Chapter 10
Antimicrobial Drug Efflux Pumps in *Salmonella*

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Abstract *Salmonella* species are causative organisms of salmonellosis, and the prevalence of multidrug-resistant *Salmonella* has increased dramatically. These multidrug-resistant isolates have been found in both humans and animals and thus pose a major public health concern. Drug resistance in *Salmonella* has been shown to be largely attributable to multiple target gene mutations and to active efflux by pumps. At least ten drug efflux system genes in the genome of this organism have been experimentally identified to date, and some efflux pump genes encoded in plasmids have been also identified. This chapter describes the drug resistance and virulence roles of efflux pumps and their regulation in *Salmonella*.

Keywords *Salmonella* • Antimicrobial resistance • Efflux • RND efflux pumps • Plasmid • Virulence • AcrAB • TolC • RamA • RamR

10.1 Introduction

Salmonella species exist all over the world and are responsible for causing acute gastroenteritis and typhoid/paratyphoid [1]. *Salmonella enterica* serovar Typhimurium is contagious in rodents, including mice, causing a systemic infectious disease, closely resembling human typhoid [2, 3]. In humans, it produces acute gastroenteritis and is a cause of food poisoning. Fluoroquinolones represent the drug of choice for the treatment of a wide range of human infectious diseases, and they were also introduced into veterinary medicine in Europe in the late 1980s through the early 1990s and the USA in 1995. Following their introduction, fluoroquinolone-resistant strains of *Salmonella* started to emerge [4]. Fluoroquinolone resistance in *S. enterica* serovar Typhimurium has been shown to be largely attributable to multiple target gene mutations and to active efflux by

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multidrug transporters [5, 6]. Also, the increasing prevalence of multidrug resistance has been found in *Salmonella* isolates from both humans and animals and thus poses an important public health concern [7, 8].

The genome sequences of *Salmonella* spp. indicate the presence of numerous efflux pump genes that encode transporters of various superfamilies and families [9, 10]. At least ten drug efflux pump genes in the genome of *S. enterica* serovar Typhimurium have been experimentally identified to date [11–15]. Some efflux pump genes encoded on plasmids have been also identified [16–18]. In addition to their roles in drug resistance, it was shown that the efflux pumps contribute to *Salmonella* virulence [13, 15, 19, 20]. Physiological functions of efflux pumps in *Salmonella* have been also reported with roles in metal resistance [21, 22], biofilm formation [23], colonization [11], adhesion, and cell invasion [19]. In this chapter, the roles of *Salmonella* efflux pumps in drug resistance and their physiological functions and regulation are described.

10.2 The AcrAB Efflux Pump in *Salmonella*

S. enterica serovar Typhimurium Tn*phoA* mutants with increased susceptibility to biological and chemical detergents were reported [24], and it was found that one mutant LX1054 had a defect in a multidrug resistance pump AcrB [11]. Nikaido et al. [12] found that the previously reported drug-susceptible *S. enterica* serovar Typhimurium [25] carried a mutation in the *acrAB* operon. The mutant of *acrAB* exhibited increased susceptibility to a wide range of antimicrobial agents including antibiotics, bile salts, dyes, detergents, and disinfectants as shown in Table 10.1 [12, 13]. AcrA and AcrB in *S. enterica* serovar Typhimurium strain LT2 exhibit the amino acid identities of 92 and 95 % with those in *Escherichia coli* [13]. High-level fluoroquinolone resistance in *S. enterica* serovar Typhimurium phage type DT204 has been previously shown to be essentially due to both multiple target gene mutations and active efflux by the AcrAB–TolC efflux system [5, 6]. In other drug-resistant isolates of *Salmonella*, overexpression of *acrB* is also reported [29], and antimicrobial treatment of *Salmonella* results in the increased expression of *acrB* [30, 31]. A post-therapy isolate of *S. enterica* serovar Typhimurium (after treatment with fluoroquinolones and β-lactams) was found to carry a Gly288Asp substitution in AcrB [32]. This residue substitution is located in AcrB drug-binding pocket and significantly affects the structural and dynamic properties of AcrB, resulting in alternated substrate specificity (i.e., reduced susceptibility to fluoroquinolone but increased susceptibility to doxorubicin and minocycline) [32]. Low-level exposure of *S. enterica* serovar Typhimurium to a biocide, either a quaternary ammonium compound, an oxidative compound, or a halogenated tertiary amine compound, in the laboratory selected mutants that were cross-resistant to nalidixic acid, ciprofloxacin, chloramphenicol, tetracycline, and/or triclosan [33]. Among multiple mutations carried by these mutants, derepression of AcrAB–TolC expression was observed [33].

