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Molecular characterization of the genes encoding DNA gyrase and topoisomerase IV of *Listeria monocytogenes*

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The genes encoding subunits A and B of DNA gyrase and subunits C and E of topoisomerase IV of *Listeria monocytogenes*, *gyrA*, *gyrB*, *parC* and *parE*, respectively, were cloned and sequenced. Compared with the sequences of quinolone-susceptible bacteria, such as *Escherichia coli* and *Bacillus subtilis*, the quinolone resistance-determining region (QRDR) of DNA gyrase subunit A was altered; the deduced amino acid sequences revealed the substitutions Ser-84→Thr and Asp/Glu-88→Phe, two amino acid variations at hot spots, commonly associated with resistance to quinolones. No relevant divergences from QRDR consensus sequences were observed in GyrB or both topoisomerase IV subunits. Thus, it could be argued that the amino acid substitutions in GyrA would explain the intrinsic resistance of *L. monocytogenes* to nalidixic acid. In order to analyse the actual role of the GyrA alterations, a plasmid-encoded *gyrA* allele was mutated and transformed into *L. monocytogenes*. However, these heterodiploid strains were not affected in their resistance to nalidixic acid. The effects of the mutant plasmids on ciprofloxacin and sparfloxacin susceptibility were only modest.

Introduction

The Gram-positive, human-pathogenic, facultative intracellular bacterium *Listeria monocytogenes* is the causative agent of listeriosis, one of the major food-borne diseases in the last two decades.[1,2] Although *L. monocytogenes* is susceptible to many common antibiotics, it is intrinsically resistant to nalidixic acid[3] and shows a decreased susceptibility to therapeutically important fluoroquinolones such as ciprofloxacin.[4] Therefore, this class of powerful antimicrobial agents is at present not a treatment option for this pathogen.

Quinolones act on the type II topoisomerases DNA gyrase (encoded by genes *gyrA* and *gyrB*) and topoisomerase IV (encoded by genes *parC* and *parE*), enzymes that control DNA topology. Single point mutations in the N-terminal domains of subunit A of DNA gyrase or subunit C of the homologous topoisomerase IV, in the so-called quinolone resistance-determining regions (QRDRs), have been found to be responsible for resistance to fluoroquinolones in *Escherichia coli* and a wide variety of pathogenic bacteria.[5,6] In addition, QRDRs were also detected in the B and E subunits of DNA gyrase and topoisomerase IV, respectively[7,8] (for a review see Drlica & Zhao[9]). The aim of this genetic study was to investigate whether alterations were present in the QRDRs of type II topoisomerases of *L. monocytogenes* and, if so, were also responsible for the quinolone-resistant phenotype of *L. monocytogenes.*

Materials and methods

Bacterial strains

L. monocytogenes EGD (SLCC 5853) was from the Special Listeria Culture Collection (Institute of Medical Medicine Mannheim, University of Heidelberg, Germany). This strain was the focus of this study, including cloning and sequencing

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of the gyrase and topoisomerase genes (source of DNA), as well as mutagenesis of *gyrA* (donator of DNA) and the complementation experiments (recipient strain).

E. coli DH5α was from Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD, USA. *E. coli* TOP10 was from Invitrogen BV, Groningen, The Netherlands.

Culture media and antibiotics

L. monocytogenes was grown in brain-heart infusion (BHI) broth (BD/Difco, Franklin Lakes, NJ, USA), *E. coli* strains were grown in Luria–Bertani (LB) broth, both at 37°C. Antibiotics for selection of transformants were used at the following concentrations (Sigma-Aldrich Corp., St Louis, MO, USA): ampicillin, 100 mg/L (*E. coli*); erythromycin, 50 mg/L (*E. coli*) or 5 mg/L (*L. monocytogenes*); chloramphenicol, 10 mg/L (*E. coli* and *L. monocytogenes*).

Determination of MICs

MICs were determined in duplicate using Etest (AB Biodisk, Solna, Sweden; purchased from Viva Diagnostika GmbH, Hürth/Köln, Germany) on Mueller–Hinton agar plates according to the manufacturer's instructions. Test strips covered the following MIC ranges: 0.002–32 mg/L for ciprofloxacin and sparfloxacin, 0.016–256 mg/L for nalidixic acid.

