate, was shown to bind BrtA and cause its dissociation from the *mdrT* promoter, resulting in the induction of *mdrT* transcription [44]. Moreover, MdrT was shown to transport cholic acid out of the cells [44]. There is significant cross-regulation among these genes. A *ladR* mutant upregulates not only *mdrL* but also *mdrM* [42]. In response to the bile acid and cholic acid, BrtA upregulates not only *mdrT* but *mdrM* as well [44].

The most fascinating function of MdrM and MdrT was described in the Portnoy laboratory [42, 45]. The authors showed that these multidrug transporters control the magnitude of the host cytosolic innate immune response to *L. monocytogenes*. On the entry into the host cytosol, *L. monocytogenes* activates host response that leads to transcription of dozens of genes, including robust expression of interferon beta (IFN-β) [46, 47]. MdrM and MdrT expression was shown to affect the induction of IFN-β in infected macrophages [42, 48, 49]. Disruption of *mdrM* [42] or *mdrT* in the strain with mutated *brtA* [49] decreased IFN-β production, while overexpression of either MdrM or MdrT resulted in increased induction of IFN-β in infected macrophages [42]. The molecule that triggers the cytosolic host response was shown to be the cyclic dinucleotide c-di-AMP [45]. This molecule is produced by many bacteria and is a second messenger that is implicated in a variety of

functions including cell wall metabolism, potassium homeostasis, DNA repair, and control of gene expression [50]. C-di-AMP in *L. monocytogenes* is secreted by MdrM and MdrT [45]. It is sensed by the cytosolic innate immune receptor, STING [51]. Stimulation of this pathway results in the activation of the interferon regulatory factor-3 and nuclear factor-kB transcription factors and, ultimately, to host transcriptional activation of IFN-β [46, 51]. While innate immune system is indispensable for defense against microbial pathogens, paradoxically, the production of IFN-β increases the bacterial burden and lethality of *L. monocytogenes* infection in mouse models [52–54], through mechanisms that are not well understood, but may involve the enhanced susceptibility of lymphocytes to apoptosis in response to a pore-forming toxin and a major virulence factor of *L. monocytogenes*, listeriolysin O [53, 54].

Finally, AnrAB is an ABC-type multidrug transporter that was isolated by screening for nisin-sensitive mutants of *L. monocytogenes* [55]. A mutant strain exhibited enhanced susceptibility to nisin, gallidermin, cefuroxime, cefotaxime, ampicillin, penicillin G, and bacitracin.

8.5 *Lactococcus lactis*

L. lactis is broadly used for food manufacturing. Despite a few case reports of *L. lactis* being an opportunistic pathogen [56], it is a generally regarded as safe organism. However, the "resistance gene reservoir" hypothesis suggests that beneficial and commensal bacterial populations in gastrointestinal tract may play a role in horizontal transfer of antimicrobial resistance to pathogenic microorganisms [57].

Initially, only secondary, proton motive force-driven multidrug transporters were described in bacteria, when, in 1994, Bolhius et al. [58] reported isolation of three mutants of *L. lactis*, selected for resistance to high concentrations of ethidium bromide, daunomycin, or rhodamine 6G. These mutants were found to be cross-resistant to a number of structurally and functionally unrelated drugs, such as quinine, actinomycin D, and gramicidin D. The drug resistance of these strains was due to energy-dependent efflux and was inhibited by reserpine, a multidrug efflux pump inhibitor. Efflux was also inhibited by orthovanadate (an inhibitor of ATP-dependent efflux activity characteristic of ABC transporters) in one of the strains, and in two others, it was partially inhibited both by orthovanadate and by nigericin (an ionophore). This observation suggested that a proton motive force-dependent and ATP-dependent systems were involved in drug efflux. A year later, a lactococcal proton motive force-dependent multidrug efflux pump, LmrP, was characterized in the same laboratory [59]. *LmrP* was cloned in *E. coli* and was shown to belong to the MFS. In *E. coli*, its substrates included ethidium bromide, daunomycin, and tetraphenylphosphonium, which were transported in a proton gradient-dependent manner. Overexpression of *lmrP* in *L. lactis* resulted in elevated resistance to ethidium bromide; however, an *lmrP* deletion mutant was only slightly more susceptible to ethidium bromide than the wild-type strain. The resistance of the *lmrP*-deficient strain to ethidium bromide could be significantly decreased by treating the cells

