Role of novel gyrA mutations in the suppression of the fluoroquinolone resistance genotype of vaccine strain Salmonella Typhimurium vacT (gyrA D87G)

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Introduction

Contaminated food from animal sources is a major cause of enteric salmonella infections.1,2 To combat zoonotic salmonella infections the application of protective live vaccines for food-producing animals is a promising approach to reduce the risk of development of antibiotic resistance inevitably following antibiotic treatment.3,4 Several attenuated live vaccine strains of Salmonella have been developed either by random or by site-directed mutagenesis of genetic loci associated with basic metabolic pathways,5 but developed either by random or by site-directed mutagenesis of antibiotic resistance inevitably following antibiotic treatment.3,4 Animals is a promising approach to reduce the risk of development of antibiotic resistance inevitably following antibiotic treatment.

Several attenuated live vaccine strains of Salmonella have been developed either by random or by site-directed mutagenesis of genetic loci associated with basic metabolic pathways,5 but only a few such strains have proven to be safe and effective. One example is the attenuated live vaccine strain ‘TAD Salmonella Typhimurium vacT’ (vacT, Lohmann Animal Health, Cuxhaven, Germany) licensed for use in chicken.

Three genetic loci have been described for vacT which are thought to contribute to attenuation and/or to serve as epidemiological markers. However, their molecular basis remains to be elucidated.6 These loci include: (i) rtt (reversion to tensid tolerance), a poorly characterized mutation associated with increased susceptibility to macrolides; (ii) rpoB, which encodes a mutated β subunit of RNA polymerase conferring rifampicin resistance to vacT; and (iii) nalA, an allele of gyrA, which codes for a quinolone-resistant A subunit of DNA gyrase.

For both rpoB and gyrA mutations, a reduction of fitness as an effect of antibiotic resistance have been described in Salmonella (for review see ref.7). Moreover, the accumulation of gyrA mutations in laboratory mutants of Escherichia coli has been associated with reductions in DNA supercoiling degree and fitness,....

Objectives: This study was aimed at characterizing the gyrA locus and determining its impact on fluoroquinolone susceptibility, DNA supercoiling degree and growth rate of Salmonella Typhimurium live vaccine strain vacT in comparison with its parent M415. Furthermore, the role of multiple drug resistance efflux in the susceptibility of vacT to fluoroquinolones and macrolides was investigated.

Methods: DNA sequences were determined for genes gyrA, gyrB, parC and parE of M415 and three consecutive mutants Nal2ori, Nal2passage and vacT. The impact of gyrA mutations on the fluoroquinolone susceptibilities and relative DNA supercoiling degrees was investigated by a complementation assay using wild-type gyrA (gyrA*) and a reporter gene system, respectively. Doubling times of the strains and MICs of different antibiotics in the absence and presence of an efflux pump inhibitor (EPI) were determined.

Results: Besides the gyrA mutation D87G, two novel mutations (G75A and A866S) were identified in the three mutants and a third novel mutation W59R in vacT. Fluoroquinolone susceptibilities and DNA supercoiling degrees of all three mutants were reduced compared with those of M415. Introduction of the gyrA mutation(s) and novel gyrA mutation(s) as the basis for the unusual fluoroquinolone susceptibility of vacT.

Conclusions: The data point to a combination of at least one non-gyrA mutation and novel gyrA mutation(s) as the basis for the unusual fluoroquinolone susceptibility of vacT.

Keywords: fitness, DNA supercoiling, multiple drug resistance, efflux
Fluoroquinolone-susceptible gyrA mutant

determined as growth rate. Thus, it is tempting to speculate that gyrA mutation(s) play(s) a role in the attenuation of virulence and fluoroquinolone resistance of vacT.

