J Antimicrob Chemother 2013; **68**: 1066–1070
doi:10.1093/jac/dks528 Advance Access publication 1 February 2013

Journal of Antimicrobial Chemotherapy

Cooperation of the multidrug efflux pump and lipopolysaccharides in the intrinsic antibiotic resistance of *Salmonella enterica* serovar Typhimurium

Seiji Yamasaki[1,2]†, Saya Nagasawa[2]†, Aiko Fukushima[1], Mitsuko Hayashi-Nishino[1] and Kunihiko Nishino[1]*

[1]*Laboratory of Microbiology and Infectious Diseases, Institute of Scientific and Industrial Research, Osaka University, 8–1 Mihogaoka, Ibaraki, Osaka 567–0047, Japan;* [2]*Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita 565-0871, Japan*

*Corresponding author. Tel: +81-6-6879-8546; Fax: +81-6-6879-8549; E-mail: nishino@sanken.osaka-u.ac.jp
†The first two authors contributed equally to this study.

Received 10 October 2012; returned 25 October 2012; revised 5 December 2012; accepted 17 December 2012

Objectives: In Gram-negative bacteria, drug susceptibility is associated with multidrug efflux systems and an outer membrane (OM) barrier. Previous studies revealed that *Salmonella enterica* serovar Typhimurium has 10 functional drug efflux pumps. Among them, AcrB is a major factor to maintain the intrinsic drug resistance in this organism. The lipopolysaccharide (LPS) content of OM is also important for resistance to lipophilic drugs; however, the interplay between the multidrug efflux pump and LPS in the intrinsic antibiotic resistance of *Salmonella* remains to be studied in detail. The aim of this study was to investigate the relationship between AcrB and LPS in the intrinsic drug resistance of this organism.

Methods: The genes encoding LPS core biosynthetic proteins and AcrB were disrupted from the wild-type *S. enterica* strain ATCC 14028s. The plasmid carrying *acrB* was transformed into these mutants and then the drug susceptibilities of the mutants and transformants were determined.

Results: Our results showed that the levels of *Salmonella* intrinsic antibiotic resistance were decreased when the length and branches of core oligosaccharide were lost. Furthermore, the deletion of *acrB* reduced multidrug resistance of all LPS mutants and AcrB production from the plasmid complemented this phenotype. However, AcrB production could not completely compensate for LPS function in intrinsic resistance.

Conclusions: Both pump inactivation and shortened LPS enhanced drug susceptibility, although the maximum susceptibility was achieved when the two were combined. Hence, these results indicated that the multidrug efflux system and OM barrier are both essential for maintaining intrinsic antibiotic resistance in *Salmonella*.

Keywords: AcrB, LPS, multidrug resistance

Introduction

Multidrug efflux pumps cause serious problems in cancer chemotherapy and in the treatment of bacterial infections. In bacteria, drug resistance is often associated with multidrug efflux pumps, which decrease cellular drug accumulation.[1,2] In Gram-negative bacteria, pumps belonging to the resistance–nodulation–division family are particularly effective in generating resistance, because they form a tripartite complex with the periplasmic proteins of the membrane fusion protein family and the outer membrane (OM) channels, ensuring that drugs are pumped out directly to the external medium.[3] High-level fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium phage type DT204 has been shown to be primarily due to multiple target gene mutations and active efflux by the AcrAB-TolC efflux system belonging to the resistance–nodulation–division family.[4]

S. enterica is a pathogen that causes a variety of diseases in humans ranging from gastroenteritis to bacteraemia and typhoid fever. Previous studies have shown that *S. enterica* serovar Typhimurium has 10 functional drug efflux pumps: AcrAB, AcrD, AcrEF, MdtABC, MdsAB, EmrAB, MdfA, MdtK, MacAB and SmvA.[5,6] Among these, AcrAB is constitutively expressed and is the most effective in intrinsic drug resistance in *Salmonella*.