with orthovanadate. This observation confirmed that an ATP-dependent multidrug transporter was functional in *L. lactis*.

In 1996, LmrA, the first bacterial ATP-dependent multidrug transporter, was characterized in the same laboratory [22]. *LmrA* was homologous to the human *mdr1*, which encoded the P-glycoprotein and, moreover, complemented MDR1 in human lung fibroblast cells [60]. LmrA was targeted to the plasma membrane and conferred typical multidrug resistance in these human cells. Blockers of P-glycoprotein-mediated multidrug resistance also inhibited LmrA-dependent drug resistance. Like P-glycoprotein, LmrA removed drugs from the inner leaflet of the cytoplasmic membrane [61]. The expression of *lmrA* in a hypersensitive *E. coli* strain increased resistance to the very wide variety of drugs, including aminoglycosides, chloramphenicol, β-lactams, lincosamides, macrolides, quinolones, streptogramins, and tetracyclines [62].

LmrA is equivalent to half of the P-glycoprotein and functions as homodimer. Later, however, a functional heterodimeric ABC-type multidrug transporter LmrCD was described in *L. lactis* [63]. LmrC and LmrD were copurified as a heterodimer, and overexpression of both LmrC and LmrD in LmrA-negative strain of *L. lactis* demonstrated ATP-dependent efflux of ethidium bromide, BCECF-acetoxymethyl ester, daunomycin, and Hoechst 33342. As a corollary, the cells did not show drug extrusion when either gene was overexpressed singly. LmrCD is also responsible for bile resistance [64]. The expression of *lmrCD* is controlled by transcriptional repressor LmrR, encoded upstream of the *lmrCD* [65, 66]. LmrR also autoregulates its own expression. LmrR binds the LmrCD substrates: Hoechst 33342, daunomycin, and rhodamine 6G [65, 67]. Drug binding to LmrR relieves the LmrR-dependent repression of the *lmrCD* genes [68]. Interestingly, when four mutant multidrug-resistant strains of *L. lactis* selected by challenging with increasing concentrations of daunomycin, ethidium bromide, rhodamine 6G, or cholate were analyzed, only *lmrCD* multidrug transporter genes were significantly and strongly upregulated in all four strains [69]. These data suggested that LmrCD was a major determinant of multidrug resistance in *L. lactis*. This study, however, did not address the expression of other putative multidrug transporters in mutant strains. Finally, in 2013, CmbT was characterized as an MFS-type multidrug transporter [70]. Overexpression of *cmbT* in *L. lactis* resulted in marginally increased resistance to cholate, ethidium bromide, Hoechst 33342, lincomycin, puromycin, rifampicin, streptomycin, sulbactam, sulfadiazine, and sulfamethoxazole (IC_{50} increased approximately 1.2–3 times). Overexpressed CmbT mediated extrusion of ethidium bromide and Hoechst 33342, and ionophores inhibited the CmbT-mediated transport of Hoechst 33342. Based on the increased level of thiol groups in supernatant of strain overproducing CmbT, the authors hypothesized possible involvement of CmbT in sulfur metabolism [70]. However, this observation was dependent on methionine and cysteine content of the medium and was not further investigated in this report. In addition to LmrP, LmrA, LmrCD, and CmbT, the genome of *L. lactis* contains 36 putative multidrug transporters; however, they are still to be characterized experimentally. Additionally, a multidrug transporter of the ABC superfamily, LmrB, was identified on a plasmid carried by a natural isolate of