An additional factor contributing to quinolone resistance in Salmonella as well as in E. coli is the increased expression of the multiple drug resistance (MDR) efflux pumps. Among these the tripartite RND-type efflux pump AcrAB-TolC, which shares high homologies to MDR efflux pumps in other enterobacterial species and consists of the MDR efflux pump AcrB, the membrane fusion protein AcrA and the outer membrane porin TolC, seems to play a major role in Salmonella. In vitro mutants lacking either a functional acrB or tolC gene show reduced MICs of different antibiotics including fluoroquinolones. Moreover, in fluoroquinolone-resistant mutants, increased expression of acrAB genes was detectable together with target mutation(s). However, so far no mutation affecting the regulation of either acrAB or tolC has been identified in clinical isolates. Instead, a soxR-constitutive mutation has been reported in a clinical isolate of Salmonella Typhimurium to be associated with a multiple antibiotic resistance (mar) phenotype including fluoroquinolones, but the underlying resistance mechanism in Salmonella has not yet been elucidated. In contrast, the susceptibility of vacT to macrolides and fluoroquinolones is increased, suggesting a role of reduced efflux pump activity as an alternative or additional mechanisms leading to fluoroquinolone susceptibility. Recently, the rtt phenotype has been suggested to be associated with the cpxA locus coding for a global response regulator involved in the control of cell wall permeability. However, no DNA sequence data supporting this hypothesis of a cpxA-mediated increase in fluoroquinolone susceptibility have been presented. Thus, the unique drug resistance phenotype of vacT showing low-level resistance exclusively to nalidixic acid while retaining susceptibility to fluoroquinolones has led us to investigate the role of target mutations.

Material and methods

Antibiotics
Antibiotics used were ciprofloxacin (Bayer AG, Leverkusen, Germany), sparfloxacin (Rhône-Poulenc-Rorer, Cologne, Germany), erythromycin and clarithromycin (Abbott, USA), obtained from the manufacturers, and ampicillin, kanamycin, nalidixic acid, norfloxacin, rifampicin and tetracycline, purchased from Sigma, Munich, Germany. Tylosin was a gift from Dr Stefan Schwarz (FAL, Celle, Germany).

Bacterial strains
Bacterial strains are summarized in Table 1. These include Salmonella Typhimurium DT009 strain M415 (obtained from Dr W. Rabsch, German National Reference Centre for Salmonella, Robert-Koch-Institute, Branch Wernigerode, Germany) as the parent for the vaccine strain ‘TAD Salmonella Typhimurium vacT’ (Lohmann Animal Health, Cuxhaven, Germany) and mutants Nal2ori and Nal2passage isolated in intermediate selection steps (kindly provided by Dr J. Beer, University of Leipzig, Leipzig, Germany), as well as Salmonella ATCC 14028. Single-step gyrA mutant M415-87 (gyrA D87Y) was selected from M415 on agar containing 0.125 mg/L ciprofloxacin. Recombinant plasmids used are summarized in Table 2. All plasmids are derivatives of plasmid pBP507, a broad-host-range vector based upon plasmid RSF1010. Plasmid pBP517 contains the wild-type allele gyrA of E. coli. Plasmids pBP507 and pBP517 were derived from plasmids pBP521 and pBP522 described previously by replacing an ~400 bp NsiI-fragment of the pBP507 part with an ~2 kb PstI fragment. These fragments carry a gene fusion of the reporter gene luc for luciferase from Photorhabdus pyralis with either promoter ptopA of topoisomerase I (ptopA-luc in pPHB94) or promoter pgyrA of gyrase A subunit (pgyrA-luc in pPHB95).

Susceptibility testing
The MICs were determined in the absence and presence of efflux pump inhibitor (EPI) phenylalanyl-arginyl-β-naphthylamide (Sigma-Aldrich, Steinheim, Germany) by a micro-broth dilution method in unsupplemented Mueller-Hinton broth (Difco, Detroit, MI, USA) according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) either by using preprefabricated microtitre plates (Microsant-S, Merlin-Diagnostika, Germany) or by manually preparing twofold dilutions. Owing to the strain-specific susceptibility to EPI alone, susceptibility tests for the different antibiotics were performed in the presence of EPI at 0.25× MIC. Assuming a methodological variation in the MIC determination of ±1 dilution step an MIC increase of >1 dilution step determined in the presence of EPI was taken as an indicator for an active efflux system.

For the complementation assay, susceptibility testing was performed in the presence of kanamycin at a final concentration of 50 mg/L to maintain selective pressure for the plasmids pBP507 and pBP517.

PCR amplification and DNA sequencing
Chromosomal DNA for PCR amplification was isolated by rapid boiling lysis. The primers used are listed in Table 3. The PCR products were purified using a QIAquick-spin PCR Purification Kit (Qiagen, Dusseldorf, Germany). Cycle sequencing was performed in a total volume of 20 μL containing 4 μL of Ready Reaction Mix (Perkin Elmer, Foster City, A, USA), 10–100 ng of purified PCR product and 10 pmol of the respective primer. PCR products were loaded onto an ABI PRISM 310 Genetic Analyzer (Perkin Elmer) for separation.