Isolation of DNA

E. coli plasmid DNA was isolated with a QIAspin Miniprep Kit (Qiagen N.V., Venlo, The Netherlands).

L. monocytogenes EGD chromosomal DNA was isolated with QIAamp and DNeasy Tissue Kits (Qiagen) according to the manufacturer's protocols for bacteria. For inverse PCR, chromosomal DNA was isolated by proteinase K and SDS lysis as described by Leimeister-Wächter & Chakraborty,[10] and cleaned up with phenol/chloroform extraction; the total amount of DNA isolated from 25 mL of overnight culture was resuspended in 600 μL of sterile distilled water. For both chromosomal DNA isolation procedures, the cell walls were initially disrupted with mutanolysin (Sigma) (cell pellet resuspended in 1 mL of 100 mM sodium phosphate buffer pH 7.0, containing 250 U of mutanolysin) for 1–3 h.

Recombinant DNA techniques

Recombinant DNA techniques were applied according to standard protocols.[11] For cloning, amplicons were ligated blunt-ended into vector pUC18, pre-digested with *Sma*I (pUC18 Ready-To-Go; Amersham Pharmacia Biotech AB, Uppsala, Sweden). For complementation and mutagenesis, the *gyrBA* genes were PCR amplified with primers GC-3 and GC-4R (Table 1) and ligated into the multiple cloning site (internal of cat68::*lacZ*α, constitutively transcribed from promoter P59) of vector pHPS9 (cm^R, er^R).[12] The vector was cut with *Nhe*I and *Sma*I (*gyrBA* divergently orientated to promoter P59, pNT) or with *Bam*HI and *Sma*I (*gyrBA* in the same orientation as P59, pW1-18).

PCR

Goldstar DNA polymerase (Eurogentec Bel SA, Seraing, Belgium) was used applying standard parameters for concentrations: 1× reaction buffer as supplied by the manufacturer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 12.5 pmol of each primer (Table 1) and 1 U of DNA polymerase per kb of amplicon in 50 μL total reaction volume. The concentration of degenerate primers in the PCR mixture was increased to match the degree of degeneration (e.g. two-fold increased concentration for an oligonucleotide with two degenerate nucleotide positions). The final $MgCl_2$ concentration was raised to 3 mM for amplification of *parC* QRDR with primers PAC-1 and PAC-2R and to 2 mM for amplification of *gyrB* (primers GYB-1X and GYB-2R) and *parE* (primers PAE-1 and PAE-2RX). Cycling parameters were: 2.5 min at 95°C as an initial denaturation step; 30 cycles of 30 s at 95°C, 30 s at the appropriate annealing temperature (5°C below T_m of oligo), and 1 min of extension per kb of amplicon at 72°C; followed by a last extension step of 2 min at 72°C. For inverse PCR,[13] 20 μL of chromosomal DNA were digested overnight with an appropriate restriction enzyme (*Eco*RI, *Hin*dIII; 150 U in 100 μL reaction volume), extracted with phenol/chloroform, religated (5 U ligase in 400 μL reaction volume) and, after precipitation, resuspended in 60 μL of sterile distilled water. Aliquots of 2.5–10 μL were used as a template in the PCR. For cloning of the full-length genes, mutagenesis and re-sequencing PCRs, proofreading DNA polymerases *Pfu* turbo (Stratagene Inc., La Jolla, CA, USA) or *Pwo* (Roche/Hoffmann-La Roche Ltd, Basel, Switzerland) were used. Amplification products were purified with the QIAquick PCR purification kit (Qiagen).

Site-directed mutagenesis

Site-directed mutagenesis was achieved by applying recombination PCR (RPCR)[14] to the *gyrA*-QRDR (PCR-amplified with primers QRD-1 and QRD-2R) of *L. monocytogenes* EGD, cloned into pUC18. Primers for mutagenesis were AMP-2 and AMP-1R from the pUC18 amp^R gene in combination with FE-1 and FE-2R, TS-1 and TS-2R, TF-1 and TF-2R, or MS-1 and MS-2R (Table 1). Following mutagenesis in pUC18, QRDR DNA fragments were exchanged between plasmids after restriction with *Sma*I and *Bst*XI (unique restriction sites in *gyrBA*, flanking the QRDR).