In addition to drug efflux pumps, OM is also important for intrinsic antibiotic resistance. Gram-negative bacteria, which have an OM barrier, are usually much more resistant than Gram-positive

bacteria to a wide range of drugs.[7] In particular, lipopolysaccharides (LPS), located exclusively in the outer leaflet of OM, prevent the easy entry of lipophilic agents.[8] The LPS molecule comprises three parts: lipid A, core oligosaccharides and the O-antigen (Figure 1). Lipid A anchors the LPS molecule into the bacterial OM. The core oligosaccharides and O-antigen are located in the outer domain of the LPS molecule (Figure 1). LPS is only found in the OM of Gram-negative bacteria and many genes required for its synthesis and modification have been identified.[8]

LPS is important for intrinsic antibiotic resistance[9–11] and previous studies have shown that the AcrB efflux pump is related to both the intrinsic and the acquired multidrug resistance of *Salmonella*.[4,5,12] In *Francisella* sp., another Gram-negative bacterium, it has been reported that both the LPS and the AcrAB efflux pump system play a role in azithromycin susceptibility.[13] However, the synergistic interplay between AcrB and LPS of *Salmonella* remains to be studied in detail. In the present report, we examined the interplay between the AcrB efflux pump and LPS by determining the drug susceptibilities of mutants with varying LPS lengths and by investigating the effect of the *acrB* deletion in LPS mutants.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1 (available as Supplementary data at *JAC* Online). The *S. enterica* serovar Typhimurium strains were derived from the wild-type strain ATCC 14028s.[14] Bacterial strains were grown at 37°C in lysogeny broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl).[15]

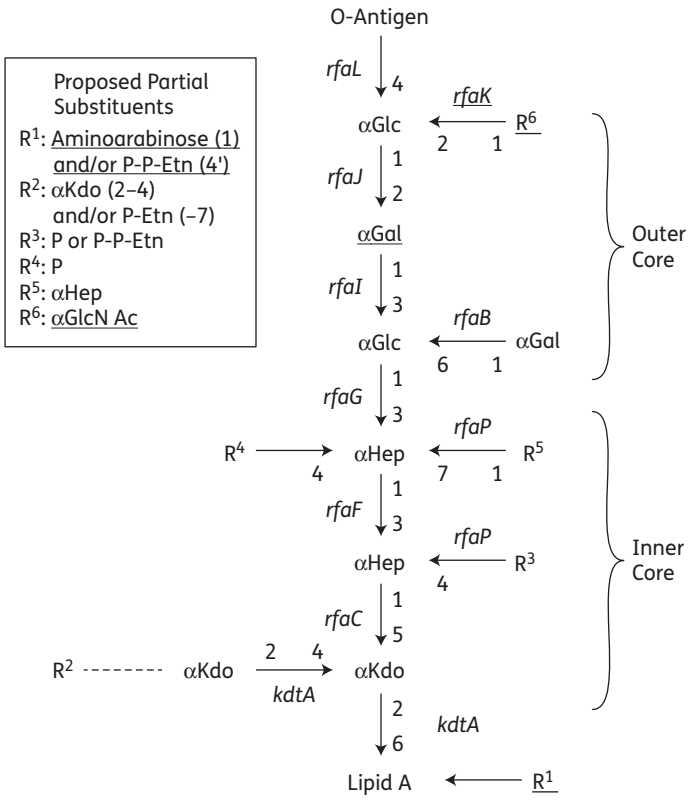

Figure 1. LPS in *S. enterica* serovar Typhimurium. Genes encoding LPS biosynthetic proteins are listed for each synthetic route. This figure has been modified from *EcoSal* with permission.[21]

Construction of gene deletion mutants

To construct all mutants, gene disruption was performed as described by Datsenko and Wanner.[16] Primers used for the construction of the mutants are listed in Table S2 (available as Supplementary data at *JAC* Online). The chloramphenicol resistance gene *cat* or the kanamycin resistance gene *aph*, flanked by Flp recognition sites, was PCR amplified and the resulting products were used to transform the recipient ATCC 14028s strain harbouring plasmid pKD46, which expresses Red recombinase. The chromosomal structure of the mutated loci was verified by PCR, as described previously.[16] Both *cat* and *aph* were eliminated using plasmid pCP20, as previously described.[16]

