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Mutations in the *gyrB*, *parC*, and *parE* Genes of Quinolone-Resistant Isolates and Mutants of *Edwardsiella tarda*

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The full-length genes *gyrB* (2,415 bp), *parC* (2,277 bp), and *parE* (1,896 bp) in *Edwardsiella tarda* were cloned by PCR with degenerate primers based on the sequence of the respective quinolone resistance-determining region (QRDR), followed by elongation of 5' and 3' ends using cassette ligation-mediated PCR (CLMP). Analysis of the cloned genes revealed open reading frames (ORFs) encoding proteins of 804 (GyrB), 758 (ParC), and 631 (ParE) amino acids with conserved gyrase/topoisomerase features and motifs important for enzymatic function. The ORFs were preceded by putative promoters, ribosome binding sites, and inverted repeats with the potential to form cruciform structures for binding of DNA-binding proteins. When comparing the deduced amino acid sequences of *E. tarda* GyrB, ParC, and ParE with those of the corresponding proteins in other bacteria, they were found to be most closely related to *Escherichia coli* GyrB (87.6% identity), *Klebsiella pneumoniae* ParC (78.8% identity), and *Salmonella* Typhimurium ParE (89.5% identity), respectively. The two topoisomerase genes, *parC* and *parE*, were found to be contiguous on the *E. tarda* chromosome. All 18 quinolone-resistant isolates obtained from Korea thus far did not contain subunit alternations apart from a substitution in GyrA (Ser83→Arg). However, an alteration in the QRDR of ParC (Ser84→Ile) following an amino acid substitution in GyrA (Asp87→Gly) was detected in *E. tarda* mutants selected *in vitro* at 8 μg/ml ciprofloxacin (CIP). A mutant with a GyrB (Ser464→Leu) and GyrA (Asp87→Gly) substitution did not show a significant increase in the minimum inhibitory concentration (MIC) of CIP. None of the *in vitro* mutants exhibited mutations in *parE*. Thus, *gyrA* and *parC* should be considered to be the primary and secondary targets, respectively, of quinolones in *E. tarda*.

Keywords: *Edwardsiella tarda*, *gyrB*, *parC*, *parE*, quinolone, *in vitro*

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Edwardsiella tarda, a Gram-negative bacterium of the *Enterobacteriaceae* family known to be one of the most important fish pathogenic agents, has been demonstrated to induce hemorrhagic septicemia (edwardsiellosis), resulting in extensive economic losses to the aquaculture industry. Currently, in many Asian countries, quinolones including oxolinic acid (OA) and ciprofloxacin (CIP) are frequently employed for the treatment of piscine *E. tarda* infections owing to the increased resistance to tetracycline exhibited by this bacterium [1, 41]. The bactericidal activity of quinolones is due to their binding to DNA gyrase and topoisomerase IV, which are essential for DNA replication and chromosome partitioning and have been shown to harbor homologous amino acid sequences. Several strains of *E. tarda* isolated from diseased fish have been found to show resistance to quinolones [1], but the mechanism has yet to be determined.

Changes in the active sites of the target enzymes DNA gyrase and topoisomerase IV are the principal mechanisms of quinolone resistance in other bacteria. DNA gyrase consists of four subunits, two GyrA and two Gyr B subunits, encoded by *gyrA* and *gyrB*, respectively. Topoisomerase IV also consists of four subunits arranged in a tetramer structure, two ParC and two ParE subunits, encoded by *parC* and *parE*, respectively [13]. Several previous reports have described the effects of mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* on the degree of resistance in a variety of Gram-

negative bacteria [22, 28, 33]. DNA gyrase is the primary target in *E. coli*, where *parC*-mediated resistance is found only in *gyrA* mutants and at high concentrations of fluoroquinolones [9, 24]. By way of contrast, topoisomerase IV is the primary target in *Staphylococcus aureus* [34], presumably due to the reversed susceptibility to quinolones of the two target enzymes relative to that in *E. coli* [13]. Thus, double *gyrA-parC* mutants in species including *E. coli*, *Haemophilus influenza,* and *Neisseria gonorrhoeae* (or *parC-gyrA* in species such as *S. aureus*) generally exhibit higher levels of resistance to fluoroquinolones compared with those of single *gyrA* (or *parC*) mutants. Although the contribution of *gyrB* mutations to quinolone resistance remains to be thoroughly elucidated, low-level resistance has been detected in *gyrB* mutants of *N. gonorrhoeae* [40] and *E. coli* [36]. In another study, the possible influence of a double *gyrA-gyrB* mutation on high-level quinolone resistance in *S.* Typhimurium was also suggested [18].