L. lactis [71]. LmrB was shown to be an active multidrug transporter capable of the extrusion from the cell of ethidium bromide and Hoechst 3342. Interestingly, two genes encoding polypeptidic bacteriocins LsbA and LsbB are located on the same plasmid as LmrB, in the immediate vicinity of the multidrug transporter gene. LmrB was shown to render the cells immune to both bacitracins, and to mediate their secretion into the medium. In this function, LmrB could be complemented by LmrA but not LmrP [71]. The location of the *lmrB* gene on a plasmid may facilitate transfer of this multidrug transporter from *L. lactis* to pathogenic bacteria and may deserve further investigation.

8.6 *Enterococcus* spp.

The enterococci are commensal bacteria that normally populate the human intestine. Over the last two decades, enterococci were identified as causative agents of nosocomial infections with increasing frequency. Infections caused by enterococci include urinary tract infections, nosocomial bacteremia, intra-abdominal infections, and endocarditis. Most enterococci have intrinsic resistance to various antimicrobial agents. However, increasingly frequent isolation of enterococci with acquired resistance to most commonly used drugs has been observed in recent years. As early as 1997, Lynch et al. [72] hypothesized that intrinsic resistance of enterococci to various antimicrobial agents, in the absence of outer membrane, is due, at least in part, to active efflux system(s). They examined four wild-type strains of *E. faecalis* and a strain of *Enterococcus faecium* and found that all strains showed energy-driven efflux of chloramphenicol, and all but one strain of *E. faecalis* extruded norfloxacin. In contrast, active efflux did not play a role in resistance to β-lactams. In this work, genetic determinants of these efflux pumps were not identified. Four years later, enterococcal genome-scanning identified a potential multidrug transporter EmeA [73] due to its homology to NorA from *S. aureus*, MFS-type multidrug transporter covered in-depth in Chap. 7 in this book. Deletion of this gene in *E. faecalis* resulted in an approximately twofold increase in susceptibility to acriflavine, ethidium bromide, clindamycin, erythromycin, novobiocin, ciprofloxacin, and norfloxacin compared to the wild-type strain. Functional complementation with wild-type plasmid-expressed *emeA* restored the resistance to ethidium bromide and resulted in the resistance to norfloxacin fourfold higher than in the wild-type strain. This resistance was due to energy-dependent efflux. Incubation with reserpine (competitive multidrug transporter blocker), verapamil (a calcium channel blocker), or lansoprazole (a H^+ and K^+-ATPase pump inhibitor) decreased resistance of both wild-type and complemented strains. The resistance of the mutant strain was unaffected by these agents, except for resistance to ethidium bromide which was lowered twofold by reserpine. These data allowed the authors to conclude that EmeA was the main enterococcal pump for these agents.

Later, Lee et al. [74] cloned EfrAB, an ABC multidrug transporter from *E. faecalis*, by using a drug-hypersusceptible mutant of *E. coli* host. When expressed in *E. coli*,

EfrAB conferred resistance to norfloxacin, ciprofloxacin, doxycycline, arbekacin, novobiocin, daunorubicin, doxorubicin, acriflavine, 4′,6-diamidino-2-phenylindole, ethidium bromide, safranin O, and tetraphenylphosphonium. Furthermore, EfrAB demonstrated energy-dependent efflux of acriflavine. This efflux was inhibited by verapamil, reserpine, and sodium orthovanadate (an ATPase inhibitor). Similar to other two-component ABC multidrug transporters, both EfrA and EfrB were required for resistance. The expression of EfrAB is induced by subinhibitory concentrations of chloramphenicol, gentamicin, and streptomycin [75]. In the same laboratory, *E. faecium* multidrug transporter belonging to the MFS, EfmA, was cloned in a similar fashion [76]. *E. coli* harboring EfmA showed energy-dependent efflux of 4′,6-diamidino-2-phenylindole and tetraphenylphosphonium, as well as norfloxacin/H^+ antiport. EfmA was found to be constitutively expressed by *E. faecium*. Overall, 34 putative multidrug transporters in *E. faecalis* have been identified from genome sequencing [8]. However, the majority of them are still experimentally unexplored.