Plasmid construction

acrB was PCR amplified from ATCC 14028s genomic DNA with primers *acrB*-F and *acrB*-R (Table S2, available as Supplementary data at *JAC* Online) which introduced restriction enzyme sites of BamHI and XbaI at both ends of the amplified fragments. The PCR fragments were cloned into the corresponding sites of the vector pTrcHis2B (Invitrogen) to produce the plasmid p*acrB*.

LPS analysis

LPS was purified as described previously.[17] Culture samples were adjusted to an optical density of 1.0 at 600 nm in a final volume of 100 μL, and LPS

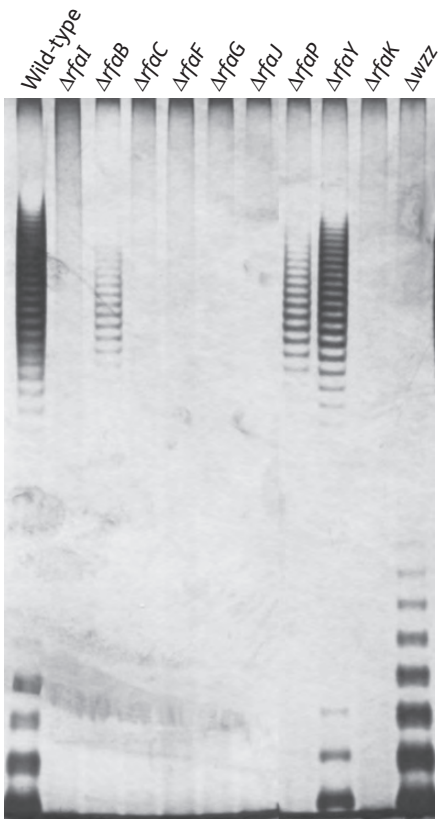

Figure 2. SDS-PAGE analysis of LPS. LPSs were isolated from the wild-type strain (ATCC 14028s), Δ*rfaI* (NKS363), Δ*rfaB* (NKS365), Δ*rfaC* (NKS366), Δ*rfaF* (NKS367), Δ*rfaG* (NKS368), Δ*rfaJ* (NKS369), Δ*rfaP* (NKS371), Δ*rfaY* (NKS372), Δ*rfaK* (NKS375) and Δ*wzz* (NKS877) strains.

samples normalized to the number of cells were separated on 12% acrylamide gels using Tris–Glycine/SDS buffer systems and stained using a modification of the conventional silver staining method.[18]

Determination of MICs of toxic compounds

The antibacterial activities of various agents were determined on lysogeny broth agar plates containing oxacillin, cloxacillin, nafcillin, erythromycin, rhodamine 6G, crystal violet, ethidium bromide, novobiocin, benzalkonium chloride, SDS or deoxycholic acid (Sigma, St Louis, MO, USA) at various concentrations. Agar plates were prepared by the 2-fold agar dilution technique, as described previously.[19] To determine MICs, bacteria were grown in lysogeny broth at 37°C overnight, diluted with the same medium and then tested at a final inoculum of 10^5 cfu/μL using a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan) after incubation at 37°C for 20 h. The MIC was the lowest compound concentration to inhibit cellular growth.

