Chapter 27
Involvement of Antimicrobial Drug Efflux Systems in Bacterial Fitness and Virulence

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Abstract Multidrug efflux pumps play an important role in antimicrobial resistance and also serve other functions that are related to bacterial cell communication, stress responses, fitness, and virulence. Although it is challenging to define the natural functions of drug efflux pumps, accumulating evidence has revealed both direct and indirect involvement of multidrug efflux systems in these cellular processes. There is also an intertwined regulation of drug efflux and other cellular systems implying various shared regulators. These features explain diverse effects of multidrug efflux pump status on bacterial functions, including interactions between bacterial species and their hosts. Drug efflux pump contribution to improved fitness and increased virulence of pathogens is supported by numerous examples. This chapter describes the current understanding of the roles of drug efflux pumps (in particular those of Gram-negative bacteria) in bacterial pathogenicity, which further underscores the clinical significance of drug efflux phenomena.

Keywords Multidrug efflux pumps • Biological burden • Biofilm • Fitness • Colonization • Pathogenesis • Quorum sensing • Virulence • RND efflux pumps • AcrAB-TolC

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27.1 Introduction

Multidrug efflux pumps, in particular, those of the resistance-nodulation-cell division (RND) superfamily, play an important role in antimicrobial resistance in bacteria. Accumulating evidence has also revealed both direct and indirect involvement of these efflux pumps in other functions that go beyond drug resistance [1–3]. Because of this, the ability of drug efflux pumps to extrude antimicrobial agents has been merely regarded as "incidental" to other functions [2, 4]. Indeed, multidrug efflux systems are ubiquitously distributed in bacteria [5], and their natural substrates have been demonstrated to include bacterial metabolites, whose accumulation inside bacterial cells can be deleterious [6–8]. Studies have also established the substrate profiles of drug efflux pumps that include non-antibiotic substances such as bile salts, phenolic agents, and cell communication molecules, which are encountered by microbes in their natural habitats [9–11]. Furthermore, multidrug efflux systems are now known to interconnect numerous microbial cellular processes such as drug resistance, cell communication, stress response, fitness, colonization, and virulence [3]. This phenomenon is consistent with the fact that intertwined regulation involving various regulators and pathways is often required for coordinated differential expressions of drug efflux and other functional systems. For example, regulators that control directly or indirectly the expression of multidrug transporters also influence quorum sensing, biofilm formation, stress responses, and virulence factor production [12]. This chapter provides an up-to-date overview of the relationship between the status of drug efflux pumps and features related to bacterial biological burden, cell communication, fitness, colonization, and virulence. Since bacterial pathogenicity is not only species specific, it also depends on the host and environmental niches. Thus, discussions are also made on several relevant cell processes such as resistance to bile salts and antimicrobial peptides as well as responses to nitrosative and oxidative stress. Overall, numerous findings support the contribution of multidrug efflux pumps to improved fitness and increased virulence of pathogens via a variety of direct and indirect mechanisms, thus highlighting the clinical significance of hijacking efflux mechanisms as an important therapeutic intervention strategy.

27.2 Bacterial Metabolic Burden and Fitness Associated with Antimicrobial Resistance and Multidrug Efflux Pumps

Bacterial fitness as expressed by growth rate and competitive ability *in vitro* and *in vivo* in the absence of antibiotics is widely supposed to be compromised by expression of multidrug transporters. The association between multidrug resistance and reduced fitness has fueled the hope that a reduction in the use of antibiotics would lead to a reduction in frequency in resistant bacteria. However, the experience shows that multidrug resistance, once acquired, is often eliminated so slowly

that it is unlikely to be of practical importance. Mathematical modeling shows that the fitness cost of drug resistance has to be quite high in order to result in elimination of resistant bacteria in a reasonable amount of time [13], which is often not the case. Thus, when the loss of plasmid-encoded tetracycline resistance from *Escherichia coli* was measured under antibiotic-free conditions, the observed dynamics was best described by a model that included a high-mutation frequency (~10^{-4} mutations per generation) and a 0.7 % reduction in the growth rate of antibiotic-resistant bacteria [13]. With these parameters, replacement of 99.9 % of the population by susceptible bacteria would take approximately 1.5 years. To reach the same replacement level in 5 weeks, the reduction in the growth rate of the resistant bacteria would need to be ~6 %, about nine times higher, which is not realistic for an induced resistance gene on a low-copy number plasmid [14], and presumably even less so for a chromosome-based resistance which is tightly regulated, as is often the case with multidrug pump-encoding genes [15]. Indeed, it has been experimentally shown that introduction of the plasmid that carries the gene encoding β-lactamase resulted in reduced growth rate in *Salmonella enterica* serovar Typhimurium. However, introduction of the regulator of the β-lactamase gene, which made β-lactamase expression inducible rather than constitutive, eliminated these fitness costs [16].

Even in the case of mutations leading to overexpression of multidrug resistance genes, the experimental data differs as to the presence of universal metabolic burden in multidrug-resistant bacteria. Some works support the notion of a fitness cost associated with overexpression of multidrug efflux genes. Thus, two *Pseudomonas aeruginosa* mutants overexpressing MexAB-OprM and MexCD-OprJ multidrug pumps of the RND superfamily were found to be impaired in their survival in water and on a dry surface, when compared to the wild-type strain [17]. In addition, these mutants exhibited impaired virulence in *Caenorhabditis elegans* model system. However, the caveat to this observation is that these mutants overexpressed the multidrug resistance genes as a consequence of mutations in the genes encoding their respective regulatory proteins, *mexR* and *nfxB*. Thus, the expression of other non-related genes could have been affected. Another example of decreased fitness associated with the expression of multidrug efflux pumps comes from *Stenotrophomonas maltophilia*. The strain overexpressing multidrug efflux pump SmeDEF was displaced by the wild-type strain in coculture experiments [18].

On the other hand, other studies failed to detect any metabolic burden that constitutive expression of multidrug efflux pumps might create. Thus, several *Neisseria gonorrhoeae* mutants overexpressing MtrCDE efflux pump to various degrees were identical to a wild-type strain in their growth and survival in *in vitro* competition assays [19].