8.7 *Streptococcus* spp.

8.7.1 Streptococcus pneumoniae

S. pneumoniae is the main bacterial cause of community-acquired pneumonia and represents a major disease burden worldwide [77]. Despite the recent introduction of the heptavalent pneumococcal conjugate vaccine, antimicrobial resistance is an increasing problem in this organism due to the spread of multidrug-resistant clones and increases in antimicrobial resistance among nonvaccine serotypes [78].

The initial report of the multidrug transporter in *S. pneumoniae* did not identify the transport protein associated with the phenotype [79]. A few years later, PmrA, a multidrug efflux pump of the MFS family, was identified using *S. pneumoniae* genomic sequence as homologous to *norA* of *S. aureus* [80]. The gene was overexpressed in *S. pneumoniae* and found to confer resistance to norfloxacin, ciprofloxacin, acriflavine, and ethidium bromide [80]. In later reports, a knock-out of *pmrA* [81, 82] did not result in increased susceptibility to drugs, indicating that PmrA is not intrinsically active in *S. pneumoniae*.

By 2006, the overexpression of the ABC superfamily efflux proteins PatA and PatB was found to be responsible for the multidrug-resistant phenotype of a mutant of *S. pneumoniae* selected after exposure to ciprofloxacin [83]. Disruption of *patA* and *patB* resulted in increased sensitivity to acriflavine, ethidium bromide, berberine, erythromycin, oxolinic acid, norfloxacin, ciprofloxacin, and novobiocin [81, 82] thus demonstrating that PatAB is normally expressed by *S. pneumoniae*. Each subunit consists of a nucleotide-binding domain and a membrane spanning domain, and heterodimerization of PatA and PatB is required to form a functional transporter [84]. Expression of *patAB* is induced by subinhibitory concentrations of fluoroquinolones [85, 86]. In clinical fluoroquinolone-resistant isolates of *S. pneumoniae*, whose resistance is ascribable to the overexpression of multidrug transporters, either PmrA [87] or PatA/PatB [88], were found to be responsible for the phenotype.

Similar to other Gram-positive examples covered in this chapter, additional putative multidrug transporters exist in the genome of *S. pneumoniae*, but so far, no phenotype was associated with them [82].

8.7.2 Streptococcus agalactiae

S. agalactiae causes neonatal sepsis, pneumonia, meningitis, as well as infections of the bovine udder. *S. agalactiae* produces α-hemolysin, which is an important virulence factor. It is capable of damaging erythrocytes, lung epithelial cells [89], and brain microvascular endothelial cells [90], which is regarded as an initial step in invasive disease. *cylA* and *cylB* were identified as genes essential for the production of the *S. agalactiae* hemolysin [91] and encode an ABC-type transporter. These genes are part of the 12-gene *cyl* operon, which contains, in addition to *cylA* and *cylB*, 5 genes similar to fatty acid biosynthesis enzymes (*cylD*, *cylG*, *acpC*, *cylZ*, and *cylI*), one similar to an aminomethyltransferase (*cylF*), one carrying the conserved domain of a glycosyltransferase (*cylJ*), a gene predicted to encode an acetyl coenzyme A carboxylase (*cylX*), a putative phosphopantetheinyl transferase (*cylK*), and a putative acyl-coA acyltransferase (*cylE*) [92]. *cylA* and *cylB* deletion mutants resulted in a nonhemolytic phenotype [93]. *cylA* mutant was shown to still harbor intracellular hemolytic activity, which was released by sonication. Since CylAB contained the signature sequence of a multidrug resistance transporter, wild-type and nonhemolytic *cylA* mutant were exposed to known substrates of multidrug transporters. Deletion of *cyl*A resulted in significant increase in susceptibility to daunorubicin, doxorubicin, and rhodamine 6G. Furthermore, the *cylA*-negative strain displayed a markedly reduced capacity to export doxorubicin. Growth in the presence of reserpine resulted in a dose-dependent decrease of extractable hemolytic activity, supporting the hypothesis that hemolysin is transported out of the cell by a multidrug transporter. At the time, the nature of the *S. agalactiae* hemolysin was unknown, and it was believed to be a pore-forming protein toxin. However, Gottschalk et al. [93] raised doubts in the protein nature of hemolysin based on the described work. Multidrug transporters were known to transport small molecules, rather than proteins. Indeed, in 2013, Whidbey et al. [94] showed that an ornithine rhamnolipid pigment known as granadaene [95] is responsible for the hemolytic activity of the bacterium. This is perhaps one of the few cases where the nature of the natural substrate of the multidrug transporters is proven very strongly.