Results and discussion

Effects of the length and branches of LPS core oligosaccharides on intrinsic antibiotic resistance

To investigate whether the length and branches of LPS core oligosaccharides affect intrinsic antibiotic resistance in *S. enterica* serovar Typhimurium, genes encoding LPS core biosynthetic proteins (Figure 1) were deleted (Table S1, available as Supplementary data at *JAC* Online). In addition to the mutants of the genes presented in Figure 1, the deletion mutants of the *wzz* gene, encoding the O-chain length determinant, were constructed (Table S1, available as Supplementary data at *JAC* Online). The deletion mutant of the *rfaY* gene, which is necessary for phosphorylating the Hep(II) heptose in the core region of the LPS, was also constructed (Table S1, available as Supplementary data at *JAC* Online). To confirm the effects of deletions of these genes on the LPS structure, we analysed the LPS profiles in silver-stained polyacrylamide gels. LPS profiles of the deletion mutants were all different from that of the wild-type strain (Figure 2). For the MIC measurement, the AcrB substrates were chosen to compare the effect of deletion of the genes involved in the LPS biosynthesis with the effect of deletion of the *acrB* gene on drug susceptibilities. Compared with the wild-type strain, the Δ*rfaK*, Δ*wzz* and Δ*rfaJ* strains maintained intrinsic resistance to all antimicrobial agents and chemical compounds tested; however, the Δ*rfaI*, Δ*rfaB*, Δ*rfaY*, Δ*rfaP*, Δ*rfaG*, Δ*rfaF* and Δ*rfaC* strains showed increased susceptibility to almost all drugs as the length and branches of LPS core oligosaccharides were lost (Table 1). Interestingly, deletion of *rfaB*, which encodes a protein that adds a galactose moiety to produce one branch of the LPS core oligosaccharide, had no impact on novobiocin resistance; however, the strains that lost a core oligosaccharide phosphorylation gene (i.e. *rfaY* or *rfaP*) were more susceptible to novobiocin than Δ*rfaC*. The electric charge produced by the

Table 1. Susceptibility of *S. enterica* serovar Typhimurium *acrB* and/or LPS mutants to toxic compounds

	MIC (mg/L)										
Strain	OXA	CLO	NAF	ERY	R6G	CV	EB	NOV	BENZ	SDS	DOC
Wild-type	1024	1024	2048	512	4096	256	>2048	512	512	>32768	>32768
Δ*acrB*	4	4	16	8	16	4	128	4	8	1024	>32768
Δ*rfaK*	1024	1024	2048	512	4096	256	>2048	512	512	>32768	>32768
Δ*rfaK*Δ*acrB*	4	4	16	8	16	4	128	16	8	128	>32768
Δ*wzz*	1024	1024	2048	512	4096	256	>2048	512	512	>32768	>32768
Δ*wzz*Δ*acrB*	4	4	16	8	16	4	128	8	8	2048	>32768
Δ*rfaJ*	1024	1024	2048	512	4096	256	>2048	512	512	>32768	>32768
Δ*rfaJ*Δ*acrB*	4	4	16	8	16	4	128	16	8	128	>32768
Δ*rfaI*	512	512	1024	512	4096	64	>2048	64	32	>32768	>32768
Δ*rfaI*Δ*acrB*	4	4	16	4	16	4	64	4	4	16384	>32768
Δ*rfaB*	512	512	1024	512	4096	64	>2048	512	32	>32768	>32768
Δ*rfaB*Δ*acrB*	4	4	16	4	16	4	64	8	4	64	32768
Δ*rfaY*	512	512	1024	256	4096	32	>2048	16	64	>32768	>32768
Δ*rfaY*Δ*acrB*	2	4	8	4	8	2	64	0.5	4	128	8192
Δ*rfaP*	256	256	512	128	4096	8	2048	4	8	2048	>32768
Δ*rfaP*Δ*acrB*	2	2	8	2	8	1	32	0.125	4	32	512
Δ*rfaG*	256	256	256	64	4096	8	2048	32	4	256	>32768
Δ*rfaG*Δ*acrB*	1	2	4	1	8	1	32	0.5	2	64	512
Δ*rfaF*	128	128	256	16	1024	8	2048	32	8	256	>32768
Δ*rfaF*Δ*acrB*	4	8	32	8	16	4	128	16	8	256	>32768
Δ*rfaC*	128	64	128	16	128	4	1024	32	4	128	2048
Δ*rfaC*Δ*acrB*	1	1	2	<0.5	2	<0.25	32	0.5	2	16	128