DNA sequencing and analysis

DNA was sequenced at MWG (MWG-Biotech AG, Ebersberg, Germany). DNA and protein sequences were analysed with DNASIS (Hitachi Software Engineering Co., Ltd, Yokohama, Japan) and the HUSAR analysis package via the inter-

Table 1. Primers used in this study[a]

Name	Sequence[b]	Comment[c]
ART-3	5′-GCAGAAACACCAAATCA-3′	
AMP-1R	5′-CAGCAATAAACCAGCCAGCCG-3′	pUC18, amp^R gene
AMP-2	5′-CGGCTGGCTGGTTTATTGCTG-3′	pUC18, amp^R gene
BRT-1	5′-TACATTGGTTCAACTAGC-3′	
BRT-2R	5′-TTCCATATCCATTACTAC-3′	
FE-1	5′-AGCGGTTTAT**GAA**ACAATGGTTCGT-3′	Phe-88→Glu
FE-2R	5′-ACGAACCATTGT**TTC**ATAAACCGCT-3′	Phe-88→Glu
FE-TST	5′-ATACGAACCATTGT**TTC**-3′	Phe-88→Glu screening
GB-1R	5′-TGCTTGAGAATCCAGTA-3′	
GB-2	5′-CAATCACGGATAAGCTA-3′	
GC-3	5′-CATCGGGAT**CC**TTCTCTGTTTTA-3′	pos. 1–23 (*Bam*HI site)
GC-4R	5′-TAATAA**G**C**TA**GCATAGTTGGATAAT-3′	pos. 5194–5170 (*Nhe*I site)
GP-1X	5′-CCIGGIATGTAYATHGG-3′	
GYA-1	5′-TWYYTIGAYTAYGCIATG-3′	
GYA-2	5′-TGAACGTGAGCCAGTTA-3′	
GYA-3R	5′-ATCATIARRTCYTCYTC-3′	
GYA-5R	5′-CGAAGTTCTACAGCACGA-3′	
GYA-7	5′-TATCCGCTTCGTAATCGT-3′	
GYB-1X	5′-CAYTAYGARGGIGGIAT-3′	
GYB-2R	5′-ATRTGIGCICCRTCIAC-3′	
IGA-F	5′-ATTAGTCAATGGTTCTA-3′	
IGA-R	5′-AAATAACTGGCTCACGT-3′	
IPC-R	5′-CTCACTAGACGTATCAT-3′	
MS-1	5′-TTGATGGCGAT**TC**GGCAGCAGCGAT-3′	Met-117→Ser
MS-2R	5′-ATCGCTGCTGCC**GA**ATCGCCATCAA-3′	Met-117→Ser
MS-TST	5′-TTCGGTTGATGGCGAT**TC**-3′	Met-117→Ser screening
P9-FO	5′-TAGCAAAAGCAGCAACCTA-3′	pHPS9 specific
P9-RE	5′-AAGGCGATTAAGTTGGGTA-3′	pHPS9 specific
PAC-1	5′-TTTGGIMGITAYWSIAARTA-3′	
PAC-2R	5′-TGIGGIGGDATRTCIGTIGC-3′	
PAC-3R	5′-RTCYTTCCAICKDATRTC-3′	
PAC-5	5′-ATCGACCAGTGCATGAGT-3′	
PAC-6R	5′-GCTCGTGAATAACAGGAG-3′	
PAE-1	5′-AAYGTIMGIACIAARGA-3′	
PAE-2RX	5′-CATYTCICCIARICCYTT-3′	
PC-1	5′-CTTGGCAT**GC**CTAATCAGGA-3′	pos. 1416–1435 (*Sph*I site)
PC-2R	5′-ATCTC**TC**T**A**GACGCCCACTT-3′	pos. 6305–6286 (*Xba*I site)
PE-1R	5′-TCACCTTCGACTAGATA-3′	
PE-2	5′-GTCGTTTCCAAGCTATA-3′	
PE-3R	5′-ATACTAAATGGTGCAGCC-3′	
QRD-1	5′-ATTCATCGAAGACAACG-3′	
QRD-2R	5′-ATCGTGACTAAGTGCTA-3′	
REF	5′-GTIGGICCICAYMGIGA-3′	
TF-1	5′-ACGGCGATA**GT**GCGGTTTAT**GAA**ACAATGGTT-3′	Thr-84→Ser, Phe-88→Glu
TF-2R	5′-AACCATTGT**TTC**ATAAACCGC**AC**TATCGCCGT-3′	Thr-84→Ser, Phe-88→Glu
TS-1	5′-ACGGCGATA**GT**GCGGTTTAT-3′	Thr-84→Ser
TS-2R	5′-ATAAACCGC**AC**TATCGCCGT-3′	Thr-84→Ser
TS-TST	5′-TGTAAAATAAACCGC**AC**-3′	Thr-84→Ser screening

[a]IUPAC abbreviations; I, inosine.
[b]Mismatches, introduced to create mutational or restriction sites, are in bold.
[c]Nucleotide positions according to the published sequence. Introduced restriction sites are in parentheses.

net (German Cancer Research Center, Heidelberg, Germany; HUSAR is based on the GCG Package, Genetics Computer Group, Inc.). FASTA searches were carried out at the EMBL European Bioinformatics Institute (Cambridge, UK).