8.7.3 Streptococcus mutans

S. mutans is a major causative agent in human caries and forms biofilm known as dental plaque [96, 97]. Although *S. mutans* strains are generally susceptible to antimicrobial agents [98], prolonged antimicrobial exposure can select antimicrobial resistance [99, 100]. Involvement of multidrug transporters in drug resistance has

been reported [101, 102]. Genome of *S. mutans* UAB159 [103] shows the presence of 71 putative ABC transporters and 10 putative MFS transporters (TransportDB at http://www.membranetransport.org).

Increased susceptibility to methyl viologen (paraquat), benzyl and ethyl viologens, and quaternary ammonium compounds was observed in mutant strains that were deficient in a function of ABC transporter complex, VltAB, which is encoded by an operon (*SMU.905-906*) [104]. The same laboratory also reported another putative ABC transporter complex, SmbFT, which is not present in strain UAB159 [105] and is encoded by genes located in the same locus and provides protection against lantibiotics Smb and haloduracin, but not against other lantibiotics (e.g., nisin) and several peptide antibiotics such as bacitracin, polymyxin B, and vancomycin [106]. A newer study also described two ABC transporter systems, SMU.654-655-656-657 and LctFEG (SMU.1148-1149-1150), which are, respectively, encoded by the genes linked to the two-component regulatory genes *nsrRS* (located downstream of *SMU.654-655-656-657*) and *lcrRS* (located upstream of *lctFEG*). Inactivation of *nsrRS* or *nsrS* (but not *SMU.654-655-656-657*) rendered the mutant strains more susceptible to nisin A (16-fold MIC reduction), while disruption of *lcrRS*, *lcrS,* or *lcrFEG* increased the susceptibility of the mutants to nukacin (eightfold MIC decrease) [101], suggesting involvement of LctFEG transporter in nukacin resistance. In *S. mutans* and *Streptococcus gordonii* (a commensal species), the gene locus *rcrRPQ* encodes an MarR-like transcriptional regulator (RcrR) and an ABC efflux complex (RcrPQ), which are linked to stress tolerance [107–109]. Inactivation of *rcrP* in *S. mutans* or *rcrR* in *S. gordonii* rendered the mutant cells more susceptible to lower pH or oxidative stress agents such as H_2O_2 and methyl viologen [107, 109]. Either overproduction of or deficiency in RcrPQ impaired the biofilm formation in *S. mutans* [107], suggesting an optimal status of this ABC exporters is essential for biofilm formation. In this regard, another exporter, the NrgA ammonium transporter, is also essential for biofilm formation in *S. mutans* [110]. Similar to that observed in *B. subtilis* [29], an ABC transporter named BceAB is encoded by part of the four-gene operon *bceABR*S that also encodes the BceRS two-component regulatory system, and BceABRS contributes in response to bacitracin-induced cell envelope stress [111]. Recently, the *copYAZ* operon (SMU.424-426-427) that encodes CopA ABC copper exporter and CopYZ regulators was demonstrated to play an important role in copper homeostasis, stress tolerance, and biofilm formation [112].