OXA, oxacillin; CLO, cloxacillin; NAF, nafcillin; ERY, erythromycin; R6G, rhodamine 6G; CV, crystal violet; EB, ethidium bromide; NOV, novobiocin; BENZ, benzalkonium chloride; DOC, deoxycholic acid.
MIC determinations were repeated at least three times.

phosphate group seems to be effective in inhibiting the entry of aminocoumarin antibiotics. These data indicate that the length and branches of LPS core oligosaccharides play a role in the maintenance of intrinsic resistance of *S. enterica* against multiple drugs.

Effect of acrB deletion on multidrug resistance of the LPS mutants

In *Salmonella*, the AcrAB-TolC efflux system is constitutively expressed and effective in intrinsic drug resistance.[5] To investigate the function of multidrug efflux systems in LPS mutants, we disrupted *acrB* from the genomic DNA (Table S1, available as Supplementary data at *JAC* Online). The Δ*acrB* strain was more susceptible to oxacillin, cloxacillin, nafcillin, erythromycin, rhodamine 6G and ethidium bromide than any other single LPS mutant and almost all drug susceptibilities of the Δ*rfaK*Δ*acrB*, Δ*wzz*Δ*acrB*, Δ*rfaJ*Δ*acrB*, Δ*rfaI*Δ*acrB* and Δ*rfaB*Δ*acrB* double mutants were comparable to that of Δ*acrB*. The Δ*rfaF*Δ*acrB* strain was more susceptible to SDS than Δ*acrB*. The Δ*rfaY*Δ*acrB* strain was more susceptible to novobiocin, SDS and deoxycholic acid than Δ*acrB*. The Δ*rfaP*Δ*acrB* was more susceptible to erythromycin, crystal violet, ethidium bromide, novobiocin, SDS and deoxycholic acid than Δ*acrB*. The Δ*rfaG*Δ*acrB* and Δ*rfaC*Δ*acrB* strains were more susceptible to almost all drugs than the Δ*acrB* strain (Table 1). These results indicate that AcrAB-TolC plays a role in drug resistance even if the LPS function is weakened.

Effect of acrB overexpression on drug susceptibilities of the LPS mutants

To investigate the effect of *acrB* overexpression on drug susceptibilities of the LPS mutants, *acrB* in *S. enterica* ATCC 14028s was cloned into the vector pTrcHis2B and then the constructed plasmid was transformed into LPS mutants lacking *acrB*. Overexpression of *acrB* conferred multidrug resistance to all of the mutants (Table 2). These data indicate that the AcrB efflux pump can function in bacteria with imperfect LPS. However, overexpressed *acrB* did not completely restore multidrug resistance of some LPS mutants to the wild-type level, e.g. Δ*rfaF*Δ*acrB* and Δ*rfaC*Δ*acrB* (Table 2). These results indicate that the multidrug efflux system cannot recover the loss of LPS required for maintenance of intrinsic *Salmonella* resistance.

Concluding remarks

Herein, we investigated the interplay between the multidrug efflux system and the OM barrier in intrinsic *Salmonella* antibiotic resistance at the genetic level. The results showed that the length and branches of LPS core oligosaccharides and the AcrB

Table 2. Susceptibility of *S. enterica* serovar Typhimurium strains to toxic compounds