Nucleotide sequence accession number

The nucleotide sequence data reported here will appear in the GenBank nucleotide sequence databases with the following accession numbers: AF084042 for the *gyrBA* genes and AF084044 for the *parEC* genes.

Gyrase homology modelling

The sequences of *E. coli* and *L. monocytogenes* EGD GyrA were aligned with the program XSAE (C. Broger, personal communication). The program MOLOC[15] was used for on-screen modelling based on the sequence alignment and on the published X-ray structure of the 59 kDa N-terminal fragment of *E. coli* GyrA.[16]

Transforming plasmids into L. monocytogenes

L. monocytogenes EGD from mid-log growth phase (OD_{550} = 0.6–0.7) was pelleted at 3000**g**, washed once in 1/10 vol. 3.5 SMEM (952 mM sucrose/3.5 mM $MgCl_2$) and resuspended in 1/100 vol. 3.5 SMEM (cells were kept at 4°C during all steps). Using a Gene Pulser II unit with Pulse Controller Plus (Bio-Rad Laboratories Inc., Hercules, CA, USA), 100–200 µL cell aliquots were electroporated with 10 µg of DNA in 0.2 cm cuvettes (Gene Pulser settings: 2.5 kV, 25 µF, 100 Ω). After pulsing, the bacteria were pre-cultured in 1 mL of BHI for 3 h at 37°C before streaking them on selective BHI plates. Transformants were obtained after 2–5 days of incubation at 37°C. Transformants were PCR-screened with plasmid/insert-specific and *L. monocytogenes iap*-specific[17] primer pairs.

RNA extraction and RT–PCR

Total RNA was isolated from *E. coli* using an RNeasy Kit (Qiagen) and on-column DNase (Qiagen) treatment according to the manufacturer's instructions.

Total RNA (1 µg) was reverse transcribed with primer IGA-R (*gyrA*) or BRT-2R (*gyrB*) using a 'First Strand cDNA Synthesis Kit for RT–PCR' (Roche) according to the manufacturer's protocol. cDNA (5 µL) was used in the subsequent PCRs (50 µL reaction volume, 1× reaction buffer, 1.5 mM $MgCl_2$, 12.5 pmol each primer, 1.5 U Goldstar DNA polymerase; 45°C annealing temperature) with ART-3/IGA-R (*gyrA*) and BRT-1/BRT-2R (*gyrB*) (Table 1).

Results

PCR amplificaton of the QRDRs

The GyrA and ParC protein sequences of *Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *E. coli* were aligned using DNASIS. Pairs of degenerated PCR primers were selected from conserved regions, GYA-1 and PAC-2R from GyrA, PAC-1 and PAC-2R from ParC (Figure 1). These primers were designed so that they would not amplify *gyrA* and *parC* of *L. monocytogenes* EGD