At the very least, the fitness cost is habitat dependent. Thus, deleterious effect of the constitutive MexEF-OprN pump overproduction in *P. aeruginosa* was possible to detect only under anaerobic conditions [20]. In aerobiosis, overexpression of this pump does not produce a metabolic burden in competition tests. In this case, it appears that *P. aeruginosa* compensates the fitness cost by adjusting its metabolism. MexEF-OprN is a proton antiporter, and its overexpression should lead to an increase in influx of protons. To prevent acidification of the cytoplasm, the mutant

increases oxygen consumption [20]. Increased oxygen consumption may lead to the drop of environmental oxygen in the culture. This situation is rectified by turning on nitrate respiratory chain genes, which are normally not expressed under aerobic conditions, and increasing nitrate consumption and nitric oxide production. As a result of this chain of events, there is no metabolic burden to *P. aeruginosa* overexpressing MexEF-OprN pump under aerobic conditions. Under anaerobic conditions, excess protons are not consumed in the process of respiring nitrate, unlike in oxygen respiration. Cytoplasmic pH of the mutant strain was found to drop to values close to 6.0, presumably contributing to the deleterious impact of MexEF-OprN overexpression observed under these conditions [20]. Another example is *S. maltophilia* strain with mutations in multidrug efflux pump genes *smeIJK*, which displayed a compromised growth in Mueller-Hinton medium, but not in Luria-Bertani medium [21]. It was determined that the defect was due to the low osmolarity of the Mueller-Hinton medium and to the presence of casein hydrolysate as one of its components.

27.3 Effect of Multidrug Efflux Pumps on Virulence

Studies of bacterial fitness in their ecological niches provide necessarily considerably more complicated and difficult to interpret data than those done under controlled conditions. Free-living bacteria are exposed to a chemically and physically complex environment and engage in constant competition with other bacteria. Pathogens are additionally a target of the whole arsenal of antimicrobial devices that their hosts possess as a result of evolution. Nevertheless, it is the fitness of the bacteria in their ecological niche that is, ultimately, the only relevant characteristic. The data on the role of multidrug efflux pumps in fitness *in vivo* and virulence of pathogenic bacteria is somewhat conflicted, but overall the inclination is to give multidrug efflux pumps credit in increasing bacterial virulence and survival in the host or to emphasize the need for precise regulation, when both overexpression and deletion of a pump prove detrimental. In many studies, virulence was investigated in conjunction with observing the involvement of the pump in a specific characteristic of the cell, such as resistance to bile, antimicrobial peptides, etc. While there is necessarily a logical leap in connecting *in vitro* and *in vivo* results, such data will nevertheless be discussed in corresponding sections of this chapter. Here, we will review several studies that concentrated heavily on *in vivo* experiments.

27.3.1 Positive Role of Multidrug Efflux Pumps in Virulence and Survival

Multiple observations suggest that multidrug efflux pumps are necessary for maximum virulence of bacterial pathogens. Thus, Buckley et al. [22] determined virulence of *S. enterica* serovar Typhimurium strains with deletions of the genes

involved in multidrug efflux. The mutants that lacked *acrB* were significantly less able to adhere, invade, and survive in mouse monocyte macrophages. In the human embryonic intestine cells, disruption of *acrB* had no effect on adherence, but bacteria were unable to invade or survive intracellularly. Disruption of *tolC* abolished the ability to adhere, invade, and survive in both cell types. A competitive index assay was used to determine the ability of mutants to colonize and persist in the avian gastrointestinal tract in competition with the parental strain. Mutants with disrupted *tolC* colonized and persisted in the avian digestive tract poorly, while mutants with disrupted *acrB* were able to colonize, but did not persist gastrointestinally.

The genetic background of *Salmonella* strains seems to play an important role in the outcome of these experiments. Indeed, in another work, in which three multidrug-resistant strains of *S. enterica* serovar Typhimurium isolated from cattle, as opposed to the laboratory strain used in the previous work, were used, the results were somewhat different. *acrB* mutants demonstrated a reduced adhesion ability but no change in invasion when human adenocarcinoma cell line HT-29 was used [23]. *tolC* mutants, similar to the previous report, demonstrated lower adhesion and lower invasion rates [23]. Moreover, the authors showed that the low invasion of these *tolC* mutants results from a downregulation of expression of the type III secretion system 1. This system is encoded on the *Salmonella* pathogenicity island 1 and is involved in bacterial entry into cells of the intestinal epithelium. It allows the translocation of a large set of effector proteins from the bacterial cytoplasm into the cytosol of the host cell. These effector proteins induce local cytoskeleton rearrangements leading to membrane ruffling, micropinocytosis, and finally internalization of *Salmonella* [24].

It is interesting that *acrB* and *tolC* mutants have different effects on virulence. In another study, *tolC* mutants of multidrug-resistant *S. enterica* serovar Typhimurium phage type DT104 and DT204 were 64- to 256-fold more susceptible to bile salts than *acrB* mutants, and in contrast to *acrB* mutants, the *tolC* mutants were unable to colonize the cecum, spleen, and liver after 1 week of infection in a day-old chicken model [25]. This may be the result of TolC involvement in other processes, but it can also reflect the global effect of *acrB* or *tolC* deletion on expression levels of other genes. Indeed, the Piddock group later further elaborated on the role of AcrAB-TolC pump in *Salmonella* virulence. Using transcriptomic comparison, they showed that the disruption of *acrA*, *acrB*, or *tolC* resulted in altered expression of multiple genes involved in pathogenesis [26]. Besides confirming the downregulation of *Salmonella* pathogenicity island-1-encoded type III secretion system genes, the affected genes included chemotaxis and motility genes, as well as the genes involved in anaerobic metabolism. In addition, the authors, prompted by somewhat different results reported by Virlogeux-Payant et al. [23], confirmed the altered gene expression in two other *S. enterica* serovar Typhimurium backgrounds. The mechanism of altering gene expression for *acrAB-tolC* mutants is not known. The profound effect of the deletion of *acrA*, *acrB*, or *tolC* on expression of multiple genes underscores the difficulties in the interpretation of data on the involvement of multidrug efflux pumps into bacterial pathogenicity.

N. gonorrhoeae mutants overexpressing MtrCDE pump were shown to have an advantage over wild-type parent strain in competitive infections in mouse [19]. This advantage paralleled *in vitro* resistance to CRAMP-38, a murine antimicrobial peptide, as well as the degree of MtrCDE overexpression.

The disruption of *acrA* or of *tolC* component of the pump AcrAB-TolC in two clinical isolates of *Enterobacter cloacae* was shown to result in a decrease in virulence in a mouse model of systemic infection [27]. However, in this work, various drug resistance cassettes were used to produce gene knockouts, which might have skewed the observed results.