Complementation assay
This test is based on the genetic dominance of the gyrA* gene of a quinolone-susceptible strain of E. coli over the respective quinolone-resistant allele gyrA from E. coli and many other Gram-negative bacteria including Salmonella. Recombinant plasmids pBP517 (gyrA*) and pBP507 (vector control) were transferred separately by conjugational mobilization from an E. coli donor (C600SN/RP1H) to the respective Salmonella strain (recipient). Transconjugants were selected on M9 minimal medium containing glucose (0.2%) and kanamycin (50 mg/L).

Supercoiling assay
The relative degree of supercoiling was determined as the supercoiling quotient Qsc defined as the luciferase activity determined for a strain containing pPHB94 divided by that of the same strain containing plasmid pPHB95. Thus, luciferase expression from plasmid pPHB94 increases with increased supercoiling degree, but decreases with reduced supercoiling degree from plasmid pPHB95.

Doubling time
Stationary phase cells grown in Luria–Bertani (LB) medium were diluted 1:1000 and incubated at 37°C under agitation (225 rpm). Every 15 min, samples were taken for determining the viable cell count by plating aliquots of serial 10-fold dilutions on LB agar.
Table 1. Genotypic and phenotypic characteristics of *Salmonella* Typhimurium M415 and its derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lyso (LT) and biotype (BT)</th>
<th>MIC of ciprofloxacin (mg/L)</th>
<th>gyrA mutations</th>
<th>Supercoiling (Qsc)a</th>
<th>Qsc (%)</th>
<th>Doubling time (min)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>M415</td>
<td>LT: DT009</td>
<td>0.03</td>
<td></td>
<td>4.5 ± 0.12</td>
<td>100 ± 2.9</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>BT: a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M415-87</td>
<td>LT: DT009</td>
<td>0.5</td>
<td>Asp-87 (GAC) → Tyr (TAC)</td>
<td>3.73 ± 0.22</td>
<td>89.9 ± 5.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BT: a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nal2ori</td>
<td>LT: DT009</td>
<td>0.125</td>
<td>Gly-75 (GGT) → Ala (GCT)</td>
<td>2.9 ± 0.13</td>
<td>57.6 ± 3.1</td>
<td>44.2</td>
</tr>
<tr>
<td></td>
<td>BT: b</td>
<td></td>
<td>Asp-87 (GAC) → Gly (GGC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ala-867 (GGG) → Ser (TCG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nal2passage</td>
<td>LT: DT009</td>
<td>0.125</td>
<td>Gly-75 (GGT) → Ala (GCT)</td>
<td>2.56 ± 0.21</td>
<td>61.7 ± 5.1</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>BT: a</td>
<td></td>
<td>Asp-87 (GAC) → Gly (GGC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ala-867 (GGG) → Ser (TCG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vacT</td>
<td>LT: DT009</td>
<td>0.03</td>
<td>Trp-59 (TGG) → Arg (AGG)</td>
<td>2.62 ± 0.18</td>
<td>58.2 ± 3.9</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>BT: a</td>
<td></td>
<td>Gly-75 (GGT) → Ala (GCT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Asp-87 (GAC) → Gly (GGC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ala-867 (GGG) → Ser (TCG)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

*aQsc, quotient of supercoiling = ptopA activity/pgyrA activity.*

*bData are shown for a typical set of experiments.

Table 2. List of plasmids

<table>
<thead>
<tr>
<th>Name of the plasmid</th>
<th>Relevant genetic markersa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBP507</td>
<td>IncQ, amiR, genR, mob*</td>
<td>14</td>
</tr>
<tr>
<td>pBP517</td>
<td>IncQ, amiR, genR, mob*, gyrAeco</td>
<td>14</td>
</tr>
<tr>
<td>pPHB94</td>
<td>IncQ, amiR, genR, mob*, ptopA-luc</td>
<td>The present study</td>
</tr>
<tr>
<td>pPHB95</td>
<td>IncQ, amiR, genR, mob*, pgyrA-luc</td>
<td>The present study</td>
</tr>
</tbody>
</table>

*aIncQ, plasmid incompatibility group Q; amiR, amikacin resistance mediated by an aminoglycoside (6') acetyltransferase; genR, gentamicin resistance mediated by an aminoglycoside (2') adenyltransferase; mob*, mobilizable by IncP plasmids such as RPl.