Table 10.1 Substrate profiles of characterized *Salmonella* efflux pumps

Transporter family/efflux pump	Substrates	Reference
RND		
AcrAB	ACR, BAC, CAR, CEF, CHL, CHO, CLX, CTX, CV, DOC, DOR, EB, ERY, FOX, FUA, MB, NAF, NAL, NOR, NOV, PEN, R6G, RIF, SDS, SUL, TET, TPP, TRI, TRX	[11–13, 26]
AcrD	AZT, CAR, DOC, NAF, NOV, OXA, SDS, SUL	[13, 27]
AcrEF	ACR, CHL, CV, DOC, DOR, EB, ERY, NAL, NOR, MB, NOV, R6G, SDS, TET, TPP, TRI	[13, 26]
MdsABC (GesABC)	ACR, BAC, CHL, CLX, CV, EB, MB, NAF, NOV, THL, TPP	[13, 22, 28]
MdtABC	DOC, NOV, SDS	[13]
MFS		
EmrAB	NAL, NOV, R6G, SDS, TRI	[13, 26]
MdfA	CHL, DOR, NOR, TET	[13]
SmvA	ACR, EB, MG, NAL, PQ, PY	[14]
MATE		
MdtK	ACR, DOR, NOR	[13]
ABC		
MacAB	ERY	[13]

ACR acriflavine, *AZT* aztreonam, *BAC* benzalkonium chloride, *CAR* carbenicillin, *CEF* cephalothin, *CHL* chloramphenicol, *CHO* cholate, *CLX* cloxacillin, *CTX* cefotaxime, *CV* crystal violet, *DOC* deoxycholate, *DOR* doxorubicin, *EB* ethidium bromide, *ERY* erythromycin, *FOX* cefoxitin, *FQ* fluoroquinolones, *FUA* fusidic acid, *MB* methylene blue, *MG* malachite green, *NAF* nafcillin, *NAL* nalidixic acid, *NOR* norfloxacin, *NOV* novobiocin, *OQX* olaquindox, *OXA* oxacillin, *PEN* penicillin G, *PQ* paraquat (methyl viologen), *PY* pyronine B, *R6G* rhodamine 6G, *RIF* rifampicin, *SDS* sodium dodecyl sulfate, *SUL* sulbenicillin, *TET* tetracyclines, *THL* thiamphenicol, *TPP* tetraphenylphosphonium, *TRI* triclosan, *TRX* Triton X-100

10.3 The *Salmonella* Drug Efflux Pumps Identified by Genomic Information

Genomic analyses revealed that *Salmonella* strains possess five putative RND efflux systems (http://www.membranetransport.org). Four of them, AcrAB (AcrA, membrane fusion protein; AcrB, RND transporter), AcrD, AcrEF (AcrE, membrane fusion protein; AcrF, RND transporter), and MdtABC (MdtA, membrane fusion protein; MdtB and MdtC, RND transporters), have homologs in *E. coli* with approximately ~90 % amino acid identity (Table 10.1) [13]. MdtB and MdtC are each an RND pump and usually function as one drug efflux system [34]. The last putative RND system is the *Salmonella*-specific MdsABC (MdsA, membrane fusion protein; MdsB, RND transporter; MdsC, outer membrane protein). In addition to the RND pumps, efflux systems belonging to the major facilitator superfamily (MFS) (EmrAB, MdfA, and SmvA), multidrug and toxic compound extrusion (MATE)

family (MdtK), and the ATP-binding cassette (ABC) superfamily (MacAB) transporter families were also experimentally identified (Fig. 10.1) [13, 14, 35].

The genes of *acrAB*, *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, and *macAB* were cloned into the multicopy number plasmid, and their ability to confer drug resistance upon the *Salmonella acrB* mutant was investigated (Table 10.1) [13]. The plasmids carrying efflux operons or genes that confer multidrug resistance phenotypes against various antimicrobial compounds are shown in Table 10.1. It was also reported that the deletion mutant of the *smvA* gene showed increased susceptibility to a range of cytotoxic agents (Table 10.1) [14]. Overproduction of SmvA provided acriflavine resistance in the *Salmonella acrB* mutant (unpublished data). A recent study also showed that *Salmonella* EmrAB and AcrEF pumps may have additive effects with the major efflux system AcrAB in decreased susceptibility to triclosan [26]. Deletion of the *tolC*, *acrB*, or *acrAB* genes resulted in strains with increased susceptibility to various compounds, and the *acrB*, *acrAB*, and *tolC* mutant strains have overlapping substrate susceptibility profiles, which is in agreement with the notion that the encoded proteins interact as a tripartite efflux complex system. The *tolC* mutant was more susceptible to certain compounds including novobiocin, deoxycholate, and sodium dodecyl sulfate than the *acrAB* mutant [13, 36] – suggesting a functional role in other efflux systems. And a strain with nine drug exporters (*acrAB*, *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, and *macAB*) deleted was shown to be more susceptible to novobiocin, deoxycholate, and sodium dodecyl sulfate, compared to the Δ*acrAB* mutant. On the other hand, strains deleted for the *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, or *macAB* genes exhibited the same drug susceptibility as the wild-type strain [13]. These two lines of data suggest, similar to *E. coli*, a predominant if not overwhelming role of the AcrAB in the drug resistance phenotype. Furthermore, that other pump expression is minimal and/or their functions are masked by overlapping substrate repertoires with AcrAB. The expression levels of drug transporter genes under laboratory conditions were investigated by streaking out onto X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) LB agar plate strains in which the *E. coli lacZY* genes replaced the chromosomal copy of the drug efflux genes in *Salmonella* [13]. The *tolC-lacZY* and *acrA-lacZY* strains were blue on plates, whereas the *acrD-lacZY*, *emrA-lacZY*, *mdfA-lacZY*, *mdtK-lacZY*, and *macA-lacZY* strains were only faint blue. Thus, the AcrAB–TolC efflux system is expressed in the complex laboratory media, whereas the other efflux systems appear to require additional cues for expression [13].