Legionella pneumophila is a pathogenic organism which survives and replicates as an intracellular parasite within free-living amoeba and initiates pneumonia in humans after inhalation of contaminated aerosols. In *L. pneumophila*, the deletion of *tolC* resulted not only in an increase in susceptibility to various drugs but also in a significant attenuation of virulence toward amoebae and macrophages [28]. However, the role of *tolC* was not further dissected in this work, and it is known to be involved in both expression and function of multiple systems.

The effect of six RND efflux pumps on virulence was studied in *Vibrio cholera* [29]. A strain lacking VexB, VexD, and VexK multidrug efflux pumps was attenuated in the infant mouse model, but the production of virulence factors was reported unaffected. In contrast, a strain with all six RND pumps deleted produced significantly less cholera toxin and fewer toxin-coregulated pili than the wild-type strain and was unable to colonize the infant mouse.

27.3.2 Negative Effect of Multidrug Efflux Pump Overproduction on Virulence

Among the observations of the negative impact of multidrug efflux pumps on virulence is the study in which overexpression of MexEF-OprN multidrug efflux pump due to a constitutive activation of the transcriptional activator MexT impaired virulence of *P. aeruginosa* in a *C. elegans* model [30]. The authors ascribed this effect to the activity of the efflux pump and not of MexT, despite the fact that the latter is involved in the regulation of type III and type VI secretion systems and early surface attachment independently of MexEF-OprN [31]. Overexpression of MexEF-OprN resulted in reduced expression of several quorum-sensing-regulated genes. This phenotype was traced back to a delay in the production of quorum-sensing signaling compound due to the extrusion of its precursor through the efflux pump [30]. However, these results may rather highlight the importance of fine-tuning the expression of multidrug efflux pumps. Indeed, in case of *mexAB-oprM* pump genes, it has been shown that both the deletion of the operon and overexpression of the pump genes result in the reduced capacity to invade or transmigrate across canine kidney epithelial cell monolayer and to kill mice [32]. In fact, *mexAB-oprM* deletion mutant exhibited much more dramatic deficiencies than the mutant overexpressing this operon [32].

27.3.3 Plant Pathogens

The interaction between plants and their pathogens and epiphytic bacteria through chemical means is intense. From the point of view of intellectual curiosity, it is regrettable that the volume of research on drug transporters of plant pathogens and epiphytes is quite low. A few published studies examine the role of multidrug efflux pumps in virulence of plant pathogens.

Plants respond to microbial attack with sophisticated defenses that include the production of antimicrobial peptides and phytoalexins, the latter including mostly terpenoids, glycosteroids, and alkaloids. Epiphytic bacteria produce a variety of antibiotics with which they antagonize phytopathogenic bacteria. Both factors are important in fitness and virulence of the plant pathogens. Several works demonstrated that efflux pumps are essential for plant-bacterium interactions. Efflux pump IfeAB of *Agrobacterium tumefaciens* was shown to be important for alfalfa root colonization in a competition experiment between wild-type strain and *ifeAB* mutant [33]. Expression from the operon promoter was induced by alfalfa isoflavonoids formononetin, medicarpin, and coumestrol. Accumulation of coumestrol in mutant cells was abnormal, consistent with coumestrol being exported by the pump. Mutants of *Rhizobium etli* deficient in multidrug efflux pump genes *rmrA* and *rmrB* had enhanced susceptibility to phytoalexins, flavonoids, and salicylate [34]. *rmrA* was inducible by bean-root-released flavonoids; mutants of *rmrA* formed 40 % less nodules in beans. A *Pseudomonas syringae* strain with mutations in the RND pump PseABC exhibited reduced virulence for immature cherry fruit compared to parental strain [35]. *Erwinia amylovora* requires AcrAB for successful colonization and pathogenesis in apple rootstock [36]. An *acrB*-deficient mutant was susceptible to the apple phytoalexins phloretin, naringenin, quercetin, and (+)-catechin, and the expression of *acrAB* was upregulated by the first two compounds. *Ralstonia solanacearum* mutants in multidrug efflux pumps AcrAB and DinF exhibited reduced ability to grow in the presence of phytoalexins and were significantly less virulent on the tomato plant [37].

27.4 Resistance to Bile Acids

Enteric bacteria found in the gastrointestinal tract need to be able to survive antimicrobial effects of up to 20 mM concentrations of bile salts and their free acids [38, 39]. Bile salts are amphipathic, water-soluble, steroidal surfactants, synthesized in the liver from cholesterol and secreted into the bile. They aid emulsification and enzymatic digestion of dietary lipids in the small intestine [40]. In humans, bile salts are secreted by hepatocytes in a form conjugated to glycine or taurine [40]. After a series of events in which intestinal microflora plays a prominent role, they are transformed to unconjugated biliary bile acids consisting primarily of cholate, chenodeoxycholate, and deoxycholate, present in approximately equal

amounts [41]. While over the physiological pH range conjugated bile salts are fully ionized and require the OmpF porin in order to traverse the outer membrane of Gram-negative bacteria [39], the unconjugated bile salts are weakly acidic molecules and in their uncharged, protonated state can diffuse through both bacterial membranes and accumulate in cytoplasm. Bile salts exert a cytotoxic effect on bacteria by way of disruption of cell membrane integrity, promotion of RNA secondary structure formation, DNA damage, denaturation of cellular proteins, and oxidative stress [42]. While Gram-positive bacteria are particularly sensitive, bile acids can also kill Gram-negative bacteria [42].

Multidrug efflux pumps are well known now to participate in providing resistance to these compounds. Bile sensitivity and reduced capacity to colonize the intestinal tract of mice was reported to be associated with *S. enterica* serovar Typhimurium gene *acrB* as early as 1996 [43]. In 1997, it was shown that AcrA of RND-type AcrAB-TolC multidrug efflux pump and, to a lesser extent, EmrB of the EmrAB-TolC of the major facilitator superfamily (MFS) were involved in active removal of chenodeoxycholate from *E. coli* cells [39]. The role of the individual components of transporters was not addressed in this work, but since then, bile salts and acids have been shown to be substrates for multiple multidrug efflux pumps in various bacteria. Moreover, bile salts were shown to induce transcription of multidrug pump genes in a number of cases. Thus, in *E. coli*, bile salts, primarily unconjugated (deoxycholate and chenodeoxycholate), bind to Rob transcription regulator to induce the transcription of *acrAB* [44]. Other pumps that have been shown to play a role in bile resistance in *E. coli* include a single component MFS pump MdtM. MdtM was shown to be involved in resistance to cholate and deoxycholate [45]. Bile salts did not upregulate *mdtM* transcription, but "housekeeping" levels of MdtM were sufficient to contribute effectively to the resistance [45].