Doubling times during log phase were determined from the linear part of a semi-logarithmic plot of the number of cfu/mL against time.

Results

Susceptibilities to different antibiotics

The vaccine strain vacT and its ancestors M415, Nal2passage and Nal2ori were characterized phenotypically for their susceptibilities to different antibiotics. The results are summarized in Table 4. Compared with its parent M415, strain vacT has a reduced susceptibility to nalidixic acid and rifampicin, but is susceptible to ciprofloxacin, sparfloxacin (Table 4) and many fluorinated quinolones (data not shown) as well as to the macrolides erythromycin, clarithromycin and tylosin, the latter licensed for veterinary use (Table 4). The intermediate derivatives Nal2ori and Nal2passage have slightly reduced susceptibilities to sparfloxacin, but are fully susceptible to ciprofloxacin. For comparison, the single-step gyrA mutant M415-87 shows an increase in the MICs of nalidixic acid (four dilution steps), ciprofloxacin (three dilution steps) and sparfloxacin (one dilution step), but not of macrolides or tetracycline (Table 4).

The strains show different susceptibilities to the EPI (Table 4). Strain vacT is inhibited by 16 mg/L EPI whereas its parent M415 and mutant M415-87 are inhibited by 256 mg/L. Thus, for determining the impact of EPI on antibiotic susceptibilities under comparable conditions the inhibitor was used at 0.25-fold of the respective MIC. For the strains M415, M415-87, Nal2ori and Nal2passage the presence of EPI resulted in reductions in the MICs of macrolides (by 4–10 dilution steps) and nalidixic acid and ciprofloxacin (by up to 2 dilution steps). The respective reductions for vacT were 3–4 dilution steps (macrolides), three dilution steps (nalidixic acid) and one dilution step (ciprofloxacin and sparfloxacin). MICs of tetracycline were generally less affected (Table 4).

Identification of target mutations leading to quinolone resistance

Determination of the DNA sequences of the entire gyrA genes and ~80% of other quinolone target genes gyrB, parC and parE (data not shown) including the quinolone resistance-determining regions (QRDRs)19,20 revealed four gyrA mutations in vacT compared with M415: TGG→AGG (W59R), GGT→GCT (G75A), GAC→GCC (D87G) and GCC→TCG (A867S) (Table 1). In strains Nal2ori and Nal2passage only the last three gyrA mutations were detected. Mutant M415-87 contains a single gyrA mutation GAC→TAC (D87Y). The DNA sequences of the QRDRs of the other target genes were identical in all strains.

Complementation assay

Conjugational transfer of the recombinant plasmid pBP517 (gyrA*) into M415 and its mutants resulted in a reduction of the MICs of fluoroquinolones for all mutants compared with the gyrA-free control plasmid pBP507. A gyrA* allele in mutant M415-87 restored the fluoroquinolone susceptibility of the parent M415 (Table 5).
Supercoiling assay
To investigate whether the accumulation of up to four gyrA mutations affects the supercoiling activity of gyrase, the global supercoiling degree of the DNA was determined for the different mutants by use of the supercoiling-sensitive luc reporter gene system. The results of the Qsc determination revealed a reduction of the Qsc of strains Nal2ori, Nal2passage and vacT to 60% (Table 1) compared with parent strain M415. Qsc of mutant M415-87 was close to that of M415 (Table 1).

Doubling time
The doubling times of three mutant strains investigated differed by factors between 0.5 (Nal2passage) and 3.1 (Nal2ori) (Table 1). In comparison with its parent M415, vacT had a nearly twofold longer doubling time.