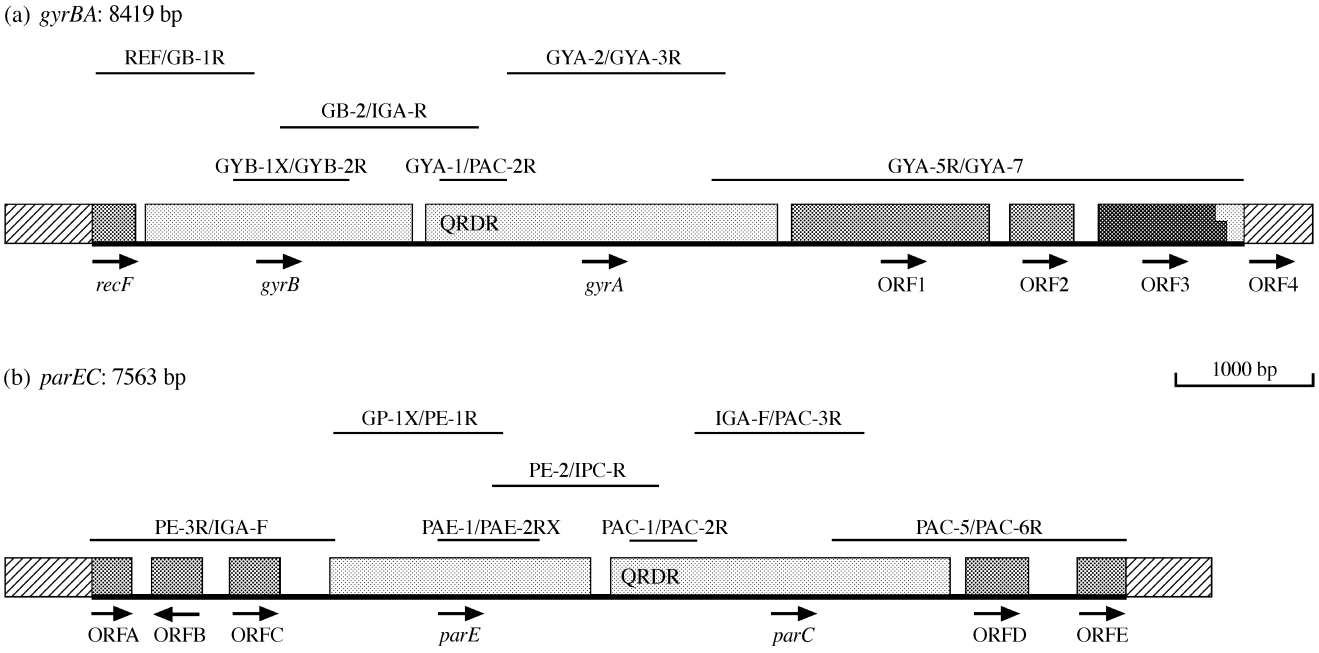

Figure 1. *L. monocytogenes* SLCC 5853 genomic DNA regions containing the*gyrBA* (a) and *parEC* (b) operons. PCR products and primers used for cloning are shown above the genes.

simultaneously (although PAC-2R has binding sites in both genes). Internal parts of *gyrB* (primers GYB-1X and GYB-2R) and *parE* (primers PAE-1 and PAE-2RX) were PCR amplified in the same way.

PCR amplification of the full-length genes

The missing DNA regions between *gyrB* and *gyrA* as well as between *parE* and *parC* were amplified with primers derived from the sequences already obtained (primers GB-2 and IGA-R for the *gyrBA* genes, PE-2 and IPC-R for *parEC* genes), demonstrating that *gyrB* and *gyrA* as well as *parE* and *parC* are contiguous in *L. monocytogenes* EGD. The missing 5′ and 3′ regions were amplified either by inverse PCR or by using degenerate primers derived from conserved amino acid stretches again (Figure 1 and Table 1).

In the case of *gyrB*, a degenerate primer (REF) for *recF* was chosen, assuming that *recF* precedes *gyrB* in *L. monocytogenes*, as it does in *B. subtilis*. The *gyrA* 3′ region was PCR amplified, first using primer GYA-2 in combination with the degenerate primer GYA-3R and subsequently by an inverse PCR with primers GYA-5R and GYA-7 (*Eco*RI cut DNA).

The *parE* 5′ region was PCR amplified, first with primers PE-1R and GP-1X (degenerate), followed by inverse PCR with primers PE-3R and IGA-F (*Hin*dIII cut DNA). The *parC* 3′ region was PCR amplified using primers IGA-F and primer PAC-3R (degenerate) and completed with inverse PCR using primers PAC-5 and PAC-6R (*Hin*dIII cut DNA).

The obtained sequences span 8419 bp (*gyrBA* plus flanking regions) and 7563 bp (*parEC* plus flanking regions). The organization of both chromosomal regions is shown in Figure 1.

Each locus was PCR amplified again for cloning and resequencing with proofreading DNA polymerases in order to minimize misincorporations. *gyrBA* was PCR amplified with primers GC-3 and GC-4R (Table 1) and cloned into vector pHPS9 for sequencing and complementation studies. As *parEC* (PCR-amplified with primers PC-1 and PC-2R; Table 1) from *L. monocytogenes* could not be cloned in *E. coli*, the amplicon was resequenced directly.