Additionally, a multidrug transporter, MdeA of the MFS predicted with 12 transmembrane domains, conferred resistance to ampicillin, oxacillin, nalidixic acid, ciprofloxacin, kanamycin, tetracycline, acriflavine, and rhodamine 6G (4- to 32-fold increase in MIC values) when expressed on a plasmid in a hypersusceptible *E. coli* host [102]. It is also noted that several regulatory systems such as LytST and ScnRK have been reported to contribute to tolerance to oxidative stress [113, 114]. Whether these regulatory systems are linked to any multidrug transporters remains to be investigated (Table 8.1).

Table 8.1 Summary of characterized drug efflux pumps in Gram-positive bacteria

Species/transporter family	Pump	Substrates[b]	Reference
B. subtilis			
ABC	BceAB	BCT	[29]
ABC	BmrA	AAD, DOR, HO	[25, 26]
ABC	BmrCD	BCECF, BCT, DOR, HO, MTO	[27, 115]
MFS	Blt	ACD, EB, DOR, FQ, R6G, TPP	[18, 21]
MFS	Bmr	ACD, EB, DOR, FQ, R6G, SPD, TPP	[18, 21, 116]
MFS	Bmr3	FQ, PUR	[31, 32]
MFS	LmrB	DOR, FQ, LIN, PUR	[117, 118]
MFS	MdtP	ACT, FUA, NOV, STR	[33]
RND	YerP	ACR, EB, SUR	[9]
SMR	EbrAB	ACR, EB, PY, SO	[34]
C. difficile			
MATE	CdeA	ACR, EB	[36]
MFS	Cme	EB, ERY, SO	[37]
E. faecalis			
ABC	ABC7	DAU, DOR, EB, OFX	[8]
ABC	ABC11	CHX, PTD	[8]
ABC	ABC16	AZI, CLA, ERY	[8]
ABC	ABC23	QD, VIR	[8]
ABC	EfrAB	ACR, CIP, DAP, DAU, DOR, EB, FQ, NOV, NOR, SO, TET, TPP	[74, 75]
ABC	Lsa	CLI, QD	[119]
MFS	EmeA	ACR, CLI, EB, ERY, FQ, NOV	[73]
E. faecium			
ABC	MsrC	ACR, DA, DP, DR, FQ, TC, TPP	[74]
L. lactis			
ABC	LmrA	DAU, DOR, EB, LIN, ML, R6G, TET, VIN, VIT	[22, 61, 62]
ABC	LmrB[a]	EB, HO	[71]
ABC	LmrCD	CHO, DAU, EB, HO, R6G	[63–65, 69]
MFS	CmbT	EB, HO	[70]
MFS	LmrP	DAU, EB, TPP	[59]
MFS	MdtA[a]	LIN, ML, STG, TET	[120]
L. monocytogenes			
ABC	AnrAB	AMP, BCT, CXM, CTX, GAL, NIS, PEN	[55]
MFS	Lde	ACR, BAC, EB, FQ	[121–123]
MFS	MdrL	CTX, EB, ML	[40, 43]
MFS	MdrM		[42]
MFS	MdrT	CHO	[42, 44]

(continued)

Table 8.1 (continued)

Species/transporter family	Pump	Substrates[b]	Reference
S. agalactiae			
ABC	CylAB	DAU, DOR, R6G	[91, 93]
MFS	MefB, MefG	ML	[124]
MFS	MreA	AZI, ERY, SPI	[125]
S. mutans			
ABC	BceAB	BCT	[111]
ABC	CopA	Cu	[112]
ABC	LctFEG	NUK	[101]
ABC	SmbFT	HAL, SMB	[106]
ABC	SMU.654-655-656-657	NIS	[101]
ABC	RcrPQ	H_2O_2 , PQ	[107, 109]
ABC	VltAB	BV, EV, PQ, QAC	[104]
MFS	MdeA	ACR, AMP, CIP, KAN, OXA, NAL, R6G, TET	[102]
S. pneumoniae			
ABC	PatAB	FQ	[81, 83, 85, 126, 127]
MFS	PmrA	FQ	[80]
MFS	MefE	ML	[128]