In *S. enterica* serovar Typhimurium, the transcription of the genes encoding the AcrAB-TolC multidrug efflux pump was also found to be upregulated by bile. Two mechanisms, not necessarily mutually exclusive, were proposed to be responsible for *acrAB-tolC* activation. Transcriptional activator RamA regulates the expression of *acrAB-tolC* genes in *Salmonella. ramA* transcription itself is normally repressed by the product of the divergently transcribed *ramR* gene. In one work, the authors found that bile prevented the association of RamR with the *ramA* promoter, thereby upregulating the transcription of *ramA* [46]. In another article, the authors did not see increased expression of *ramA* in response to bile salts, but observed the binding of cholic acid to RamA and proposed that this leads to altered conformation and activity of RamA [47]. It seems that significantly lower concentrations of bile salts were used in the second work. It is possible that both mechanisms are involved, and the concentration of bile salts in the second work was insufficient to induce the transcription of *ramA*. Additionally, the altered activity of RamA bound to cholic acid was not demonstrated. Overexpression of *ramA* from the plasmid was found to induce the transcription of *acrA*, *acrB*, and *tolC* in yet another work [48], so at least when greatly overexpressed, RamA does not need an inducer to activate these genes; however, it is not known how RamA behaves when it is present at physiological levels.

In *Vibrio parahaemolyticus*, which is an enteric Gram-negative bacterium that can cause acute gastroenteritis in humans, RND-type multidrug efflux pumps VmeAB, VmeCD [49], and VmeTUV [50] are involved in resistance to bile acids (cholate and deoxycholate).

In *Campylobacter jejuni*, the leading bacterial cause of food-borne enteritis in humans, inactivation of multidrug efflux pump CmeABC drastically decreases resistance to various bile salts [51]. It was also found that the *cmeABC* mutants failed to colonize chickens, and the minimal infective dose for the *cmeABC* mutant was at least 2.6×10^4-fold higher than that of the wild-type strain. Moreover, bile salts drastically elevated the expression of the *cmeABC* operon via binding to a transcriptional repressor CmeR and reducing its affinity to the *cmeABC* promoter [52, 53]. CmeR is encoded by a gene located immediately upstream of the *cmeABC* operon, and its loss changes the expression of a number of genes [54]. Loss of function mutation of *cmeR* severely reduced the ability of *C. jejuni* to colonize chickens [54]. This is not the result expected as a consequence of derepression of the *cmeABC* operon, but CmeR is a pleiotropic regulator, and other genes whose expression level change in response to the deletion of *cmeR* might be responsible for the observed phenotype.

In *Klebsiella pneumoniae*, the efflux pump KpnEF of the small multidrug resistance (SMR) family has been shown to be involved in resistance to bile [55]. The ability of the Δ*kpnEF* mutant to grow in the presence of bile was compromised, as well as its stress response, as discussed later in this chapter. *Yersinia enterocolitica* multidrug efflux pump genes *acrAB* were found to be induced by bile salts [56].

Listeria monocytogenes is a Gram-positive intracellular pathogen causing significant mortality and morbidity, especially among neonates, elderly, and pregnant women [57]. It is food-borne and encounters bile in the gut during initial infection, in the liver where it replicates robustly, and in the gallbladder, from which it can return to the intestine and thence to the environment. *L. monocytogenes* uses its multidrug efflux pump MdrT to protect itself against cholic acid [58]. Cholic acid disrupts binding of the transcriptional repressor BrtA to the *mdrT* promoter, thereby inducing transcription of *mdrT* [58]. The deletion of *mdrT* significantly sensitized the cells to cholic acid. The authors also determined that in a mouse intravenous infection model, bacterial abundance in the liver four days postinfection was tenfold attenuated for Δ*mdrT* cells and 100-fold attenuated for *ΔmdrTmdrM* cells, while the abundance of the wild-type and *ΔmdrM* cells was similar. It suggests that while dispensable in wild-type cells, *mdrM* becomes essential for virulence in the tested situation in *ΔmdrT* background.

27.5 Response to Nitrosative Stress

The gut is mostly an anaerobic environment. Consequently, facultative anaerobic pathogens such as *E. coli* have to switch to the anaerobic physiology when in the gut environment. Since oxygen is limited, the cell must utilize alternative terminal

electron acceptors, with nitrate being the preferable electron acceptor [59]. The generation of reactive nitrogen species during this physiological process results in nitrosylation and subsequent condensation of indole molecules and, ultimately, in significant accumulation of nitrosyl indole derivatives (e.g., indole red) in anaerobically grown *E. coli* when the bacterium respires nitrate [60]. These compounds are toxic to the cell. It has been shown that an RND efflux pump MdtEF actively removes the nitrosyl-damaged cellular components out of the cell [61]. *mdtEF* expression is activated under anaerobic conditions by two global transcription regulatory factors that mediate the transition from aerobic to anaerobic lifestyle in *E. coli*, anaerobic respiration control (ArcA) and, to a lesser extent, fumarate nitrate reduction (FNR) [61]. Deletion of *mdtEF* resulted in a significantly slower growth and increased sensitivity to indole red under anaerobic conditions.

