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SmvA, and not AcrB, is the major efflux pump for acriflavine and related compounds in *Salmonella enterica* serovar Typhimurium

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***Objectives*: The aim was to study the role played by SmvA pump in the efflux of quaternary ammonium compounds (QACs) in *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium).**

***Methods*: Mutants in the *smvA*, *acrB* and *tolC* genes were constructed by the red swap method. P22 was used to transduce *tolC* to *acrB* and *smvA* mutant strains. The susceptibility of these strains to acriflavine and a variety of QACs was determined by MIC assays.**

***Results*: In comparison with the *Salmonella* Typhimurium wild-type strain, the *smvA* mutant was more susceptible to QACs than the *acrB* mutant strain. A *tolC* single mutant was more susceptible than an *acrB* mutant to QACs, acriflavine, ethidium bromide, malachite green and pyronin B. The *tolC–acrB* double mutant was as susceptible as the single *tolC* mutant to QACs. Additionally, the *smvA* mutant strain was more susceptible to acriflavine than the *acrB* mutant (MICs = 31.3 versus 125 mg/L, i.e. 4-fold). Finally, the *tolC–smvA* double mutant (3.9 mg/L) was approximately 10 times more susceptible to acriflavine than either *smvA* (31.3 mg/L) or *tolC* (31.3 mg/L) single mutants.**

***Conclusions*: It is the SmvA efflux pump, and not AcrB, that plays the major role in the efflux of acriflavine and other QACs from *Salmonella* Typhimurium. This apparently conflicting report is due to the fact that in *Escherichia coli* the *smvA* gene does not exist. Our results suggest that *tolC* and *smvA* genes encode components of two different efflux systems with overlapping specificities that work in parallel to export acriflavine and other QACs.**

Keywords: multidrug resistance, quaternary ammonium compounds, TolC, enteric bacteria

Introduction

Salmonella enterica is a pathogen that causes a variety of diseases in humans ranging from gastroenteritis to bacteraemia and typhoid fever.[1] Emerging resistance to antibiotics in *Salmonella* has been found in both humans and animals, and is thus a potentially serious public health problem.[2] *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) is an important cause of food poisoning and is the second most common cause of bacterial diarrhoea in the Western world. The fatality rate is dependent upon the country and the serovar, but is usually 1% to 4%. This incidence is increased at least 2-fold when antibiotic-resistant strains are involved.[3]

Salmonella Typhimurium resistance to antibiotics arises by the enzymatic inactivation of drugs, the alteration of antibiotic targets by mutation and by the active antibiotic efflux. Efflux involves complexes of proteins that function to decrease the concentration of specific drug or multiple toxic substrates by transporting these substrates across the inner and outer membranes into the external medium.[4] To date, five major families of efflux systems have been identified in Gram-negative bacteria, including: the ATP-binding cassette, small multidrug resistance, major facilitator superfamily (MFS), multidrug and toxic compound extrusion and resistance-nodulation-division (RND) efflux pumps.[4]

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Multidrug efflux pumps, especially those belonging to the RND family, play a major role in establishing intrinsic or developed resistance of Gram-negative bacteria to a wide range of toxic compounds, including antibiotics. A well-studied example is the AcrAB–TolC multidrug resistance tripartite pump system of *Escherichia coli*, which confers resistance to a wide variety of lipophilic and amphiphilic compounds, including the quaternary ammonium compounds (QACs) and acriflavine.[5] The presence and impact of the AcrAB–TolC systems in multidrug resistance have been reported for other members of Enterobacteriaceae.[5,6] In the case of *Salmonella* Typhimurium, there are several significant publications on the roles of AcrB and TolC in multidrug resistance to detergents, bile salts, quinolones, fluoroquinolones, β-lactams, tetracycline and other antimicrobials.[6–8]