Sequence analysis

The DNA gyrase subunits GyrA (842 aa) and GyrB (646 aa) show the highest similarity to GyrA (71.4% identity) and GyrB (71.2% identity) from *B. subtilis*. Preceding *gyrB*, a gene similar to *recF* can be found. The deduced amino acid sequence (only partial, 106 aa, C-terminus) shows 77.1% identity to RecF from *B. subtilis*. Downstream of *gyrA*, four open reading frames (ORFs) are located. ORF1 (504 aa) codes for a protein with similarity to cardiolipin synthase (*cls*) from *Bacillus firmus* (37.5% identity). The deduced amino acid sequence of ORF2 (172 aa) is similar to spermidine *N*1-acetyltransferase (*speG*) from *E. coli* (70.6% identity). ORF3 codes for a protein (327 aa) showing similarity to mevalonate kinase (*mvaK1*) from *Enterococcus faecium* (40.8% identity), and ORF4 codes for a protein (only partial, 79 aa, N-terminus) with similarity to mevalonate diphosphate decarboxylase (*mvaD*) from *Staphylococcus haemolyticus* (59.5% identity). Interestingly, ORF3 overlaps ORF4, the start codon of ORF4 being located 41 bp upstream of the stop codon of ORF3.

The topoisomerase IV subunits ParC (819 aa) and ParE (655 aa) show highest similarities to their counterparts ParC (64.0% identity) and ParE (76.0% identity) from *B. subtilis*. Three ORFs are located upstream of *parE*. The first (ORFA, only partial, 103 aa, C-terminus) codes for a protein with similarity to LacX from *Lactococcus lactis* subsp. *lactis* (32.0% identity). ORFB (198 aa) and ORFC (135 aa) code for proteins with similarity to *B. subtilis* YneS (58.6% identity) and YneT (61.8% identity), respectively. Downstream of *parC* ORFD (155 aa) codes for a protein with similarity to autoinducer-2 production protein LuxS from *Helicobacter pylori* (68.5% identity). No significant similarity to proteins in the databases was found for ORFE (only partial, 187 aa, N-terminus).

Sequence analysis hints at a putative but perfect –10 box preceding *gyrB* (TATAAT, nt 332–337 in the published sequence). However, no matching –35 consensus sequence is found here (consensus sequences without –35 boxes have been described for gyrase genes). No promoter consensus sequences and no terminator-like structures seem to be present in the intergenic space between *gyrB* and *gyrA*. In contrast, a terminator-like structure is located downstream of *gyrA* (nt 4933–4989). The situation is similar for *parE* and *parC*: a terminator-like structure is situated downstream of *parC* (nt 6127–6166) and a rather weak putative promoter is located upstream of *parE* [TTGTCA-$(N)_{15}$-ATTAAT, nt 1582–1608 in the published sequence].

A QRDR alignment of *gyrA* of *L. monocytogenes* and of other quinolone-susceptible bacterial species (Figure 2) revealed two altered residues, commonly associated with resistance to fluoroquinolones[5] (for an overview see Piddock[18]). At position 88 (87 in *E. coli* numbering) the aromatic amino acid phenylalanine is found instead of the negatively charged aspartate/glutamate residue. At position 84 (83 in *E. coli*) threonine replaces serine. A third possible variation of importance was discovered in the N-terminus of GyrA of *L. monocytogenes* by superimposing three-dimensional models of the N-terminal portions of GyrA of *E. coli* and *L. monocytogenes* using Swiss-PdbViewer (Glaxo Wellcome/GlaxoSmithKline, UK). The side-chain of Met-117 (Ser-116 in *E. coli*) is bulkier than usually found at this position and points towards the two other altered side-chains. All three side-chains are located in close proximity to each other. In 1999, Pestova *et al.*[19] reported on *S. pneumoniae* gyrase mutants with decreased susceptibility to fluoroquinolones. Beside alterations in ParC

