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

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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 bacteremia 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, AcrB 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).\(^15\)

**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 pacrB.

**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 was precipitated with ethanol.

**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.\(^21\)

**Figure 2.** SDS-PAGE analysis of LPS. LPSs were isolated from the wild-type strain (ATCC 14028s), Δ*rafB* (NKS363), Δ*rafB*Δ*rafC* (NKS365), Δ*rafA*Δ*rafC* (NKS366), Δ*rafB*Δ*rafC*Δ*rafA* (NKS367), Δ*rafA*Δ*rafC*Δ*rafB* (NKS368), Δ*rafA*Δ*rafC*Δ*rafB*Δ*rafP* (NKS371), Δ*rafY* (NKS372), Δ*rafK* (NKS375) and Δwzz (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.\textsuperscript{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.\textsuperscript{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 $10^8$ CFU mL\textsuperscript{-1} 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 \(\alpha r\)\(f\)\(Y\) 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 \(\Delta rfaK\), \(\Delta wzz\) and \(\Delta rfaJ\) strains maintained intrinsic resistance to all antimicrobial agents and chemical compounds tested; however, the \(\Delta rfaL\), \(\Delta rfaB\), \(\Delta rfaY\), \(\Delta rfaP\), \(\Delta rfaG\) and \(\Delta rfaC\) strains showed increased susceptibility to almost all drugs as the length and branches of LPS core oligosaccharides were lost (Table 1). Interestingly, deletion of \(\alpha r\)\(f\)\(B\), 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. \(\alpha r\)\(f\)\(Y\) or \(\alpha r\)\(f\)\(P\)) were more susceptible to novobiocin than \(\Delta rfaC\). The electric charge produced by the

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

| 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 |
| \(\Delta acrB\) | 4 | 4 | 16 | 8 | 16 | 4 | 128 | 4 | 8 | 1024 | >32768 |
| \(\Delta rfaK\) | 1024 | 1024 | 2048 | 512 | 4096 | 256 | >2048 | 512 | 512 | >32768 | >32768 |
| \(\Delta rfaK\Delta acrB\) | 4 | 4 | 16 | 8 | 16 | 4 | 128 | 16 | 8 | 128 | >32768 |
| \(\Delta wzz\) | 1024 | 1024 | 2048 | 512 | 4096 | 256 | >2048 | 512 | 512 | >32768 | >32768 |
| \(\Delta wzz\Delta acrB\) | 4 | 4 | 16 | 8 | 16 | 4 | 128 | 8 | 8 | 2048 | >32768 |
| \(\Delta rfaJ\) | 1024 | 1024 | 2048 | 4096 | 256 | >2048 | 512 | 512 | >32768 | >32768 |
| \(\Delta rfaJ\Delta acrB\) | 4 | 4 | 16 | 8 | 16 | 4 | 128 | 16 | 8 | 128 | >32768 |
| \(\Delta rfaL\) | 512 | 512 | 1024 | 512 | 4096 | 64 | >2048 | 64 | 32 | >32768 | >32768 |
| \(\Delta rfaL\Delta acrB\) | 4 | 4 | 16 | 4 | 16 | 4 | 64 | 4 | 4 | 16384 | >32768 |
| \(\Delta rfaB\) | 512 | 512 | 1024 | 512 | 4096 | 64 | >2048 | 512 | 32 | >32768 | >32768 |
| \(\Delta rfaB\Delta acrB\) | 4 | 4 | 16 | 4 | 16 | 4 | 64 | 8 | 4 | 64 | 32768 |
| \(\Delta rfaY\) | 512 | 512 | 1024 | 256 | 4096 | 32 | >2048 | 16 | 64 | >32768 | >32768 |
| \(\Delta rfaY\Delta acrB\) | 2 | 4 | 8 | 4 | 8 | 2 | 64 | 0.5 | 4 | 128 | 8192 |
| \(\Delta rfaP\) | 256 | 256 | 512 | 128 | 4096 | 8 | 2048 | 4 | 8 | 2048 | >32768 |
| \(\Delta rfaP\Delta acrB\) | 2 | 2 | 8 | 2 | 8 | 1 | 32 | 0.125 | 4 | 32 | 512 |
| \(\Delta rfaG\) | 256 | 256 | 512 | 64 | 4096 | 8 | 2048 | 32 | 4 | 256 | >32768 |
| \(\Delta rfaG\Delta acrB\) | 1 | 2 | 4 | 1 | 8 | 1 | 32 | 0.5 | 2 | 64 | 512 |
| \(\Delta rfaF\) | 128 | 128 | 256 | 16 | 1024 | 8 | 2048 | 32 | 8 | 256 | >32768 |
| \(\Delta rfaF\Delta acrB\) | 4 | 8 | 32 | 8 | 16 | 4 | 128 | 16 | 8 | 256 | >32768 |
| \(\Delta rfaC\) | 128 | 64 | 128 | 16 | 128 | 4 | 1024 | 32 | 4 | 128 | 2048 |
| \(\Delta rfaC\Delta acrB\) | 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-ToIC efflux system is constitutively expressed and effective in intrinsic drug resistance. 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 ∆acrB strain was more susceptible to oxacillin, claxacillin, nafcillin, erythromycin, rhodamine 6G and ethidium bromide than any other single LPS mutant and almost all drug susceptibilities of the ∆rfaK∆acrB, ∆wzz∆acrB, ∆rfaJ∆acrB and ∆rfaG∆acrB double mutants were comparable to that of ∆acrB. The ∆rfaJ∆acrB strain was more susceptible to SDS than ∆acrB. The ∆rfaY∆acrB strain was more susceptible to novobiocin, SDS and deoxycholic acid than ∆acrB. The ∆rfaG∆acrB was more susceptible to erythromycin, crystal violet, ethidium bromide, novobiocin, SDS and deoxycholic acid than ∆acrB. The ∆rfaG∆acrB and ∆rfaC∆acrB strains were more susceptible to almost all drugs than the ∆acrB strain (Table 1). These results indicate that AcrAB-ToIC plays a role in drug resistance even if the LPS function is weakened.

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

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

**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...
The overexpression of \( \text{acrB} \) biotic resistance even when most of the core region of LPS is lost. when compared with \( \text{Dide} \) (4-fold), SDS (64-fold) and deoxycholic acid (\( \text{IDB} \)). Interestingly, Giraud et al. reported that there was an increased function of LPS in the maintenance of intrinsic resistance, although functional AcrB was present in all of the LPS mutants. These data indicate that the AcrAB-ToLC efflux system is an important mechanism for resistance to acriflavine and related compounds in Salmonella enterica serovar Typhimurium. J Antimicrob Chemother 2008; 62: 1273–6.

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

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