In addition, *Salmonella* Typhimurium has another gene, *smvA*, whose function also contributes to multidrug resistance. This gene is predicted to encode an MFS efflux pump in the inner membrane.[9] Unlike the *acrRAB* operon, which is widely distributed throughout all Enterobacteriaceae genomes, homologues of *smvA* are not found in *E. coli* and *Shigella* spp. This gene appears to be present only in the genomes of *Salmonella* and *Klebsiella*. SmvA is most similar to the KmrA pump of *Klebsiella pneumoniae* (75% identity), which is involved in resistance to a variety of antimicrobial agents, and the well-characterized *Staphylococcus aureus* QacA pump (32% identity), involved in the efflux of toxic QACs. Like mutations in *qacA*, mutations in *smvA* confer increased susceptibility to methyl viologen (paraquat), a hydrophilic, doubly charged QAC.[9] The present study was performed to evaluate the role played by SmvA in the efflux of QACs in *Salmonella* Typhimurium.

Materials and methods

Bacterial strains, media and growth conditions

The *Salmonella* Typhimurium strains used in this study are derivatives of the parental wild-type strain 14028s (ATCC) and are listed in Table 1. Bacteria were grown routinely at 37°C and aerated by shaking. Rich medium for growth was Luria–Bertani (LB) broth (Bacto tryptone, 10 g/L; Bacto yeast extract, 5 g/L; and NaCl, 5 g/L) and minimal medium was M9 glucose (NaH_2PO_4, 6 mg/mL; K_2HPO_4, 3 mg/mL; NH_4Cl, 1 mg/mL; NaCl, 0.5 mg/mL; $MgSO_4$, 0.12 mg/mL; $CaCl_2$, 0.015 mg/mL; and glucose, 2 mg/mL). When required, LB medium was supplemented with ampicillin (100 mg/L), chloramphenicol (25 mg/L), kanamycin (50 mg/L), arabinose (2 mg/mL) or glucose (2 mg/mL). Solid media included Bacto agar (15 g/L). PBS buffer comprises 55 mg/mL $NaH_2PO_4{\cdot}7H_2O$, 15 mg/mL K_2HPO_4 and 4.25 mg/mL NaCl.

Construction of gene deletion mutants

Gene deletion was performed according to the method of Datsenko and Wanner,[10] with recombination between short homologous DNA regions catalysed by phage λ Red recombinase. Briefly, PCR primers (60 bp) were designed with 40 bp of 5′ homology to the *Salmonella* Typhimurium ATCC 14028s *acrB* gene and 20 bp of 3′ homology to the kanamycin or chloramphenicol cassettes present in plasmids pKD4 or pKD3 (acrB1, acaggagccgttaagacatgcctaatttctt tatcgatcgtgtaggctgga; acrB2, tatcaacagtgagcaaatcagcgatgttctgtcgaat gaccatatgaatat). Purified PCR products were used to electroporate *Salmonella* Typhimurium/pKD46 grown at 30°C in LB supplemented with 10 mM L-arabinose and 100 mg/L ampicillin. Bacteria were plated on LB agar plates (1.5% agar) supplemented with kanamycin or chloramphenicol, and were incubated at 37°C to select for the allelic exchange and cured plasmid pKD46. The presence of the Δ*acrB::kan* mutant allele was confirmed by PCR amplification, using primers flanking the sites of substitution, and backcrossed into a wild-type genetic background by generalized transduction using phage P22 HT105/1 *int201*. The Δ*smvA::cam* mutant was obtained previously in our laboratory.[9] Double mutants were obtained by generalized transduction of Δ*acrB::kan* or Δ*smvA::cam* alleles using P22 HT105/1 *int201* into the recipient Δ*tolC::*FRT mutant background. The 'FRT scar' for *tolC* was obtained by the deletion of the antibiotic-resistant cassette.[10]

Antimicrobial susceptibility test

The MICs of QACs and other antimicrobial agents for each bacterial strain were determined by microdilution in liquid medium as recommended by the CLSI,[11] with modifications. Briefly, bacteria were grown overnight in M9 glucose medium, diluted 5000-fold in fresh M9 glucose medium and aliquotted (100 μL) into the wells of sterile microtitre plates. A series of 2-fold dilutions was made along the columns of one or more microtitre dishes, and microtitre dishes were incubated at 37°C for 24 h. The lowest concentration of antimicrobial agents that inhibited growth (measured as the optical density at 640 nm) by >50% relative to growth in the absence of antimicrobial agents, respectively, was defined as the MIC.