TolC is required for the function of seven drug efflux systems AcrAB, AcrD, AcrEF, MdsAB, MdtABC, EmrAB, and MacAB in *S. enterica* serovar Typhimurium [27]. Therefore, plasmids carrying the *acrAB*, *acrD*, *acrEF*, *mdsAB*, *mdtABC*, *emrAB*, or *macAB* genes do not confer resistance to the *tolC* mutant, whereas they conferred drug resistance in the *acrB* mutant. Plasmids carrying *mdsABC*, *mdfA*, or *mdtK* provide resistance to the *tolC* mutant, indicating that these three efflux systems function without TolC. The crystal structure of TolC (i.e., ST50) from *Salmonella* Typhi was recently reported, showing the structural basis for TolC role in multidrug efflux pumps across the outer membrane [37]. The *Salmonella*-specific

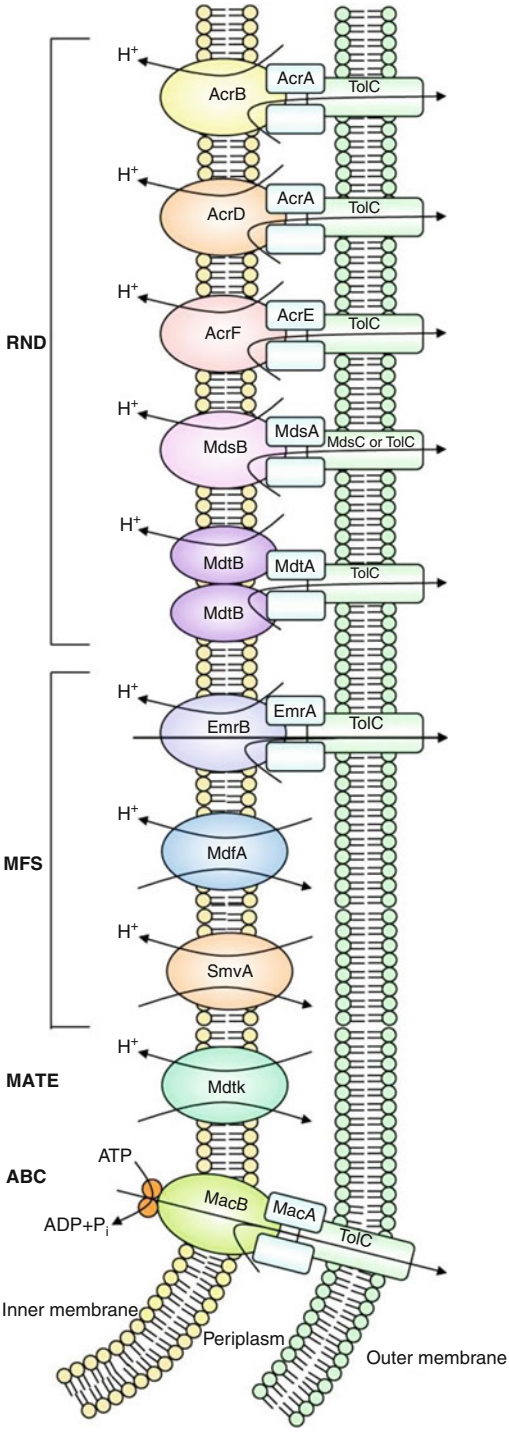

Fig. 10.1 Diagrammatic representation of the structure of multidrug efflux pumps in *Salmonella* and their location on the membranes (Modified from Horiyama et al. [27])

drug efflux system *mdsABC* operon codes for a putative outer membrane protein – MdsC – which is in contrast to the other operons coding for RND-type drug transporter genes. In *E. coli*, most operons coding for RND-type drug transporter homologs lack genes for outer membrane proteins [38] because they rely on TolC as their outer membrane component [39–42]. Overexpression of both the *mdsABC* and *mdsAB* genes produced drug resistance in the Δ*acrB mdsABC* strain. On the other hand, overexpression of *mdsABC*, but not *mdsAB*, resulted in drug resistance to the Δ*acrB tolC mdsABC* strain. These findings indicate that the drug resistance phenotype conferred by the MdsAB system is dependent on the presence of either the MdsC or TolC proteins and that the MdsAB system can function with both TolC and MdsC outer membrane components [13, 27].