	MIC (mg/L)							
Strain	ERY	R6G	CV	EB	NOV	BENZ	SDS	DOC
Wild-type	512	4096	256	>2048	256	512	>32768	>32768
Δ*acrB*/pTrcHis2B	4	8	2	32	4	4	256	32786
Δ*acrB*/p*acrB*	**256**	**4096**	**128**	**2048**	**256**	**64**	**>32786**	**>32786**
Δ*rfaK*Δ*acrB*/pTrcHis2B	4	16	2	64	8	4	128	>32786
Δ*rfaK*Δ*acrB*/p*acrB*	**256**	**4096**	**128**	**4096**	**512**	**128**	**>32786**	>32786
Δ*wzz*Δ*acrB*/pTrcHis2B	4	8	2	32	8	4	256	>32786
Δ*wzz*Δ*acrB*/p*acrB*	**128**	**4096**	**128**	**4096**	**512**	**128**	**>32786**	>32786
Δ*rfaJ*Δ*acrB*/pTrcHis2B	4	16	2	64	8	4	128	32786
Δ*rfaJ*Δ*acrB*/p*acrB*	**256**	**4096**	**128**	**4096**	**512**	**64**	**>32786**	**>32786**
Δ*rfaI*Δ*acrB*/pTrcHis2B	4	8	2	32	4	4	256	>32786
Δ*rfaI*Δ*acrB*/p*acrB*	**128**	**4096**	**128**	**4096**	**128**	**64**	**>32786**	>32786
Δ*rfaB*Δ*acrB*/pTrcHis2B	4	16	1	64	4	4	64	32786
Δ*rfaB*Δ*acrB*/p*acrB*	**128**	**4096**	**32**	**4096**	**256**	**16**	**>32786**	**>32786**
Δ*rfaY*Δ*acrB*/pTrcHis2B	2	4	1	32	1	4	128	4096
Δ*rfaY*Δ*acrB*/p*acrB*	**128**	**4096**	**64**	**2048**	**32**	**32**	**>32786**	**>32786**
Δ*rfaP*Δ*acrB*/pTrcHis2B	1	4	1	32	0.25	4	32	512
Δ*rfaP*Δ*acrB*/p*acrB*	**64**	**2048**	**16**	**2048**	**8**	**8**	**256**	**>32786**
Δ*rfaG*Δ*acrB*/pTrcHis2B	1	8	1	32	4	2	32	256
Δ*rfaG*Δ*acrB*/p*acrB*	**32**	**2048**	**8**	**1024**	**16**	**4**	**128**	**>32786**
Δ*rfaF*Δ*acrB*/pTrcHis2B	4	16	2	64	16	4	128	>32786
Δ*rfaF*Δ*acrB*/p*acrB*	**16**	**256**	**4**	**512**	16	4	128	>32786
Δ*rfaC*Δ*acrB*/pTrcHis2B	0.25	2	0.5	64	0.5	4	16	128
Δ*rfaC*Δ*acrB*/p*acrB*	**8**	**64**	**2**	**1024**	**8**	4	**128**	**2048**

ERY, erythromycin; R6G, rhodamine 6G; CV, crystal violet; EB, ethidium bromide; NOV, novobiocin; BENZ, benzalkonium chloride; DOC, deoxycholic acid.
MIC determinations were repeated at least three times.
Values in bold are larger than those of the corresponding strains harbouring the vector only.

efflux pump are necessary for the maintenance of the intrinsic resistance of *S. enterica* serovar Typhimurium. The maximal susceptibility was achieved when deletions of *acrB* and genes related to LPS synthesis were combined. Additive synergistic effects were especially observed in the Δ*rfaG*Δ*acrB* and Δ*rfaC*-Δ*acrB* mutants. Compared with Δ*acrB*, Δ*rfaG*Δ*acrB* was susceptible to oxacillin (4-fold), nafcillin (4-fold), erythromycin (8-fold), crystal violet (4-fold), ethidium bromide (4-fold), novobiocin (8-fold), benzalkonium chloride (4-fold), SDS (16-fold) and deoxycholic acid (>64-fold). Δ*rfaC*Δ*acrB* was also susceptible to oxacillin (4-fold), cloxacillin (4-fold), nafcillin (8-fold), erythromycin (>16-fold), rhodamine 6G (8-fold), crystal violet (>16-fold), ethidium bromide (4-fold), novobiocin (8-fold), benzalkonium chloride (4-fold), SDS (64-fold) and deoxycholic acid (>256-fold) when compared with Δ*acrB*. These data indicate that the AcrAB-TolC efflux system is important for maintaining the intrinsic antibiotic resistance even when most of the core region of LPS is lost. The overexpression of *acrB* cannot completely compensate the function of LPS in the maintenance of intrinsic resistance, although functional AcrB was present in all of the LPS mutants. Interestingly, Giraud *et al*.[20] reported that there was an increased density of the long O-polysaccharide chains and an increased level of the AcrAB pump in *in vitro*-selected quinolone-resistant mutants of *S. enterica* serovar Typhimurium, suggesting that both efflux pump and LPS are also important for the acquired resistance. Our results support the notion that both pump inhibition and OM disruption could constitute an effective approach to increasing the drug susceptibility of multidrug-resistant strains.[9,10] In summary, we genetically determined that the AcrB multidrug efflux pump and bulkiness of LPS core oligosaccharides are essential for intrinsic antibiotic resistance in *S. enterica*.