In the case of *E. tarda*, nothing has yet been reported regarding gyrase and topoisomerase gene structure and alteration, with the exception of the recently determined sequence of *gyrA* [38]. Since the development of high-level quinolone resistance requires the contribution of multi point changes in the QRDRs of the genes encoding DNA gyrase and topoisomerase IV, determining the entire sequence and alternations of the genes encoding the remaining subunits of these two enzymes may be critical to elucidating the underlying mechanisms in *E. tarda*. Here, we cloned and determined the complete sequence and organization of *gyrB*, *parC*, and *parE* in *E. tarda* as well as changes in the QRDRs of the encoded proteins in both quinolone-resistant isolates obtained from aquatic farms over five years, as well as mutants selected *in vitro* in the presence of CIP.

Materials and Methods

Bacterial Strains

A total of 48 isolates of fish-pathogenic *E. tarda*, collected from 2005 to 2009 from different areas of Korea, were grown at 25°C in tryptose soy broth (TSB; Difco) supplemented with 1% (w/v) NaCl. These isolates were first diluted in phosphate-buffered saline (PBS) buffer (10^5 colony-forming units per 25 μl) and subsequently plated on selective tryptose soy agar (TSA; Difco) plates containing 60 μg/ml nalidixic acid (NA; Sigma). For the susceptibility test, six isolates exhibiting >20 colonies on the selective medium were found to be highly NA-resistant (minimum inhibitory concentration [MIC], 64 to 512 μg/ml) but were only modestly resistant to other quinolones (OA: MIC, 0.5 to 2 μg/ml; norfloxacin: MIC, 0.08 to 0.64 μg/ml; CIP: MIC, 0.03 to 0.13 μg/ml). All of the isolates employed herein were grown at 25°C in TSB and were subsequently stored in 20% glycerol at −72°C until use.

Purification of *E. tarda* DNA

Total *E. tarda* DNA was purified using a previously described protocol [38]. NA-sensitive *E. tarda* GE1 (wild type) grown aerobically for 18 h as described above was harvested by centrifugation at 8,000 ×*g* for 10 min. Collected cells were lysed with a solution of 5.5% sodium dodecyl sulfate and 0.125 mg/ml proteinase K (Boehringer Mannheim, Germany). Nucleic acid was obtained from the aqueous phase following extraction with a phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)] mixture, and then precipitated with ethanol, resuspended in distilled water, and stored at −20°C until further use.

Amplification and Cloning of Partial *E. tarda gyrB* and *parC* Sequences

For cloning of *gyrB* and *parC*, total DNA extracted from *E. tarda* GE1 was amplified by PCR with the degenerate primer sets UP1-F/UP2-R and DGYR1-F/DGYR3-R, respectively (Table 1). These primers were derived from the conserved nucleotide sequences of *gyrB* and *parC* in nine bacterial species (*E. coli*, *Aeromonas salmonicida*, *Pectobacterium carotovorum*, *K. pneumoniae*, *N. gonorrhoeae*, *Pasteurella multocida*, *Pseudomonas aeruginosa*,

Table 1. Primers used in this study.