P. aeruginosa is an opportunistic human pathogen that infects the lungs, urinary tract, bloodstream, and surgical wounds [62]. Individuals with cystic fibrosis are particularly susceptible to chronic lung infections caused by *P. aeruginosa* [63]. *P. aeruginosa* can adapt to existence in various niches and is able to grow either aerobically or anaerobically in the presence of alternative terminal electron acceptors, such as nitrate, nitrite, etc. In the cystic fibrosis lung, *P. aeruginosa* grows as a biofilm in stagnant mucus, which was found to be an anaerobic environment rich in nitrate and nitrite [64]. Thus, *P. aeruginosa* likely needs to respire nitrate at the site of infection. *mexEF-oprN* multidrug transporter operon of *P. aeruginosa* has been shown to respond to nitrosative stress [65]. The operon's transcription was induced by S-nitrosoglutathione (GSNO) or diethylamine triamine NONOate (DETA). Both of these compounds generate NO and produce nitrosative stress. The inducibility of *mexEF-oprN* by nitrosative stress was dependent on MexT, a LysR-family-positive regulator of transcription. The deletion of the *mexEF-oprN* operon, however, did not result in changes in the resistance to GSNO. NO nitrosylates multiple substrates in the cell, one or more of which, rather than GSNO or NO itself, may be substrates of MexEF-OprN pump. Interestingly, chloramphenicol, which is a nitroaromatic compound, was capable of rapidly inducing *mexEF-oprN* expression and was also a substrate of MexEF-OprN pump. A derivative resembling chloramphenicol but lacking the nitro-moiety failed to induce *mexEF-oprN* expression. This suggests that chloramphenicol resembles a nitrosative stress product that is deleterious to the cell and serves as a natural substrate for MexEF-OprN pump. A possible link between MexEF-OprN and anaerobic lifestyle was not addressed in this work. Indeed, the link between anaerobic lifestyle, nitrosative stress, and multidrug efflux pumps may not be as straightforward as in *E. coli*. Thus, a *mexEF-oprN*-overexpressing strain was found to have impaired fitness compared to the wild-type strain under anaerobic conditions [20]. MexEF-OprN exchanges external protons for their substrates; thus, constitutive activity of MexEF-OprN may lead to the acidification of the cytoplasm. This, indeed, is the case under anaerobic conditions [20]. When oxygen is available, it is used to consume excess of protons resulting from MexEF-OprN activity. The *mexEF-oprN*-overexpressing mutant consumes oxygen at a much higher rate than wild type, creating local microaerobic conditions, which, in turn, leads to the untimely activation of the nitrate respiratory chain in the

presence of oxygen. This data, however, does not rule out the possible role in detoxification of the cells respiring nitrate, and the fitness levels of the mutant strain overexpressing *mexEF-oprN* under anaerobic conditions may be compromised due to excessive overproduction of the pump.

The authors mention that airway epithelium cells are known to secrete NO in response to lipopolysaccharide exposure or inflammatory signals [65]. It is true that NO is produced in host tissues by inducible nitric oxide synthase (iNOS), whose expression is upregulated following inflammatory stimulus, and NO exhibits antimicrobial activity, reducing adherence of *P. aeruginosa* cells [66]. It is worth noting however that the expression of iNOS and production of NO in the respiratory epithelium of cystic fibrosis patients is markedly reduced, despite chronic severe inflammation [67, 68].

K. pneumoniae, similar to *E. coli*, can respire nitrate under anaerobic conditions [69] and, thus, can potentially encounter nitrosative stress [70]. In this bacterium, the deletion of the SMR-type multidrug efflux pump-encoding genes *kpnEF* leads to the three-times slower growth in the presence of an NO donor, sodium nitroprusside, and up to 20-times reduced growth rates in the presence of acidified nitrite [55]. These results imply that KpnEF expression is involved in nitrosative stress tolerance in *K. pneumoniae*. The deletion mutant also exhibited decreased survival under hyperosmotic conditions and at the elevated (42 °C) temperatures. These results are complicated, however, by the fact that the deletion of *kpnEF* alters the expression of other genes. In this work, the authors used RT-PCR to show that the expression of capsular synthesis genes was changed; the deletion of *kpnEF* may also result in the change in expression of other genes that have not been tested.

27.6 Response to Oxidative Stress

While oxygen provides substantial advantages in energy yield by respiration, it can become life-threatening due to reactive oxygen species (ROS) production by various mechanisms. The most common ROS include superoxide ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO). ROS are detrimental to living organisms since they react with proteins, DNA, lipids, and other biomolecules [71]. Threat from damaging ROS is ever present for both free-living and commensal or pathogenic microorganisms. ROS can be generated both endogenously and exogenously. In aerobic cells, ROS are formed in the process of consecutive addition of electrons to oxygen. H_2O_2 may be generated by various bacteria to inhibit their competitors [72]. Plant [73] and animal [74] hosts generate various ROS as a defense mechanism against microbial pathogens. Interestingly, ROS can be produced by certain bacterial pathogens as virulence factors against their host [75].

There is limited data on the involvement of multidrug transporters in bacterial resistance to ROS. Multidrug efflux pumps are known to be regulated by a global transcriptional system SoxRS in enteric bacteria [76, 77] or directly by SoxR in non-enterics [78]. SoxR-mediated transcription is activated via oxidation of the $[2Fe\text{-}2S]^{+}$ cluster in

the presence of various redox-cycling agents [79]. For a long time, it was generally accepted that redox-cycling agents acted indirectly via superoxide formation, which oxidized SoxR. However, recently it has been shown that redox-cycling agents activate SoxR directly and cause cellular toxicity independently of the production of ROS [80]. This is consistent with the fact that many genes of the SoxRS regulon in *E. coli* have no apparent function in superoxide detoxification, but rather function in export or modification of redox-cycling drugs. Interestingly, in *P. aeruginosa* SoxR is activated by a phenazine pyocyanin, which is a redox-cycling compound, but is also known to be a quorum-sensing signal [81]. Considering the apparent involvement of multidrug efflux pumps in the release of phenazines, the SoxR response may ensure proper shuttling of these compounds [78].

In *E. coli*, the NorM pump of the multidrug and toxic compound extrusion (MATE) family was found to reduce the level of intracellular ROS and protect the cell from oxidative stress via unknown mechanism [82]. Likewise, in *Salmonella*, macrolide-specific efflux pump of the ATP-binding cassette (ABC) superfamily, MacAB, is induced upon exposure to hydrogen peroxide and is critical for the survival of *Salmonella* in the presence of peroxide [83]. Furthermore, MacAB was required for intracellular replication inside wild-type macrophages, but not inside ROS-deficient macrophages. *macAB* mutants also had reduced survival in the intestine of a mouse. In *P. aeruginosa*, expression of MexXY multidrug efflux pump was induced by peroxide via ArmZ (the anti-repressor against MexZ repressor of MexXY pump); however, the MexXY efflux system did not contribute to peroxide resistance [84].