Results and discussion

To compare the relative roles of the SmvA and AcrB pumps in the efflux of antimicrobial agents from *Salmonella* Typhimurium, we first performed a quick, qualitative screening

Table 1. Strains used in this study

Strain	Genotype	Method of construction	Source or reference
ATCC 14028s	wild-type		ATCC
SC169	Δ*acrB::kan*	Datsenko and Wanner[10]	this work
SC106	Δ*smvA::cam*	Datsenko and Wanner[10]	Santiviago *et al.*[9]
SC142	Δ*tolC::kan*	Datsenko and Wanner[10]	Santiviago *et al.*[9]
SC172	Δ*acrB::kan* Δ*tolC::FRT*	transduction with P22 (Δ*acrB::kan*)	this work
SC144	Δ*smvA::cam* Δ*tolC::FRT*	transduction with P22 (Δ*smvA::cam*)	this work

Table 2. MICs of acriflavine and QACs for wild-type and mutant strains

Strain	Genotype	Antimicrobial agent (mg/L)					
		MV[a]	NAL	AC	EB	MG	PB
ATCC 14028s	wild-type	0.98	37.5	250	160	31.3	125
SC169	Δ*acrB::kan*	0.98	4.7	125	80	15.7	62.5
SC106	Δ*smvA::cam*	0.12	18.8	31.3	40	7.8	31.3
SC142	Δ*tolC::kan*	1.96	2.3	31.3	20	<1	<1
SC144	Δ*smvA::cam* Δ*tolC::FRT*	0.24	9.4	3.9	2.5	<1	<1
SC172	Δ*acrB::kan* Δ*tolC::FRT*	1.96	1.17	15.6	10	<1	<1

MV, methyl viologen; NAL, nalidixic acid; AC, acriflavine; EB, ethidium bromide; MG, malachite green; PB, pyronin B.
[a]MIC values of MV are expressed in μM for a direct comparison with values commonly reported in the literature.[9]

to determine the susceptibility of the wild-type and *smvA* mutant strains to a variety of toxic agents. To do this, both the *smvA* mutant and the wild-type strains were spread onto LB agar plates and allowed to dry. Then, crystals of various QACs (standardized by weight) were spotted on the bacterial lawns. In all cases, the dissolution and diffusion of the QACs analysed in the agar were easy to follow as most of them are coloured substances. Zones of bacterial growth inhibition were observed and compared by visual inspection after overnight incubation at 37°C.

Among the QACs tested, we found that acriflavine, ethidium bromide, malachite green, methyl viologen and pyronin B inhibit the growth of the *smvA* mutant more than its wild-type parent. To quantify these apparent differences, we then performed MIC assays. Because *E. coli acrB* mutants have been shown to be more susceptible to nalidixic acid,[8] we included this antibiotic in our assays. In addition, since the TolC protein functions together with the AcrAB proteins and other pumps in *E. coli*,[12] we also tested mutant strains of *Salmonella* Typhimurium with inactivations of *tolC* as well as *tolC–smvA* and *tolC–acrB* double mutants.

The *Salmonella* Typhimurium *smvA–acrB* double mutant was not tested because it grew extremely poorly, suggesting that these pumps are important in the efflux of toxic metabolic products generated in the bacterial cell.