Gene	Primer names	Sequence	Position (bp)	Object
gyrA	GYRQ-F	5'-GATGTTCGGGATGGCCT-3'	106–122	Detection of *gyrA* QRDR
	GYRQ-R	5'- GCCAACAGCTCATGAGCAAT-3'	407–388	
gyrB	UP1-F	5'-GAAGTCATCATGACCGTTCTGCAC(T)GCA (C,G,T)GGA(C,G,T)AAA(G)TTC(T)GA-3'	274–314	Screening of *gyrB*
	UP2-R	5'-AGCAGGGTACGGATGTGCGAGCCA(G)TC A(C,G,T)ACA(G)TCA(C,G,T)GTCAT-3'	1,529–1,486	
	GBQ-F	5'-GCACGTGAAGCTGCGCGTAA-3'	1,141–1,160	Detection of *gyrB* QRDR
	GBQ-R	5'-CTGTGGTAGCGCAGCTTATC-3'	1,475–1,456	
parC	DGYR1-F	5'-ATG(C)GGTAATTAC(T)CACCC-3'	223–239	Screening of *parC*
	DGYR3-R	5'-GCCATA(G)CCG(T,C)ACG(T,C)GCA(G)ATACC-3'	542–523	
	PCQ-F	5'-GATGTCTGAGCTGGGGCTATC-3'	156–176	Detection of *parC* QRDR
	PCQ-R	5'-GGTATAACGCATCGCCGCGA-3'	375–356	
parE	PEQ-F	5'-CAGGAAGTGATCGATAACAG-3'	112–131	Detection of *parE* QRDR
	PEQ-R	5'-GACAGGGCGTTGACTACCGA-3'	371–352	

Serratia marcescens, and *Vibrio parahaemolyticus*; X57174, L47978, X80798, X16817, U08817, AE006122, L29417, U56906, and AB023569, respectively) with the aid of gene alignment using the MACAW program (Version 2.0.5; National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). PCR amplification was carried out in a 50-μl reaction mixture, containing 100 ng of total nucleic acid, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.5% Tween-20, 200 mM each dNTP, 1 mM each antisense and sense primers, and 1.25 U Ampli *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), with a Perkin-Elmer 2400 thermal cycler (PE Applied Biosystems, Norwalk, CT, USA). Amplification consisted of 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min in 0.2-ml thin-walled tubes. Amplification products were subsequently analyzed by electrophoresis on a 1.5% agarose gel. PCR products of the expected lengths, 1,256 bp for *gyrB* and 320 bp for *parC*, were purified *via* electrophoresis on an agarose gel using the Prep-A-Gene DNA Purification system (Bio-Rad Laboratories, Hercules, CA, USA), and then cloned into TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Cloned fragments were then sequenced using a Big Dye Terminator Cycle DNA Sequencing Kit (ABI PRISM, PE Applied Biosystems, Foster City, CA, USA) and an automatic sequencer (Applied Biosystems).

Complete Nucleotide Sequencing of *E. tarda gyrB*, *parC*, and *parE*

The entire gene sequences of *gyrB*, *parC*, and *parE* in *E. tarda* were determined by 5', 3' end extension from each corresponding partial sequence using cassette ligation-mediated PCR (CLMP) as described in a previous study [38]. Briefly, total DNA from *E. tarda* GE1 was digested separately with *Pst*I/*Sal*I, *Eco*RI/*Xba*I/*Sal*I, and *Pst*I for *gyrB*, *parC*, and *parE*, respectively. To obtain each complete gene, sequential cloning steps by CLMP (5' and 3') with the appropriately restriction enzyme-digested cassette-ligated bacterial DNAs were performed in accordance with the manufacturer's instructions (Takara Shuzo, Japan) (Fig. 1).

In Vitro Selection of CIP-Resistant *E. tarda*

Mutants were selected by plating approximately 2×10^{10} CFU of strain *E. tarda* GE1 on Muller Hinton (MH) agar plates containing 60 μg/ml NA and 0.2–8.0 μg/ml CIP. A first-step resistant strain was selected at 60 μg/ml NA, a second-step resistant mutant at 0.2 μg/ml CIP, a third-step resistant mutant at 1 μg/ml CIP, and a fourth-step mutant at 8 μg/ml CIP. Two of the 8 mutants arising from each of the four steps were selected according to their resistances to six quinolones (NA, OA, norfloxacin, CIP, ofloxacin, and pefloxacin; Sigma, USA) and were employed for *gyrA*, *gyrB*, *parC*, and *parE* QRDR sequence analysis.