27.7 Combating Iron Deficiency

Another line of defense against bacterial pathogens is the withholding of nutrients to prevent bacterial outgrowth. This process is termed “nutritional immunity” with the most significant form being the sequestration of nutrient iron. The vast majority of vertebrate iron is intracellular, sequestered within the iron storage protein ferritin or complexed with porphyrin ring of heme as a cofactor of hemoglobin or myoglobin [85]. Extracellular iron is insoluble and therefore difficult to access for invading pathogens or tightly bound to the serum protein transferrin. Thus, all bacterial pathogens encounter a period of iron starvation upon entering their host. Most pathogens circumvent iron withholding through high-affinity iron uptake mechanisms that compete against host-mediated sequestration. One of such strategies involves secretion of siderophores; low molecular weight iron-binding molecules which scavenge iron from the host and later in complex with iron are imported back into the bacterial cell. There is a limited evidence of the role of multidrug transporters in the export of siderophores in some bacteria.

As far ago as 1993, genes encoding multidrug efflux system *mexAB-oprK* (i.e., *mexAB-oprM*) in *P. aeruginosa* were identified while trying to complement a mutant strain deficient in the production of siderophore ferripyoverdine receptor and incapable

of growth in an iron-deficient medium [86, 87]. The expression of the efflux operon appeared induced under iron-limiting conditions. The operon was also reported to be co-regulated with other genes of the ferripyoverdine uptake system [87].

Export of the siderophore enterobactin across the cytoplasmic membrane in *E. coli* was first described to be accomplished by a dedicated MFS transporter EntS [88], but it was noted that mutants in *entS* still secrete some enterobactin, suggesting involvement of some other mechanism. Later, enterobactin was shown to be exported through the outer membrane by TolC [89], prompting the analysis of the possibility of involvement in enterobactin export of RND multidrug efflux pumps that engage TolC as an outer membrane component. It was found that multiple deletions in *acrB*, *acrD*, and *mdtABC* resulted in a significant decrease in enterobactin export; however, single deletions of these multidrug efflux systems did not affect the ability of *E. coli* to excrete enterobactin [90].

27.8 Resistance to Host-Derived Antimicrobial Peptides

Host defense peptides are encoded in the host genome and derive from large precursors through one or more proteolytic activation steps [91]. They are known as direct antimicrobial agents as well as innate immune modulators. These peptides are mainly secreted at those anatomical sites that are routinely exposed to environmental challenges, such as skin and mucosal epithelia [92]. Additionally, they are found in the body fluids or stored in the cytoplasmic granules of professional phagocytes [93]. The antimicrobial activity of host defense peptides is the consequence of their ability to interact with and insert into biomembranes [94]. Besides bacteria, they can target fungi, yeast, viruses, and cancer cells [92]. Microbial pathogens utilize a number of resistance mechanisms to subvert the action of host defense peptides [95]. In a few instances, multidrug efflux pumps were shown to be involved in microbial resistance to antimicrobial peptides.

Thus, several mutants of *N. gonorrhoeae* overexpressing MtrCDE multidrug efflux pump exhibited markedly increased resistance to human cathelicidin LL-37, and the level of the resistance corresponded to the degree of MtrCDE overexpression [19]. These mutants also showed similarly increased resistance to CRAMP-38, the murine homolog of the human cathelicidin LL-37, and outcompeted the wild-type strain *in vivo* in a murine infection model [19]. The loss of MtrCDE resulted in increased susceptibility of *N. gonorrhoeae* to cathelicidin LL-37, as well as porcine protegrin-1 and horseshoe crab-derived tachyplesin-1 [96].

Likewise, *Neisseria meningitidis*, which is intrinsically highly resistant to human antimicrobial peptides, utilizes MrtCDE pump as one of the mechanisms of defense against these peptides. Mutants in the *mtrCDE* operon are about 15 times more susceptible to polymyxin B and about 10 times to LL-37 and protegrin-1 [97]. Multidrug efflux system RosA/RosB of *Yersinia pestis* provides bacterium with resistance to polymyxin B [98]. It was also active against cecropin P1 (from pig) and melittin (from bee venom).

AcrAB efflux pump in *K. pneumonia* has been shown to provide the organism with resistance not only to antibiotics but also to host-derived antimicrobial peptides, present among the array of antimicrobial compounds in the mucus. AcrB knockout was more susceptible both to antimicrobial properties of human bronchoalveolar lavage fluid and to such antimicrobial peptides as human neutrophil peptide-1, β-defensin 1 and β-defensin 2 [99]. *acrB* knockout also exhibited a reduced capacity to cause pneumonia in a murine model, compared to the wild-type strain [99].

27.9 Control of Host Immune Response

MdrM and MdrT of *L. monocytogenes* have a fascinating role in controlling the magnitude of the host cytosolic innate immune response to the bacteria [100, 101]. On entry into the host cytosol, *L. monocytogenes* activates a host response that leads to transcription of dozens of genes, including robust expression of interferon β (IFN-β) [102, 103]. MdrM and MdrT expression was shown to affect the induction of IFN-β in infected macrophages [100, 104, 105]. The disruption of *mdrM* [100] or *mdrT* in the strain with mutated *brtA* [104] decreased IFN-β production, while overexpression of either MdrM or MdrT resulted in increased induction of IFN-β in infected macrophages [100]. The molecule that triggers the cytosolic host response was shown to be the cyclic dinucleotide c-di-AMP [101]. 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 [106]. c-di-AMP in *L. monocytogenes* is secreted by MdrM and MdrT [101]. It is sensed by the cytosolic innate immune receptor, STING [107]. 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 [102, 107]. While the 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 [108–110]. The mechanisms of this effect 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 [109, 110].

27.10 Export of Virulence Factors

Streptococcus agalactiae, an invasive pathogen, produces β-hemolysin, which is an important virulence factor. It is capable of damaging erythrocytes, lung epithelial cells [111], and brain microvascular endothelial cells [112], which is regarded as an initial step in invasive disease. The genes *cylA* and *cylB* encoding an ABC-type

multidrug efflux pump were identified as genes essential for the production of the *S. agalactiae* hemolysin [113]. The hemolysin is now known to be an ornithine rhamnolipid pigment [114] described previously as granadaene [115]. 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 efflux pump.

27.11 Effect of Multidrug Efflux Pumps on Cell-to-Cell Communication and Quorum Sensing

Quorum sensing is a density-dependent mechanism by which bacteria coordinate expression of specific target genes in response to a critical concentration of signaling molecules. Effectively, quorum-sensing systems allow bacteria to behave somewhat in the manner of a multicellular organism when their population reaches a threshold level. Quorum sensing controls complex activities such as changes in secondary metabolism, bioluminescence, protein secretion, root nodulation, motility, virulence factor production, plasmid transfer, and biofilm maturation [116]. Quorum sensing plays a critical role in both pathogenic and symbiotic bacteria-host interactions. In pathogens, virulence factors are released and coordinated attack on the host made only when bacterial population reaches certain density and can overwhelm host responses. For symbiotic bacteria, quorum sensing allows synchronization of important cellular responses, for example, bioluminescence and root nodulation, with the host [116].