This table excludes drug efflux pumps identified in staphylococci and mycobacteria, which are described in Chaps. 7 and 21 of this book

[a]The genes encoding these pumps are located on plasmids

[b]*AAD* 7-aminoactinomycin D, *ACD* acridine dyes, *ACR* acriflavine, *ACT* actinomycin, *AG* aminoglycosides, *AMP* ampicillin, *AZI* azithromycin, *BAC* benzalkonium chloride, *BCECF* 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, *BCT* bacitracin, *BV* benzyl viologen, *CHX* chlorhexidine, *CHO* cholate, *CIP* ciprofloxacin, *CLA* clarithromycin, *CLI* clindamycin, *CTX* cefotaxime, *CXM* cefuroxime, *DAP* 4′,6-diamidino-2-phenylindole, *DAU* daunorubicin, *DOR* doxorubicin, *EB* ethidium bromide, *ERY* erythromycin, *EV* ethyl viologen, *FQ* fluoroquinolones, *FUA* fusidic acid, *GAL* gallidermin, *HAL* haloduracin, *HO* Hoechst 33342, *KAN* kanamycin, *LIN* lincosamides, *ML* macrolides, *MTO* mitoxantrone, *NAL* nalidixic acid, *NIS* nisin A, *NOR* norfloxacin, *NOV* novobiocin, *NUK* nukacin, *OFX* ofloxacin, *OXA* oxacillin, *PEN* penicillin G, *PQ* paraquat (methyl viologen), *PTD* pentamidine, *PUR* puromycin, *PY* pyronine Y, *QAC* quaternary ammonium compounds, *QD* quinupristin-dalfopristin, *R6G* rhodamine 6G, *SMB* a lantibiotic, *SO* safranin O, *SPD* spermidine, *SPI* spiramycin, *STG* streptogramin, *STR* streptomycin, *SUR* surfactin, *TET* tetracycline, *TPP* tetraphenylphosphonium, *VIN* vinblastine, *VIR* virginiamycin, *VIT* vincristine

8.8 Concluding Remarks

Gram-positive organisms are generally more susceptible to antimicrobial drugs than their Gram-negative counterparts. Typical model archetypes for drug efflux have been more well developed genetically, biochemically, and structurally in Gram-negative organisms. This research bias may be generally attributable to the relative

contributions of tripartite efflux systems to clinically significant drug resistance phenotypes as targets for inhibitory compounds with potential dramatic modulation of drug resistance phenotypes. From a historical perspective, however, it is significant that some of the first bacterial drug efflux systems were identified and characterized in Gram-positive organisms (with little clinical significance) but noteworthy genetic and functional conservation with the major mammalian multidrug transporter, P-glycoprotein. However, counterintuitive to membrane evolution and drug kinetics, Gram-positive organisms encode a similar, if not greater, genomic investment (in the case of *Bacillus subtilis*) to efflux-based transport mechanisms. This observation suggests efflux may be physiologically and functionally more essential and, hence, more intrinsically active in Gram-positive organisms to accommodate their respective environments and survival. Further research into pump regulation and expression-level comparisons may be useful to determine relative functional balance between efflux and cell energetics in context with cell physiology and metabolism. In this regard, Gram-positive organisms described herein may be instructive in identifying physiologically relevant substrates and roles for respective efflux systems in the host-bacterial interface. The increasing availability of genetic tools in these organisms will facilitate genetic manipulation required to conduct such studies and *in vivo* modeling.

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