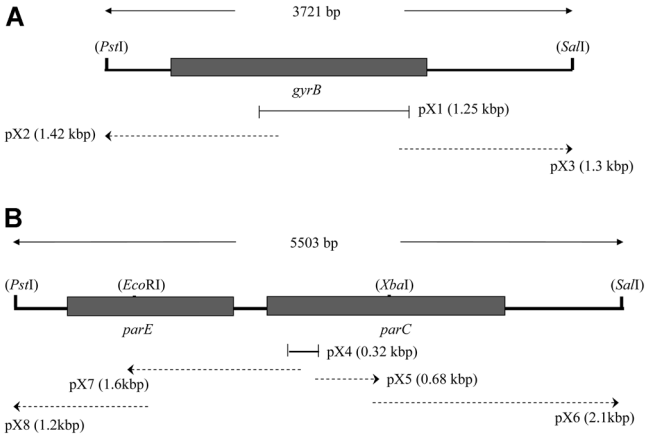

Fig. 1. Restriction maps of *gyrB* (**A**) and *parE-par*C (**B**) in *E. tarda* and alignment of plasmid clones.
The genes *gyrB*, *parC*, and *parE* are indicated by the shaded regions. Fragments pX1 and pX4 are the 1256- and 320-bp products resulting from PCR amplification of *gyrB* and *parC* sequences, respectively, using degenerate primers. Dotted arrows indicate the DNA fragments produced by cassette ligation-mediated PCR with the indicated restriction enzymes.

Amplification and Sequencing of *gyrA*, *gyrB*, *parC*, and *parE* QRDRs

The sequences of the primers used for PCR amplification of *gyrA* (GYRQ-F and GYRQ-R), *gyrB* (GBQ-F and GBQ-R), *parC* (PCQ-F and PCQ-R), and *parE* (PEQ-F and PEQ-R) are given in Table 1. These primer sets were derived from the complete nucleotide sequences of *E. tarda gyrA* [38] and *gyrB*, *parC*, and *parE* (this study). For the amplification, cloning, and sequencing of QRDRs,

Table 2. Degree of identity of the nucleotide and deduced amino acid sequences of *gyrA*, *gyrB*, *parC*, and *parE* in *E. tarda* to those of other bacterial strains.

		Strains															
		E. coli				*S.* Typhimurium				*K. pneumoniae*				*S. pneumoniae*			
		gyrA	*gyrB*	*parC*	*parE*	*gyrA*	*gyrB*	*parC*	*parE*	*gyrA*	*gyrB*	*parC*	*parE*	*gyrA*	*gyrB*	*parC*	*parE*
E. tarda	Whole gene	78.3 (85.5)	80.8 (87.6)[a]	73.6 (79.0)	80.0 (89.4)	81.0 (88.0)	80.5 (87.2)	75.3 (77.8)	80.0 (89.5)	80.0 (86.0)	81.5 (87.8)	76.1 (78.8)	87.0 (89.7)	52.0 (46.0)	57.3 (37.9)	38.3 (26.0)	37.3 (38.5)
	QRDR region	84.1 (97.5)	88.9 (96.3)	83.2 (93.0)	84.2 (95.9)	81.6 (97.5)	89.3 (96.3)	86.3 (93.0)	84.9 (93.8)	84.0 (97.0)	83.7 (98.2)	84.9 (96.2)	78.0 (87.7)	65.0 (68.0)	67.0 (51.3)	61.1 (39.5)	49.1 (41.2)

[a]Numbers in parentheses indicate identity to deduced amino acid sequence.
GenBank accession numbers for sequences of *gyrA*, *gyrB*, *parC*, and *parE* in *E. coli*, *S.* Typhimurium, *K. pneumoniae*, and *S. pneumoniae* are X57174, NC_000913, X16817, and NC_000913; NC_003197, NC_003197, M68936, and NC_003197; L29417, NC_012731, NC_012731, and NC_012731; and AY157689, NC_008534, Z67739, and NC_008534, respectively.
The nucleotide sequence of *gyrA* was determined in a previous study [38].
Abbreviations: NA (nalidixic acid), OA (oxolinic acid), NOR (norfloxacin), CIP (ciprofloxacin), OF (ofloxacin), PEF (pefloxacin).

we followed the procedures described above for cloning the partial *E. tarda gyrB* and *parC* sequences.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of *E. tarda gyrB*, *parC*, and *parE* and their flanking regions have been deposited in GenBank under accession numbers FJ158597, FJ172222, and FJ952156, respectively.