Five main categories of cell-to-cell signaling systems have been described [117, 118]. Two of these systems, which use *N*-acyl homoserine lactones (AI-1, for autoinducer 1) and AI-3, an aromatic aminated compound whose structure is presently unknown [119], are found in Gram-negative bacteria. Gram-positive cells use an autoinducer polypeptide system. The fourth system, using various furanones collectively referred to as AI-2 as signals, is found in both Gram-positive and Gram-negative cells. *P. aeruginosa* also produces a 4-quinolone molecule called pseudomonas quinolone signal (PQS) [118]. Putative biosynthetic genes homologous to the *P. aeruginosa* genes of PQS system were discovered in *Burkholderia pseudomallei* and *Burkholderia thailandensis* [117], and the production of various 4-quinolones has been detected in *Burkholderia* species and in *Pseudomonas putida* [120].

Quorum sensing is probably best studied in *P. aeruginosa*. Three molecules, 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), C4-homoserine lactone (C4-HSL), and PQS, are involved in *P. aeruginosa* quorum sensing [7]. Multidrug efflux pumps are intimately involved in the functioning of all three systems, though the details of their function may presently be unclear.

nfxC-type mutants of *P. aeruginosa* overexpress the MexEF-OprN efflux system. It has been shown that variations in the transcriptional activator gene *mexT* can lead to MexEF-OprN overexpression [121]. However, *nfxC* should be considered a

phenotype, since not all of these mutants have been molecularly characterized, and overexpression of MexEF-OprN pump can result from mutations which are not necessarily linked to *mexT*. Köhler et al. [7] reported that production of extracellular virulence factors, such as pyocyanin, elastase, and rhamnolipids, was decreased in *nfxC*-type mutants. The latter were also deficient in swarming motility (a multicellular phenomenon involving the coordinated and rapid movement of a bacterial population across a semisolid surface) [122]. This observation was in agreement with decreased production of rhamnolipids by *nfxC* mutants, since swarming was shown to depend on the properties of rhamnolipids as biosurfactants [123]. The production of these virulence factors depends on *rhl* cell-to-cell signaling system. Indeed, it was found that both the expression of the *rhlI* gene, which encodes for C4-HSL synthase, and the amount of C4-HSL autoinducer in culture supernatants were compromised in *nfxC* mutants. The interpretation of results of this study was hindered by the lack of molecular characterization of *nfxC* mutants, but it was shown that the reduction in the expression of *rhlI* and in the concentration of C4-HSL in supernatant required functional *mexEF* genes [7]. Short-chain autoinducers like C4-HSL diffuse apparently freely across the membrane [124]. It was, therefore, unlikely that MexEF-OprN pump would be involved in an active export of this signaling molecule [7]. Indeed, it was later shown that MexEF-OprN exported the precursor of PQS, 4-hydroxy-2-heptylquinolone, out of the cell, therefore diminishing its availability to intracellular enzyme PqsH which synthesizes PQS [125]. PQS, in turn, regulates *rhl* quorum-sensing system [126]; thus, decreased production of PQS leads to a decrease in expression of *rhlI* and in the production of C4-HSL.

Another multidrug efflux pump, MexAB-OprM, has also been shown to be involved in quorum sensing in *P. aeruginosa. nalB* mutants which overexpress the MexAB-OprM pump, similar to mutants overproducing MexEF-OprN, produced reduced levels of extracellular virulence factors, namely, pyocyanin, elastase, and casein protease [127]. In this case, it was shown that the effect is achieved via regulation of the *las* quorum-sensing system. This system does not directly regulate the expression of pyocyanin; however, it regulates the expression of *rhl* quorum-sensing genes, which, in turn, are responsible for the activation of production of pyocyanin by *nalB* mutants. MexAB-OprM overexpressing mutant produces considerably less 3-oxo-H12-HSL, which is a signal for *las* system. Compromised *las* system activation was proposed to result in diminished expression of *rhl* quorum-sensing genes and in decreased production of virulence factors. It has been proposed that MexAB-OprM efflux pump participates in the export of 3-oxo-H12-HSL, reducing its intracellular concentration and availability to activate LasR [127].

It appears that expressing multidrug efflux pumps would be detrimental to the virulence of *P. aeruginosa*. Nevertheless, MexAB-OprN is expressed constitutively in wild-type *P. aeruginosa*, though not to the same degree as in *nalB* mutants. Minagawa et al. [128] have shown that MexAB-OprM multidrug efflux pump ensures selectivity of the response to *P. aeruginosa*'s own quorum-sensing factor, 3-oxo-C12-HSL. Different species of bacteria use a variety of *N*-acyl-homoserine lactones with different acyl side-chain lengths. LasR, the transcriptional regulator of

the *las* quorum-sensing system, can bind and be activated by *P. aeruginosa* quorum-sensing signal, 3-oxo-C12-HSL, but in a strain with deleted *mexB* gene, it also binds a spectrum of 3-oxo-acyl-HSLs with chains with 8–14 carbons. In fact, in a *mexB* deletion strain, 3-oxo-Cn-HSLs with $n=9$, 10, and 11 are better activators of the transcription of *lasR* target gene, *lasB*, than 3-oxo-C12-HSL. As a result, *P. aeruginosa* mutant with *mexB* deletion can detect 3-oxo-C10-HSL quorum-sensing signal of *Vibrio anguillarum* and start producing pyocyanin in response [128]. In a strain with intact *mexB*, although transcription of *lasB* in response to 3-oxo-C12-HSL is somewhat dampened, it is, however, capable of discriminating between 3-oxo-C12-HSL and other homoserine lactones and is more responsive to the former [128].