Discussion
Use of attenuated live vaccine strain Salmonella Typhimurium vacT has proven to be an efficient measure to reduce the prevalence of contaminated food from chicken. In view of the increasing incidence of quinolone resistance among salmonellae the presence of a nalA genotype in vacT might be a cause of concern. However, vacT displays an unexpected high susceptibility to fluoroquinolones similar to parent strain M415, despite a reduced susceptibility to nalidixic acid. This resistance phenotype is reminiscent of a GGC!GAC mutation (G81D) mediating resistance to nalidixic acid, but not to fluoroquinolones, while all known gyrA mutations within the QRDR including those affecting serine-83 and aspartate-87 result in complete cross-resistance to all quinolones in Salmonella. DNA sequence analysis of gyrA revealed a combination of four mutations in vacT compared with M415. These include two mutations outside (W59R and A867S) and two within (G75A and D87G) the QRDR. That a mutation affecting aspartate-87 is associated with quinolone resistance is known from single-step mutants of different Salmonella serotypes described by Hansen et al., Reyna et al. and Griggs et al. and from mutant M415-87 for the specific strain M415 (Table 1). The other three mutations are novel mutations. Considering the applied strategy of random mutagenesis to isolate vacT the accumulation of several mutations in gyrA is not unexpected; however, the complete lack of

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Table 3. PCR primers used for DNA sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (nucleotide position)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>5'-GTTCACCAGTACGGGCGAATG-3' (–76 to 54)</td>
</tr>
<tr>
<td></td>
<td>5'-GACAAGAGAGAGACATGCTTGAG-3' (595–617)</td>
</tr>
<tr>
<td></td>
<td>5'-ATGACGGCAACGTGCTGC-3' (1247–1265)</td>
</tr>
<tr>
<td></td>
<td>5'-AGCCGGCTACGTTCCTATGG-3' (1292–1285)</td>
</tr>
</tbody>
</table>

Table 4. Antibiotic susceptibilities of M415, M415-87, Nal2ori, Nal2passage and vacT in comparison with ATCC 14028 determined as MICs (mg/L) in the absence and presence of efflux pump inhibitor (EPI)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ATCC 14028</th>
<th>M415 + EPI</th>
<th>M415-87 + EPI</th>
<th>Nal2ori + EPI</th>
<th>Nal2passage + EPI</th>
<th>vacT + EPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI</td>
<td>256</td>
<td>256</td>
<td>NA</td>
<td>256</td>
<td>NA</td>
<td>16</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2</td>
<td>4</td>
<td>≤0.25</td>
<td>64</td>
<td>2</td>
<td>128</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>0.03</td>
<td>0.03</td>
<td>≤0.007</td>
<td>0.06</td>
<td>0.015</td>
<td>0.125</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.03</td>
<td>0.03</td>
<td>0.007</td>
<td>0.25</td>
<td>0.03</td>
<td>0.125</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>8</td>
<td>16</td>
<td>ND</td>
<td>8</td>
<td>ND</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>64</td>
<td>&gt;64</td>
<td>0.5</td>
<td>64</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>64</td>
<td>32</td>
<td>0.125</td>
<td>32</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>Tylosin</td>
<td>128</td>
<td>256</td>
<td>4</td>
<td>ND</td>
<td>32</td>
<td>8</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined.

EPI, efflux pump inhibitor (phenylalanyl-arginyl-β-naphthylamide). EPI concentration used: M415 and M415-87, 64 mg/L; Nal2ori, 32 mg/L; Nal2passage, 16 mg/L; vacT, 8 mg/L.
mutations in >80% of other target genes gyrB, parC and parE including the QRDRs of any mutant strain points to a prominent role of the gyrase A subunit for the selection of vacT. While in high-level fluoroquinolone-resistant mutants of Salmonella a combination of specific target mutations affecting subunits A (gyrA, parC) of both DNA gyrase and topo IV, respectively, as well as subunit B of DNA gyrase has been reported, a combination of gyrA mutations in a mutant showing increased susceptibility to fluoroquinolones is new.

The accumulation in fluoroquinolone-resistant mutants of target mutations, such as those affecting S83 and D87 in gyrA, has been also shown to be associated with reduced DNA supercoiling degree. In multi-step gyrA mutants of *E. coli* concomitantly doubling time was prolonged and expression of type I fimbriae reduced. Another study reports reduced production of haemolysin in a *nalA* mutant. The DNA supercoiling degrees of mutants *nalA*2ori, *nalA*2passage and vacT were ~60% that of M415 (Table 1). Thus, it is tempting to speculate that at least one of the three gyrA mutations in these mutants caused a reduction of the DNA supercoiling activity. The finding that the Qsc value of another single-step gyrA mutant (M415-87) carrying a different gyrA mutation (D87Y) resembles that of parent M415 does not necessarily exclude a possible impact of the D87G mutation on the Qsc for the other three mutants. However, a reduced susceptibility to ciprofloxacin and sparfloxacin of *nalA*2ori and *nalA*2passage, but not of vacT, might point to a role as resistance modulator of the W59R mutation unique to vacT (Table 4). However, no obvious correlation between degree of supercoiling and doubling time was detectable in the present study.