RESULTS

Cloning of *gyrB*, *parC*, and *parE* Homologs from *E. tarda*

Five *parC* clones were randomly selected, and the expected 320-bp DNA inserts in pBluescript generated by PCR with the primers DGYR1-F/DGYR3-R were isolated. DNA sequence analysis and comparison indicated that the inserts from two clones were derived from a specific region of *gyrA* (GenBank Accession No. DQ019315), whereas those from three other clones were from an unknown region suspected to be *parC*. To obtain the full-length candidate *parC* gene and information regarding its chromosomal context, CLMP was used to isolate the overlapping clones pX5 (*Xba*I), pX6 (*Sal*I), and pX7 (*Eco*RI) (Fig. 1). The 5' end of the insert in plasmid pX7 had an incomplete ORF encoding a partial protein of 150 amino acids and showing strong nucleotide sequence homology to the C-terminal portion of *parE* in four bacterial strains: *E. coli*, *S.* Typhimurium, *K. pneumoniae*, and *Streptococcus pneumoniae* (Table 2). Along with plasmid pX7 (*Eco*RI), the overlapping clone pX8 (*Pst*I) allowed us to identify the complete *parE* gene. Fig. 1 presents the full 5.5-kb region encompassing the *parC* and *parE* loci specified by four different overlapping fragments (clones). DNA fragments (1,256 bp in length) generated by PCR with the primers UP1-F/UP2-R for *gyrB* were also cloned, sequenced, and subjected to CLMP to produce the overlapping clones pX2 (*Pst*I) and pX3 (*Sal*I), which enabled analysis of the nucleotide sequence of the full-length *gyrB* locus (Fig. 1). The nucleotide sequence of *gyrB* shared more than 80% homology with those of other Gram-negative bacteria (Table 2).

Characterization of *E. tarda gyrB*, *parC*, and *parE*

Using the BioEdit program (Version 5.0.9; Department of Microbiology, North Carolina State University, Raleigh, NC, USA), we compared the deduced protein and nucleotide sequences of *gyrB*, *parC*, and *parE* in *E. tarda* with those identified in other bacterial species. Based on the high identity of the nucleotide and deduced protein sequences of the candidate *E. tarda* genes to the corresponding genes in other Gram-negative bacteria, we assigned three cloned ORFs, encoding 804-, 758-, and 631-residue proteins, to GyrB, ParC, and ParE, respectively. Additionally, *parC* was located in the region downstream of *parE* as seen in *S. pneumoniae* and exhibited relatively high identity with the *gyrA* sequence of *E. tarda* (approximately 35%) and the *parC* sequences of other Gram-negative bacteria (>70%) (Table 2). Specifically, putative promoter regions and ribosome binding sites (Fig. 2 and 3) were found upstream of the initiation ATG codon in *gyrB*, *parC*, and *parE* (the location of the ribosome binding site in the latter was not as clear as that in *gyrB* and *parC*). Inverted repeat sequences involved in the regulation of transcription were also present upstream and downstream of each ORF for *gyrB*, *parC*, and *parE*. The deduced protein sequence of GyrB contained the conserved motifs EGDSA and YKGL at residues 424 and 739, respectively [37]. Furthermore, another 14-residue motif, EKARFDKMLSSQEV, was observed at residue 454 instead of EKARFEKMLASQEV,