The MexGHI-OpmD efflux pump appears to play a role in *P. aeruginosa* quorum sensing that is more difficult to interpret, due either to the complexity of metabolic and regulatory pathways or to insufficient data. The deletion of *mexI* or *opmD* genes resulted in the inhibition of the PQS biosynthetic genes, *phnA* and *pqsA*, and inability to produce PQS [129]. As a consequence, *mexI* and *opmD* deletion mutants failed to produce a number of virulence factors and demonstrated attenuated virulence in rat and plant infection models. These mutants were also impaired in growth. The authors suggested that mutants in *mexGHI-opmD* pump have a growth defect because they accumulate a toxic PQS precursor, anthranilate [129]. Anthranilate is produced by anthranilate synthase PhnAB and further modified by PqsA during the initial steps in PQS biosynthesis. In support of this hypothesis, introduction of *phnA* mutation, which would abolish anthranilate production, into Δ*mexI* strain restores its growth, while introduction of the mutation in *pqsA*, which would accumulate anthranilate, makes Δ*mexI* strain nearly unviable [129]. It is, however, not clear why a wild-type strain with intact MexGHI-OpmD would produce more PQS than Δ*mexI* strain. According to this hypothesis, MexGHI-OpmD pump would export anthranilate out of the cell and therefore make it unavailable for further intracellular steps in PQS biosynthesis.

The quorum-sensing systems of *B. pseudomallei* are known to produce up to six different types of acyl-homoserine lactones, the composition of which differs somewhat from strain to strain [130]. The secretion of all of them was abolished in the null mutant in *bpeAB-oprB* multidrug efflux pump [130]. Only one of the six acyl-homoserine lactones, *N*-octanoyl-homoserine lactone, was still synthesized intracellularly by this mutant. The defect in the synthesis of other acyl-homoserine lactones was suggested to result from the deficiency in the secretion of quorum-sensing compounds [130]. The lack of quorum-sensing signaling abolished the expression of autoinducer synthase BpsI and might also negatively regulate the expression of other relevant biosynthetic genes. BpeAB-OprB function is necessary for optimal production of quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and for biofilm formation. *bpeAB* mutant also exhibited significantly attenuated cell invasion and cytotoxicity toward human lung epithelial and human macrophage cells [131]. These results are however dependent on the genetic background of the *B. pseudomallei* cells. Despite dramatic results obtained for KHW strain, BpeAB-OprB pump in the strain 1026b did not play any role either in the export of acyl-homoserine lactones or in virulence factor production [132].

Multidrug transporters appear to be involved in the export of a quorum-sensing signals or signals of unknown nature in *E. coli*. It was shown that cells with deletion of genes coding for AcrAB or NorE multidrug efflux pumps grow to a higher cell density in stationary phase [133]. Overproduction of either pump caused cells to reach lower density. Conditioned medium from pump mutant cells and conditioned medium from cells overexpressing *acrAB* were, correspondingly, less and more repressive to cellular growth than medium conditioned by growth of wild-type cells. Also, expression of the *rpoS* gene encoding the stationary phase sigma factor is induced earlier in cells overexpressing *acrAB* and later in *acrAB* mutant cells. These results are consistent with AcrAB-TolC and NorE efflux pumps being involved in exporting a quorum-sensing signal out of the *E. coli* cell. Indeed, entry into stationary phase is typically controlled by quorum sensing [134], although the quorum-sensing signal or system responsible for stationary phase entry remains unidentified in *E. coli*. The authors have shown that the deletion of LuxS, which synthesizes the quorum-sensing signal AI-2 in *E. coli*, does not affect these results; therefore, some other molecule(s) must be involved.

27.12 Effect of Multidrug Efflux Pumps on Biofilm Formation

Biofilm is a structured adherent microbial community encased in extracellular matrix. Many bacteria in natural, industrial, and clinical settings predominantly live in biofilms. Biofilms constitute tremendous clinical challenge, being extremely resistant to antibiotics, host immune response, disinfectants, and some physical treatments. The process of biofilm formation is highly complex and involves multiple genes. This process is known to be regulated by cyclic diguanosine-5′-monophosphate, small RNAs, and quorum sensing. A separate chapter of this book is devoted to biofilm (see Chap. 25); here, we will discuss it briefly.

Multidrug efflux pumps appear to play an important role in biofilm formation. Matsumura et al. [135] studied biofilm formation in 22 *E. coli* mutants that were missing various multidrug efflux pumps. They found that all of the mutants showed compromised biofilm formation. The highest inhibition of biofilm formation was observed in mutants lacking *emrD*, *emrE*, *emrK*, *acrD*, *acrE*, and *mdtE* genes. Similarly, ten *Salmonella* strains with mutations in multidrug efflux systems were compromised in their ability to form biofilm [136]. Efflux inhibitors phenylalanine-arginine β-naphthylamide, carbonyl cyanide *m*-chlorophenylhydrazone, and chlorpromazine also repressed biofilm formation in *S. enterica*. Efflux inhibitors were also shown to be effective in preventing biofilm formation in *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* [137], as well as in *P. putida* [138]. Focused research on inhibition of biofilm production in *acrB* and *tolC* mutants of *S. enterica* showed that both strains exhibited transcriptional repression of the structural genes responsible for biosynthesis of a proteinaceous filament named curli, which is a major component

of the biofilm extracellular matrix [137]. Interestingly, it was demonstrated that the defect in biofilm formation does not result from the inability of *acrB* and *tolC* mutants to export a biofilm-promoting compound [137].

27.13 Concluding Remarks

Antimicrobial resistance is a key phenotype of bacterial pathogens that adversely affects the effectiveness of chemotherapy. How the resistance feature influences interaction between bacterial species and their hosts such as humans remains a topic of intense significance. In this chapter, specific examples have been provided to support the involvement of multidrug efflux pumps in bacterial pathogenicity, because of impacts of multidrug exporters on bacterial responses to diverse hostile environments (antimicrobial substances, nitrosative and oxidative conditions), fitness, colonization, and virulence production. Obviously, this relationship well reflects a general observation that the overall effects from drug resistance on bacterial pathogenicity are dependent on four major factors: the specific bacterial species, resistance and virulence mechanisms, the ecological niche, and the host [139]. However, it is evident that baseline or inducible expression of multidrug efflux pumps is often essential for bacterial virulence, suggesting that an optimized efflux pump expression has likely been evolved for bacterial survival and persistency. Although overproduction of certain drug efflux pumps comes with biological burden and fitness cost, the roles of multidrug efflux pumps in bacterial pathogenesis cannot be underestimated. This is consistent with isolation of a large number of multidrug efflux pump-overproducing strains (e.g., *P. aeruginosa*) from clinical samples. Hence, the contribution of drug efflux systems to bacterial fitness and virulence provides another strong argument for targeting efflux process for the discovery and development of antimicrobial agents or antimicrobial adjuvants.

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