Except for the *acrD* gene, all RND efflux system genes also code for a membrane fusion protein in the same operon. The overproduction of AcrD yielded multidrug resistance in the Δ*acrB* mutant against β-lactam antibiotics and other agents (Table 10.1). It was revealed that AcrD requires AcrA and TolC to function (Fig. 10.1) [27, 43]. One possibility for AcrD utilizing AcrA, coded in a different operon, is that AcrD may form a complex with AcrA and TolC when mutations occur in AcrB and compensate for the lost function of AcrAB–TolC multidrug efflux system. Another possibility is that AcrA contributes to different biological functions by forming complexes with two different RND pumps, AcrB and AcrD. Such a functional network of multidrug efflux pumps may contribute to bacterial adaptation to various environmental conditions [43].

10.4 Plasmid-Mediated Fluoroquinolone Efflux Pumps

In addition to the efflux systems encoded in the *Salmonella* genome, plasmid-mediated fluoroquinolone efflux pumps have been identified. The MFS efflux pump QepA was originally identified in *Escherichia coli* clinical isolate [44]. Resistance levels against ciprofloxacin, enrofloxacin, and norfloxacin were significantly elevated in *E. coli* transformants harboring *qepA* under AcrB–TolC-deficient conditions. The intracellular accumulation of norfloxacin was decreased in a *qepA*-expressing *E. coli* transformant [44]. In *Salmonella*, *qepA* was first detected in the clinical isolates obtained in the hospital clinic in Spain [45]. Subsequently, *qepA* was detected in several quinolone-resistant *Salmonella* spp. clinical isolates [46, 47].

Plasmid-encoded multidrug efflux genes *oqxAB* were also identified in *Salmonella* [18, 48–51]. The quinoxaline-di-*N*-oxide olaquindox has been a growth enhancer in pigs. Its antimicrobial activity is due to inhibition of DNA synthesis [52]. The *oqxAB* genes were originally identified from a conjugative plasmid isolated from *E. coli* [53]. OqxA, a membrane fusion protein, and OqxB, an inner membrane protein, are homologous to several RND family efflux systems from different species. Plasmids containing the *oqxAB* genes yielded high resistance to olaquindox in *E. coli*. The *oqxAB*-encoded pump also conferred high resistance to

chloramphenicol [53]. H^+-dependent ethidium efflux abilities of OqxAB were also confirmed in *E. coli* [53]. A derivative of the plasmid encoding OqxAB was readily transferred to enterobacterial pathogens and transconjugants showed reduced susceptibility to chloramphenicol, ciprofloxacin, and olaquindox [54]. OqxAB were found in human clinical isolates on a plasmid in *E. coli* and on the chromosome of *Klebsiella pneumoniae*. IS26-like sequences flanked the plasmid-mediated *oqxAB* genes, suggesting that they had been mobilized as part of a composite transposon [55]. After the first detection of *oqxAB* in *Salmonella* spp. isolated from food [47], the genes were identified in many *Salmonella* isolates which exhibited resistance to fluoroquinolones [48–51, 56, 57].

10.5 Virulence Roles of *Salmonella* Drug Efflux Pumps

Drug efflux systems are evolutionarily ancient and are found throughout the three domains of life [58, 59]. These systems are fundamental to the bacterial physiology and some have roles other than conferring resistance to antimicrobials. Recognizing that the AcrAB–TolC system serves as an important antimicrobial resistance determinant [11, 12], it was also reported that this efflux system is required for *Salmonella* resistance to bile salts [11, 60] which are found exclusively associated with higher vertebrates. It was shown that the *acrB* mutant of *S. enterica* serovar Typhimurium exhibited a reduced capacity to colonize the intestinal tract, and this suggests that AcrAB–TolC efflux system play an important role in mouse intestinal colonization [11]. It was also reported that the deletion of the *macAB* genes attenuated *Salmonella* virulence, and a strain lacking all drug efflux systems was avirulent when mice were inoculated by the oral route [13]. These results indicate that drug efflux genes are required for *Salmonella*'s ability to cause a lethal infection in mice. Utilizing similar approaches, Buckley et al. [19] studied the role of efflux systems on virulence of *S. enterica* serovar Typhimurium using efflux-defective mutants in a chicken model and found that mutants deficient in either *acrB* or *tolC* genes colonized poorly and did not persist in the avian gut, indicating that AcrAB–TolC system is essential for the colonization of *S. enterica* serovar Typhimurium in chickens. Experiments using BALB/c mice by the oral route with isogenic strains harboring deletions in efflux genes showed that the mutation in *tolC* of *S. enterica* serovar Typhimurium attenuated virulence [13], as reported for an *S. enterica* serovar Enteritidis *tolC* mutant [61]. Inactivation of the MarA or RamA activator (which upregulates AcrAB–TolC expression; see Sect. 10.7) reduced both the invasion and survival ability of *Salmonella choleraesuis* in the host cells and virulence in mice [62].