As observed in Table 2, we can divide the substrates tested into three different classes, depending on the susceptibilities of single and double mutants to each substrate. One class, represented by methyl viologen, appears to be exported only by the SmvA and not by the AcrAB–TolC pump, since only *smvA* mutants display increased susceptibility to methyl viologen in comparison with the wild-type strain (0.12 versus 0. 98 μM). A *tolC* mutant was more resistant to methyl viologen than the wild-type strain (1.96 versus 0.98 μM), as described previously. The simplest interpretation for this observation is that TolC facilitates the import of methyl viologen.[9]

Another class, represented by nalidixic acid (which is not a QAC), appears to be exported only by the AcrAB–TolC and not by the SmvA pump. A *tolC* mutant was slightly more susceptible than an *acrB* mutant to this substrate (2.3 versus 4.7 mg/L), and the *tolC–acrB* double mutant was as susceptible as the *tolC* single mutant (1.17 versus 2.3 mg/L). There are two possibilities why a *tolC* mutant was more susceptible to these agents than an *acrB* mutant. Either TolC functions together with other efflux pumps in the export of these substrates or *tolC* mutations have a pleiotropic effect on membrane integrity. Thus, *tolC* mutants will always be more susceptible to substrates exported solely by the AcrAB–TolC pump than *acrB* mutants.

Finally, the class represented by acriflavine, ethidium bromide, malachite green and pyronin B appears to be exported by both the SmvA and AcrAB–TolC pump systems. A *tolC* mutant was more susceptible than an *acrB* mutant to each of these substrates, and the *tolC–acrB* double mutant was as susceptible as or slightly more susceptible than the single *tolC* mutant. The *smvA* mutant was more susceptible than an *acrB* mutant, but was less susceptible than the *tolC* mutant to QACs. A *tolC–smvA* double mutant was always more susceptible than either of its single mutant parents. These results indicate that acriflavine, ethidium bromide, malachite green and pyronin B are substrates for both efflux systems, but the SmvA pump plays the major role in the efflux of QACs.

One striking observation is that the *tolC–smvA* double mutant (3.9 mg/L) was approximately 10 times more susceptible to acriflavine than either *smvA* (31.3 mg/L) or *tolC* (31.3 mg/L) single mutants and 4 times more susceptible than the *tolC–acrB* double mutant (15.6 mg/L). Based on our observations, we propose that *tolC* and *smvA* encode components that function in two different efflux systems with overlapping specificities to work in parallel to export acriflavine and other QACs.

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Transparency declarations

None to declare.

References

1. Scherer CA, Miller SI. Molecular pathogenesis of *Salmonella*. In: Groisman EA, ed. *Principles of Bacterial Pathogenesis*. New York: Academic Press, 2001; 266–333.

2. Piddock LJ. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS Microbiol Rev* 2002; **26**: 3–16.

3. Helms M, Vastrup P, Gerner-Smidt P *et al*. Excess mortality associated with antimicrobial drug-resistant *Salmonella* Typhimurium. *Emerg Infect Dis* 2002; **8**: 490–5.

4. Putman M, van Veen HW, Konings WN *et al*. Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 2000; **64**: 672–93.

5. Nikaido H, Zgurskaya HI. Antibiotic efflux mechanisms. *Curr Opin Infect Dis* 1999; **12**: 529–36.

6. Lacroix F, Cloeckaert A, Pardon P *et al*. *Salmonella* Typhimurium *acrB*-like gene: identification and role in resistance to biliary salts and detergents and in murine infection. *FEMS Microbiol Lett* 1996; **135**: 161–7.

7. Giraud E, Cloeckaert A, Kerboeuf D *et al*. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2000; **44**: 1223–8.

8. Piddock LJ, White DG, Gensberg K *et al*. Evidence for an efflux pump mediating multiple antibiotic resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2000; **44**: 3118–21.

9. Santiviago CA, Fuentes JA, Bueno SM *et al*. The *Salmonella enterica* sv. Typhimurium *smvA, yddG* and *ompD* (porin) genes are required for the efficient efflux of methyl viologen. *Mol Microbiol* 2002; **46**: 687–98.

10. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 2000; **97**: 6640–5.

11. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically—Seventh Edition: Approved Standard M7-A7*. CLSI, Wayne, PA, USA, 2006.

12. Fralick JA. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J Bacteriol* 1996; **178**: 5803–5.