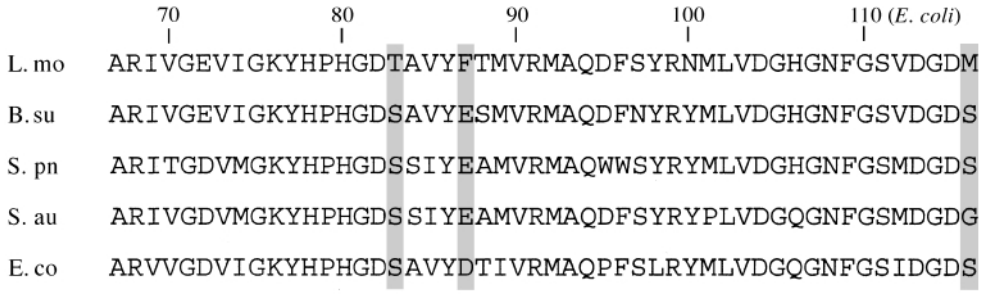

Figure 2. Comparison of the QRDRs of *L. monocytogenes* (L. mo), B. subtilis (B. su), S. pneumoniae (S. pn), S. aureus (S. au) and E. coli (E. co). Numbering of residues according to E. coli GyrA.

alone or in ParC and ParE, a Ser-114→Gly (116 in *E. coli*) amino acid substitution in GyrA was found in these mutants (although no direct role in the contribution to resistance could be assigned to this substitution in complementation experiments).

Mutagenesis and complementation

In order to investigate the role of the mentioned amino acid substitutions of gyrase of *L. monocytogenes*, *gyrA* of *L. monocytogenes* EGD was mutagenized on plasmid pW1-18 (where *gyrBA* can be transcribed from plasmidic promoter P59). Phe-88 was replaced with Glu on pW1-A, Thr-84 with Ser on pW1-B, and both positions were changed on pW1-C. Met-117 alone was replaced with Ser on pW1-D and Phe-88→Glu was introduced additionally on pW1-E. Mutations were verified by sequencing the exchanged DNA fragments; the insert of pW1-C was sequenced completely. On the basis of reports that sensitive, plasmid-borne alleles of *gyrA* are dominant over chromosomal alleles,[20–22] the plasmids were transformed into *L. monocytogenes* EGD (Table 2). The presence of transformed mutants was verified with PCR. Gyrase-specific primers (GC-3 and GC-4R) were used in combination with plasmid-specific primers (P9-FO and P9-RE, forward and reverse primers with binding sites in the multiple cloning site of vector pHPS9) to check for the presence of the plasmids carrying the mutagenized alleles. A set of primers with 3′ bases complementary to the codons of the newly introduced amino acids in *gyrA* was used to verify the presence of the amino acid substitutions: primer FE-TST for Phe-88→Glu, TS-TST for Thr-84→Ser and MS-TST for Met-117→Ser (Table 1). *L. monocytogenes* EGD wild type served as a negative control in both screening PCRs. To confirm that indeed *L. monocytogenes* was transformed, mutants were screened with *L. monocytogenes*-specific primers (MONO-A and MONO-B).[17]

Susceptibility testing

No differences in the susceptibility patterns between *L. monocytogenes* wild type and the complemented strains were detected for nalidixic acid. The strains SLCC 5853-A, -B and -C showed slightly decreased MICs of ciprofloxacin and sparfloxacin. The double mutation in SLCC 5853-C conferred the highest degree of sensitivity to ciprofloxacin (MIC 0.38 mg/L), followed by the intermediate phenotype of SLCC 5853-A (MIC 0.5 mg/L), whereas SLCC 5853-B showed the lowest reduction of the MIC (0.75 compared with 1 mg/L for the control strain SLCC 5853-18, complemented with wild-type *gyrBA*). The MIC of sparfloxacin was reduced to 1 mg/L compared with 1.5 mg/L for the control strain SLCC 5853-18 in all three cases. No differences in the MICs for all tested antibiotics were observed for SLCC 5853-D and SLCC 5853-E, again compared with SLCC 5853-18 (Table 2).

No differences in the pattern of susceptibility to nalidixic acid, either between *E. coli* DH5α (nal^R, *gyrA*96) wild type or complemented with all plasmids, or between *E. coli* TOP10 (nal^S) wild type or complemented with pW1-18 or pW1-C, were observed (data not shown).