Salmonella MacAB pump plays a role in the detoxification of reactive oxygen species, compounds that salmonellae are exposed to at various stages of infection [63]. The *macAB* operon is induced upon exposure to hydrogen peroxide and is critical for survival of *S. enterica* serovar Typhimurium in the presence of oxidative stress. Furthermore, *macAB* is required for intracellular replication inside murine macrophages but is not required for survival in reactive oxygen species-deficient

macrophages [63]. Bogomolnaya et al. [63] suggested the presence of a soluble anti-peroxide compound secreted by *Salmonella* cells through a MacAB-dependent mechanism. In *E. coli*, MacAB is involved in the secretion of heat-stable enterotoxin II [64], and MacA binds lipopolysaccharide core specifically with high affinity [65]. Also, it was recently reported that protoporphyrin is exported by MacAB–TolC in *E. coli* [66]. Because high protoporphyrin levels result in production of reactive oxygen species [67], Turlin et al. [66] proposed that MacAB is involved in the efflux of intracellular protoporphyrin which decreases reactive oxygen species formation in the bacterial cytoplasm, providing a possible explanation for the role of MacAB in *Salmonella* pathogenicity.

10.6 Physiological Functions of *Salmonella* Drug Efflux Pumps

There are several reports about the physiological functions of *Salmonella* drug efflux systems. The BaeSR two-component signal transduction system activates the *acrD* and *mdtABC* expression in response to indole, copper, and zinc. BaeSR, AcrD, and MdtABC contribute to copper and zinc resistance in *Salmonella* [21]; andiron and sodium tungstate are inducers of the BaeR regulon suggesting MdtA, AcrD, and AcrB exist for the waste disposal of tungstate from the cell [68]. Additionally, the MdsABC pump (also called GesABC) is required for gold resistance and the *mdsABC* operon is controlled by GolS which is a MerR-like sensor and highly selective for Au ions [22]. In contrast to heavy metal-specific CusCBA RND pump of *E. coli*, MdsABC, accommodates a large number of substrates including many antibiotics (Table 10.1) [28].

Recent studies have showed that defects in efflux activity impair biofilm formation. In *S. enterica* serovar Typhimurium, deletion of any efflux pump or chemical inhibition of the efflux activity results in compromised ability of *Salmonella* to form biofilm [23]. The defect of biofilm formation in efflux mutants resulted from transcriptional repression of curli biosynthesis genes and consequently inhibition of its production, but was not associated with altered aggregative ability or export of any biofilm-promoting factor [69] (also see Chap. 25).

10.7 Regulation of *Salmonella* Drug Efflux Pumps

The key to understanding how bacteria utilize multidrug efflux pumps lies in the regulation of pump expression. The data currently available show that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control. For example, expression of *macAB* is controlled by the PhoPQ system, the master regulator for the virulence of *Salmonella* (Table 10.2) [13]. A sequence

resembling the PhoP binding box exists in the upstream of the *macAB* operon [78]. DNase I footprinting analysis with the purified PhoP protein showed protection of the region upstream of the *macA* open reading frame [13], indicating that the PhoPQ two-component signal transduction system controls *macAB* directly. Analysis of mRNA levels of drug efflux genes revealed that the expression of *macAB* is induced when the organism infects macrophages [15]. A recent study also showed that hydrogen peroxide induces expression of *macAB* [63], supporting the induction of *macAB* inside macrophages and the existence of additional regulator to control the *macAB* genes responsive to hydrogen peroxide.

Moreover, positive regulation of the multidrug efflux pump *mdtABC* and *acrD* genes by the BaeSR two-component signal transduction system was found (Table 10.2) [21]. In addition to the roles of MdtABC, AcrD, and BaeSR in multidrug resistance, they contribute to copper and zinc resistance in *Salmonella* as described above. Both copper and zinc are essential for organisms but can be toxic at high levels, and microorganisms express diverse resistance mechanisms. The expression of *mdtABC* and *acrD* is induced by copper or zinc, and BaeSR is involved in this induction (Table 10.2). This finding indicates that the MdtABC and AcrD efflux systems have physiological roles in metal homeostasis beyond multidrug resistance [21]. It was also reported that GolS controls MdsABC in response to Au ions [22].