Funding

This study was supported by a research grant from the Uehara Memorial Foundation (to K. N.), the Institute for Fermentation (to M. H.-N.), Grants-in-Aid for Young Scientists and Bilateral Joint Research Projects from the Japan Society for the Promotion of Science (to M. H.-N.) and a grant NEXT Program (LS080) from the Cabinet Office, Government of Japan (to K. N.). S. Y. was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Nikaido H. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 1996; **178**: 5853–9.

2 Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* 2000; **37**: 219–25.

3 Nikaido H, Takatsuka Y. Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta* 2009; **1794**: 769–81.

4 Baucheron S, Chaslus-Dancla E, Cloeckaert A. Role of TolC and *parC* mutation in high-level fluoroquinolone resistance in *Salmonella enterica* serotype Typhimurium DT204. *J Antimicrob Chemother* 2004; **53**: 657–9.

5 Nishino K, Latifi T, Groisman EA. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* 2006; **59**: 126–41.

6 Villagra NA, Hidalgo AA, Santiviago CA *et al*. SmvA, and not AcrB, is the major efflux pump for acriflavine and related compounds in *Salmonella enterica* serovar Typhimurium. *J Antimicrob Chemother* 2008; **62**: 1273–6.

7 Vaara M. Antibiotic-supersusceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob Agents Chemother* 1993; **37**: 2255–60.

8 Nikaido H, Vaara M. Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* 1985; **49**: 1–32.

9 Nikaido H. The role of outer membrane and efflux pumps in the resistance of gram-negative bacteria. Can we improve drug access? *Drug Resist Updat* 1998; **1**: 93–8.

10 Li XZ, Zhang L, Poole K. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2000; **45**: 433–6.

11 Peterson AA, Fesik SW, McGroarty EJ. Decreased binding of antibiotics to lipopolysaccharides from polymyxin-resistant strains of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob Agents Chemother* 1987; **31**: 230–7.

12 Nikaido H, Basina M, Nguyen V *et al*. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β-lactam antibiotics containing lipophilic side chains. *J Bacteriol* 1998; **180**: 4686–92.

13 Ahmad S, Hunter L, Qin A *et al*. Azithromycin effectiveness against intracellular infections of *Francisella*. *BMC Microbiol* 2010; **10**: 123.

14 Fields PI, Swanson RV, Haidaris CG *et al*. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* 1986; **83**: 5189–93.

15 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual, 2nd edn*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.

16 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.

17 Marolda CL, Welsh J, Dafoe L *et al*. Genetic analysis of the O7-polysaccharide biosynthesis region from the *Escherichia coli* O7:K1 strain VW187. *J Bacteriol* 1990; **172**: 3590–9.

18 Fomsgaard A, Freudenberg MA, Galanos C. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *J Clin Microbiol* 1990; **28**: 2627–31.

19 Nishino K, Yamaguchi A. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. *J Bacteriol* 2004; **186**: 1423–9.

20 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.

21 Raetz CR. *Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles*. *EcoSal* Chapter 69. http://ecosal.org (29 November, 2012, date last accessed).