Expression of gyrase

RT–PCR with primers specific for *gyrA* or *gyrB* of *L. monocytogenes* revealed transcription of both genes in complemented *E. coli* DH5α, showing that the constructed plasmids were functional. Transcripts of both genes were also detected in *E. coli* complemented with pNT, where *gyrBA* is orientated divergently to plasmidic promoter P59 (data not shown). Transcription of *gyrA* and *gyrB* was tested in *E. coli*, as two alleles of these genes were present in the complemented *Listeria* strains (plasmidic on vector pHPS9 and chromosomal), which might have resulted in false positive results. The vector pHPS9 had already been used sucessfully for cloning and expression of listerial genes in *Listeria* spp.[23]

Discussion

The complete *gyrBA* and *parEC* genes were cloned and sequenced (Figure 1) and could subsequently be structurally characterized. Assignments were based on close sequence similarities to DNA gyrase and topoisomerase IV subunits of *B. subtilis*, *S. pneumoniae*, *S. aureus* and *E. coli*.

Table 2. Plasmids and determination of MICs

Plasmid/insert[a]	MIC (mg/L)[b]		
	CIP	SPAR	NA
No plasmid (*L. monocytogenes* EGD wild type)	2	2	>256
pHPS9 (cloning vector, no insert)	1	1.5	>256
pW1-18 (*gyrA* wild type)[c]	1	1.5	>256
pW1-A (Phe-88→Glu)[c]	0.5	1	>256
pW1-B (Thr-84→Ser)[c]	0.75	1	>256
pW1-C (Phe-88→Glu, Thr-84→Ser)[c]	0.38	1	>256
pW1-D (Met-117→Ser)[c]	1	1.5	>256
pW1-E (Met-117→Ser, Phe-88→Glu)[c]	1	1.5	>256

[a]*L. monocytogenes* EGD SLCC 5853 complemented with the plasmid.
[b]CIP, ciprofloxacin; SPAR, sparfloxacin; NA, nalidixic acid.
[c]Insert of the plasmid is *gyrBA* (can be transcribed from P59), mutation of *gyrA* is indicated in parentheses.

Sequence analysis of both loci indicates that *gyrBA* and *parEC* might constitute transcriptional units, i.e. operons. Terminator-like sequences are only found downstream of *gyrA* and *parC*, and promoter-like sequences seem to be located only upstream of *gyrB* and *parE*.

Further analysis of the gyrase genes confirmed the presence of Phe and Thr residues in GyrA at positions 88 and 84, respectively, in place of Asp/Glu and Ser residues conserved in many quinolone-susceptible bacteria. After site-directed mutagenesis of these residues, the recombinant *gyrBA* alleles were transformed into *L. monocytogenes*, producing heterodiploid strains for this locus. Introducing these plasmid-borne, presumably sensitive alleles of DNA gyrase had no effect on the sensitivity pattern of *L. monocytogenes* transformants to nalidixic acid. The plasmidic mutations Phe-88→Glu or Thr-84→Ser produced a slightly elevated sensitivity for fluoroquinolones (Table 2). As this effect was very modest, no definitive conclusion could be drawn about the phenotype of the *gyrA* gene, or the basis of the inherent resistance of *L. monocytogenes* to nalidixic acid and fluoroquinolones.

This raises the following possibilities regarding the nalidixic acid resistance of *L. monocytogenes*: (i) the revealed alterations of the amino acid sequence in the QRDR of GyrA are not exclusively responsible for the nalidixic acid-resistant phenotype; (ii) sensitive, plasmid-borne alleles of *gyrA* are not dominant over chromosomal alleles in *L. monocytogenes* (as described for other bacterial species[20–22]); and (iii) other mutations or factors such as efflux pumps dominate over the presumed DNA gyrase mutations. Preliminary experiments with reserpine, a known inhibitor of Gram-positive efflux pumps,[24] added to the susceptibility test agar plates[25] indicate that efflux does not contribute to the resistance to nalidixic acid, as the phenotypes of the strains tested were not changed. Also, addition of chloramphenicol or erythromycin to the agar plates, in order to prevent plasmid loss, finally had no effect (data not shown).

The quinolone class of antimicrobial drugs is not yet applicable in the treatment of listeriosis, although newer derivatives of the quinolones exhibit improved activity against *Listeriae* (and Gram-positive bacteria in general). However, quinolones would be of particular interest in the treatment of listeriosis, as they are accumulated intracellularly in the eukaryotic cell, thereby having the potential to reach the intracellular *Listeriae* in high concentrations, where other antibiotics like β-lactams (the drug of choice against listeriosis) perform inadequately.[4] But, as *L. monocytogenes* is intrinsically resistant to nalidixic acid, the elucidation of the resistance mechanism is more complicated in this case compared with naturally sensitive microorganisms, where the development of resistance (i.e. mutations) can be monitored in relation to the degree of resistance.

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