Table 10.2 The known regulators of multidrug efflux pumps in *Salmonella*

Efflux pump	Regulator	Regulator family	Inducible signal	Reference
AcrAB	RamA	AraC	Bile, indole	[70]
	RamR	TetR	Berberine, bile, crystal violet, dequalinium, ethidium bromide, rhodamine 6G	[71, 72]
	AcrR	TetR	Unknown	[73]
	MarA	AraC	Unknown	[74]
	SoxS	AraC	Paraquat	[75]
AcrEF	AcrS	TetR	Unknown	[76]
	H-NS	Histone-like protein	Unknown	[77]
AcrD	BaeSR	Two-component system	Indole, copper, iron, zinc tungstate	[21, 68]
	CpxAR	Two-component system	Indole, copper, zinc	[21]
MdtABC	BaeSR	Two-component system	Indole, copper, zinc, tungstate	[21, 68]
	CpxAR	Two-component system	Indole, copper, zinc	[21]
MdsABC	GolS	MerR	Gold	[22]
MacAB	PhoPQ	Two-component system	Magnesium	[13]

Mutations in *acrR* contribute to overexpression of *acrAB* and increases resistance to multiple drugs in *Salmonella* [73]. The histone-like protein (H-NS) modulates multidrug resistance through repression of the *acrEF* genes [77]. Eaves et al. [74] suggested that *acrB*, *acrF*, and *acrD* are coordinately regulated and that their expression is also influenced by the expression of the transcriptional activators *marA* and *soxS*. Nikaido et al. [75] found that *acrAB* induction in response to methyl viologen is dependent on SoxS. Indole, bile salts, and an *E. coli*-conditioned medium were also able to induce the expression of *acrAB* in *Salmonella*. The *acrAB* induction by these three signal sources is completely dependent on the *Salmonella*-specific regulator RamA, indicating that RamA plays a major role in inducing *acrAB* (Table 10.2) [70]. RamA belongs to the AraC transcriptional activator family, and this gene appears to be specific for *Salmonella* serovars and is absent in many other Gram-negative microorganisms; notable exceptions are *Klebsiella pneumoniae* and *Enterobacter* species [79–81]. The AcrAB induction pathway in *Salmonella* is different from that in *E. coli*. Bile induces AcrAB in both *Salmonella* and *E. coli*. In *E. coli*, the transcriptional factor Rob plays a major role in inducing *acrAB* expression in response to bile [82]. However, bile induction of *acrAB* in *Salmonella* is dependent on RamA, not Rob. Other regulators, including MarA, SoxS, SdiA, and AcrR, are not involved in AcrAB induction by indole and bile [70]. These facts suggest that RamA is the major regulator of *Salmonella acrAB* and may mask the contributions of any other *acrAB* regulators.

Abouzeed et al. [83] demonstrated that the inactivation of the *ramR* gene upstream of *ramA* resulted in an increased expression of *ramA* and the AcrAB efflux pump, indicating that RamR is a local repressor of *ramA*. Inactivation of *marR*, *marA*, *soxR*, and *soxS* did not affect the susceptibilities of the *S. enterica* serovar Typhimurium strain LT2, whereas the disruption of *ramR* resulted in a multidrug resistance phenotype with this strain. In *E. coli*, multiple regulators, including MarA, Rob, SoxS, and SdiA, work together in controlling *acrAB* expression in response to *acrAB* inducers. This may be related to the lack of RamA in *E. coli*. Indeed, overproduction of RamA has induced the drug resistance level of *E. coli* [84, 85]. There may also be different induction mechanisms for *acrAB* via the RamA regulator. Indole was shown to induce *ramA* expression, and such increased expression of *ramA* can induce *acrAB*, whereas bile binds to RamA. This is reminiscent of the binding of bile to the Rob protein involved in regulation of *acrAB* in *E. coli* [82]. It seems that RamA can be converted from a low-activity state to a high-activity state in response to bile. More recently, Baucheron et al. [71] also identified a different induction mechanism of *acrAB* in response to bile whereby the bile-mediated activation of the *acrAB* and *tolC* multidrug efflux genes occurs via transcriptional derepression of the *ramA* activator gene, likely via the RamR repressor protein controlling expression of *ramA*. Indole and bile salts are found in various internal human environments, especially in the intestine [86, 87]. Indole is produced by many enteric bacterial species [87], and bile is often present in high concentrations in the intestinal tract [86]. Therefore, RamA may be required for *Salmonella* to detect environmental signals and for subsequent induction of the AcrAB–TolC system, resulting in excretion of toxic compounds into the surrounding environment

in the above examples, the intestine. A recent study showed heterogeneity in *ramRA* mutations and its differential impact on expression of regulator genes *ramA*, *marA*, *soxS*, and *acrR* and efflux component genes *acrB*, *acrF*, *emrB*, and *tolC*, revealing deletions that affected RamR-binding site exhibiting a major impact on the *ramA* transcript level and the multidrug resistance phenotype [88].