In complementation tests using the gyrA*"* allele of *E. coli* the susceptibilities to nalidixic acid, ciprofloxacin and sparfloxacin are usually reduced to below wild-type levels. This was also detectable for mutants M415-87, *nalA*2ori and *nalA*2passage; however, for vacT MICs of fluoroquinolones were reduced below those of M415. This could plausibly be explained by an additional mutation reducing the activity of an MDR efflux pump, the second factor determining the quinolone susceptibility. This view is supported by the low MIC of EPI (16 mg/L) for vacT compared with the MICs for M415 and M415-87 (256 mg/L) and the other two mutants (64–256 mg/L) (Table 4). These data point to a reduced expression level of functional efflux pumps in vacT compared with M415.

Results of the determination of MICs in the presence of EPI (Table 4) are indicative of decreased outer membrane efflux: the amount of EPI required to reduce the MICs of erythromycin or clarithromycin to identical values differs between M415 (64 mg/L EPI) and vacT (8 mg/L). This suggests that increased macrolide susceptibility, including the 16-membered macrolide tylosin, results from decreased outer membrane efflux in vacT. Thus, the increased susceptibility of vacT to EPI indicates a reduction in the intrinsic activity of MDR efflux pumps compared with its precursors, most probably explained by mutation(s) inactivating or down-regulating MDR efflux pumps of the RND-type, the preferred substrates of EPI. Among MDR efflux systems the AcrAB-TolC pump plays a role in intrinsic resistance of *E. coli* to macrolides and, if overexpressed, as an additional mechanism leading to acquired resistance to fluoroquinolones. In *E. coli* mutations affecting local or global regulators of the AcrAB-TolC efflux pump have been shown to be associated with increased expression of *acrAB* genes alone or in combination with a decrease of outer membrane porin (OMP) F, respectively. Accumulating indirect evidence points to a similar role of AcrAB-TolC in multiple drug resistance in *Salmonella* too. From our experimental approach using EPI as an inhibitor of most RND-type efflux pumps we cannot conclude that fluoroquinolones and macrolides are substrates of the same efflux pump. In contrast to *E. coli*, the *marRAB* operon of *Salmonella* has not yet been demonstrated to be involved in a fluoroquinolone resistance phenotype. Alternatively, alterations of outer membrane constituents (porins, lipopolysaccharide) have been associated with altered fluoroquinolone susceptibility. Compared with M415, sequencing data did...
Fluoroquinolone-susceptible gyrA mutant

not reveal an alteration in acrR of Salmonella vacT (data not shown).

Recently, a cpxA mutation has been suggested as a possible candidate leading to a germination to tnsid tolerance (rtt mutation) in vacT.13 However, supporting genetic evidence has not been provided and the present study did not reveal a mutation in the respective gene of vacT compared with M415 (data not shown). Probably, beside the rtt-associated mutation additional unknown mutation(s) introduced into vacT during random mutagenesis could account for the fluoroquinolone-susceptible phenotype of the gyrA mutant vacT.

The data presented emphasize the importance of gyrA as a major factor contributing to the unique phenotype/genotype correlation of vacT characterized by a low level of resistance to nalidixic acid and a simultaneous wild-type susceptibility to fluoroquinolones and macrolides in the presence of a known fluoroquinolone resistance mutation gyrA D87G. Currently, different gyrA mutations are under investigation for their individual impact on the fluoroquinolone susceptibility and the supercoiling degree of vacT.

A combination of additional novel gyrA mutation(s) with an as-yet-undetermined mutation associated with reduced efflux seems to explain additional characteristics of vacT, such as high susceptibility to macrolides, reduced doubling time and, presumably, attenuated virulence. A detailed understanding of the mechanisms leading to attenuation will be a step forward to the design of safe and stable live vaccines in the future.

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Transparency declarations

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References

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