10.8 Structure of Multidrug Efflux Pump Regulator RamR with Multiple Drugs

As described above, RamR and RamA are important regulators for AcrAB–TolC in *Salmonella*. From the structural and biochemical analysis of RamR, a multidrug recognition mechanism of RamR occurs, whereby the DNA-binding activity is controlled by multiple drugs in order to induce *ramA* expression [72]. Yamasaki et al. [72] identified five substrates of the RamR protein, including berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G (Fig. 10.2). Similar approaches in crystallizing the TetR family regulators with multiple drugs have been also reported in QacR [89], TtgR [90], and CmeR [91]. The molecular weight of RamR in solution was calculated to be 36 kDa using gel filtration chromatography, which was conducted during the purification of the RamR protein. Dissolved RamR was found to exist in the dimer form in solution, and the molecular weight of the RamR monomer was 21 kDa [72]. The structure of RamR was initially determined at a resolution of 2.6 Å by multiple wavelength anomalous dispersion using selenomethionine modification. Subsequently, the RamR structure was determined at 2.1 Å by molecular replacement. Approximate overall dimensions of the RamR dimer were $58 \times 47 \times 44$ Å^3. RamR is composed of nine α-helices, and the three-helix bundle structures formed at the N-terminus maintain a helix-turn-helix motif conserved in DNA-binding sites. The structure of the RamR DNA-binding site is similar to that of other TetR family regulators. By the surface plasmon resonance analysis, it was found that five compounds, berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G, bind to the RamR protein. In contrast, tetracycline did not show any indication of binding to RamR. Using a *ramA* reporter plasmid, a ß-galactosidase assay showed the enhanced promoter activity of *ramA* when bacterial cells were treated with berberine, crystal violet, dequalinium, ethidium bromide, or rhodamine 6G. The crystal structures of RamR in complex with berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G were determined at a resolution of 2.4, 2.2, 2.6, 1.6, and 2.5 Å, respectively [72]. The structure reveals that RamR binds two molecules of berberine, ethidium bromide, or rhodamine 6G per dimer. And RamR binds one crystal violet or dequalinium molecule per dimer. It was originally reported that all the ligands bind to QacR with a 1:2 stoichiometry (one ligand per QacR dimer) [89], while either 1:2 or 1:1 stoichiometry has been observed for RamR. Similar observations were reported in TtgR [90]. The orientation of all agents is parallel with the Phe155 of RamR, suggesting that all these drugs bind with RamR through π–π stacking interactions. In contrast

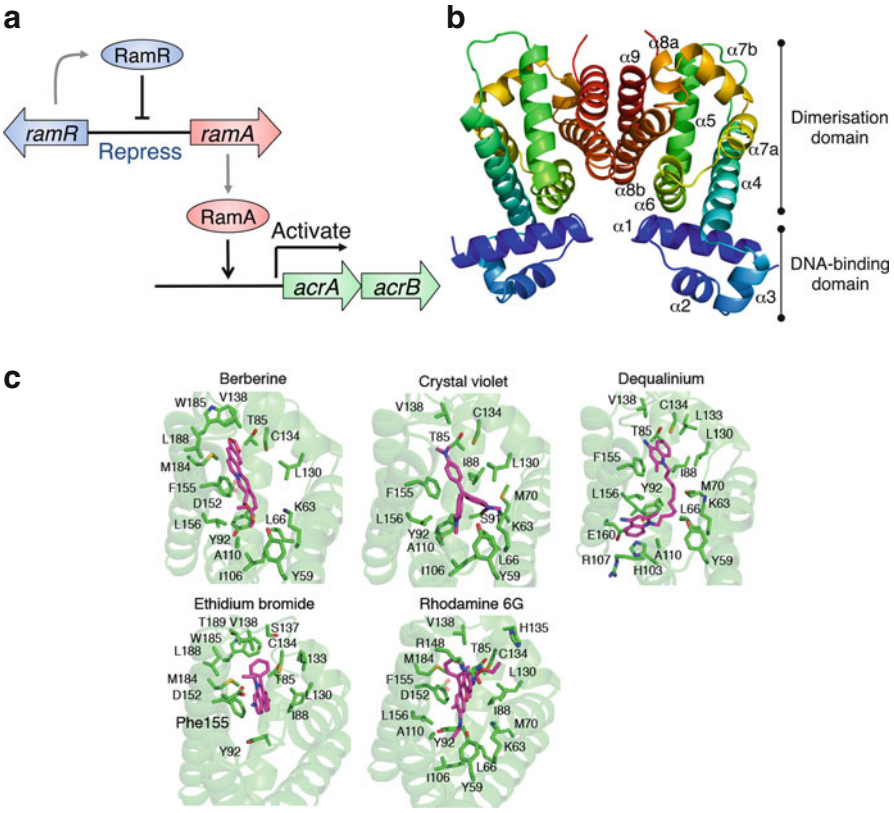

Fig. 10.2 Regulatory cascade and structure of RamR. (**a**) Model for gene regulation by RamR. RamR represses expression of the *ramA* gene, which encodes the activator protein for the *acrAB* efflux pump genes. RamR binds to the intergenic region between the *ramR* and *ramA* genes, and RamA binds to the upstream region of *acrAB*. (**b**) Crystal structure of the RamR dimer. Each monomer is colored as follows: the α-helices are represented in *blue* (*α1*), *marine* (*α2*), *sky blue* (*α3*), *cyan* (*α4*), *green* (*α5*), *limon* (*α6*), *yellow* (*α7a*), *deep olive* (*α7b*), *orange* (*α8a*), *brown* (*α8b*), and *red* (*α9*). (**c**) Multidrug recognition by RamR. Substrate binding site of RamR with a bound molecule berberine, *crystal violet*, dequalinium, ethidium bromide, or rhodamine 6G. Key residues are shown, including residue Phe155, which is involved in π–π stacking interactions with drugs. Carbon atoms of drugs and RamR are shown in *magenta* and *green*, respectively. Nitrogen, oxygen, and sulfur atoms are shown in *blue*, *red*, and *yellow*, respectively (Figure is modified from Yamasaki et al. [72])

to the common interaction of all of these drugs with Phe155, each individual drug was also found to interact with a different set of amino acid residues other than Phe155. The interaction of different sets of amino acid residues with each drug indicates that multiple drugs are recognized by the multisite binding of RamR [72]. Comparison of the liganded structures with an unliganded RamR structure reveals that drug binding triggers an expansion of the distance between the N-termini of the helix-turn-helix motifs in the RamR dimer. This expansion occurred as a result of the binding of all of the drugs examined. By the electrophoretic mobility shift assays

and surface plasmon resonance experiments, RamR substrates interact with their recognition sites to reduce the DNA-binding affinity of RamR, resulting in the induction of *ramA* [72]. Because RamA has also been reported to negatively influence virulence in *S. enterica* serovar Typhimurium by downregulating expression of the *Salmonella* pathogenicity island 1 [92], determining the crystal structure of RamR is the first step in understanding the structural basis for the function of the regulatory proteins that control both drug resistance and virulence in pathogens. This effort extended our knowledge of transcriptional regulation mediated by RamR, a regulator of multidrug resistance in several enterobacterial pathogens.

10.9 Concluding Remarks

Post-genomic research has demonstrated that bacteria possess a large number of drug efflux system genes. As described in this chapter, at least ten drug efflux systems in the genome of *S. enterica* have been experimentally identified to date. Under normal growth conditions, most of drug efflux pumps are thought to be weakly expressed [13]. Increased expression of such efflux systems is possible when mutations occur in their regulatory factors. In fact, various types of mutations in *ramR* and the *ramR–ramA* intergenic region were identified in multidrug-resistant strains of *S*. Typhimurium, other *S. enterica* serovars, and *K. pneumoniae*, which result in increased expression of *ramA* and an increase in efflux-mediated multidrug resistance [83, 93, 94]. Also, it was reported that overexpression of the multidrug efflux operon *acrEF* occurs by insertional activation with IS1 or IS10 elements in *S. enterica* serovar Typhimurium DT204 *acrB* mutants selected with fluoroquinolones [76]. A mutation in *acrR*, the local repressor of *acrAB*, was found for two ciprofloxacin-resistant selected mutants of *S. enterica* serovar Typhimurium [73]. In addition to these mutations, the structural and biochemical analysis showed that toxic compounds bind to RamR resulting in the increased efflux activity of *Salmonella* to protect this organism against the compounds [72].

Association of resistance mechanism with two-component signal transduction systems, which control the expression of drug efflux pumps, has also been identified in *Salmonella*. These findings suggest that the expression of efflux systems is transiently induced through some types of stimulation. In fact, this induction occurs as a result of various environmental stressors, such as low pH, osmotic changes, metals, and oxidative stress. The mechanism by which efflux pumps are expressed in response to the environment suggests that they might be expressed in the growth environments of bacteria such as at infection sites. It is reasonable to assume that efflux systems are induced inside hosts because these contribute not only to drug resistance but also to bacterial virulence. Therefore, it is necessary to identify the regulatory network of multidrug transporters in order to understand their physiological functions. Moreover, determining the physiological substrate of efflux systems is an important area of study, which will contribute to the understanding of the role of drug efflux systems in virulence.

The mechanism by which drug efflux pumps contribute to bacterial virulence has three features. Firstly, the efflux system has the capacity to transport substrates necessary to establish virulence, for example, toxins. Secondly, the efflux system is able to export antibacterial substances present in the host (such as bile acid and antimicrobial peptides) in order to protect the bacteria from the host environment. Thirdly, it can transport factors contributing to bacterial homeostasis or promoting bacterial regulatory functions within the host (such as autoinducers). Currently, several research groups and pharmaceutical companies are conducting research to develop drug efflux pump inhibitors. As efflux systems contribute to multidrug resistance and bacterial virulence, efflux systems are an attractive target for the development of new drugs. If an effective inhibitor is found, it could play a role in the development of new therapies that could conquer bacterial multidrug resistance and virulence.

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