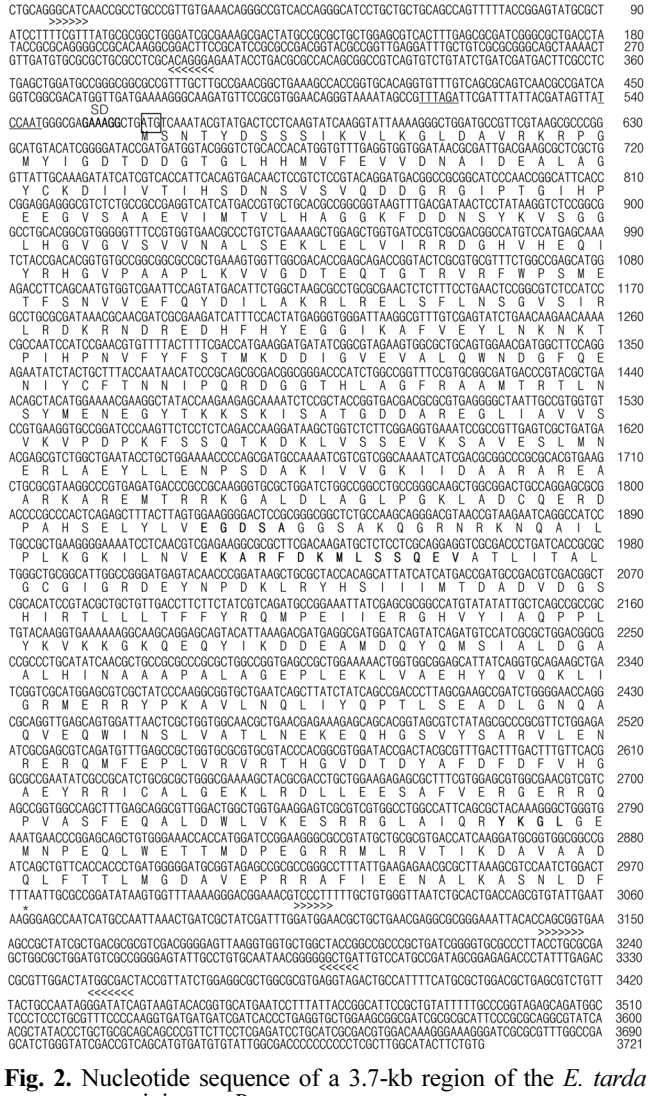

Fig. 2. Nucleotide sequence of a 3.7-kb region of the *E. tarda* genome containing *gyrB*.

The inferred amino acid sequence of the ORF is shown above the DNA sequence. A methionine initiation codon is indicated by the box, and putative -10 and -35 elements are underlined. Amino acid motifs and the Shine–Dalgarno consensus sequence are shown in bold. An asterisk indicates the translation stop codon.

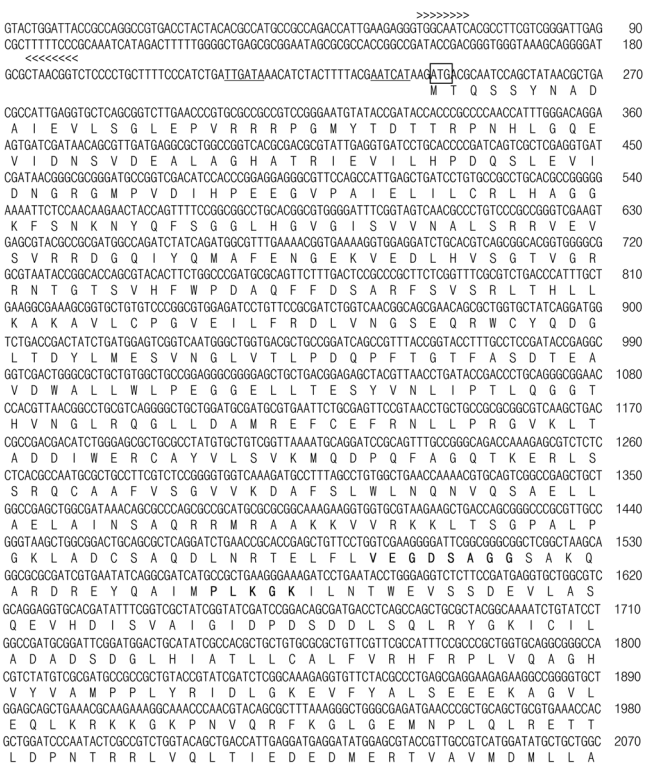

Fig. 3. Nucleotide sequence of a 5.5-kb region of the *E. tarda* genome containing *parE-parC*.
The inferred amino acid sequences of two ORFs are shown above the DNA sequence. Symbols are as defined in the legend to Fig. 2.

which has been found in 25 quinolone-resistant strains of *N. gonorrhea* (Fig. 2) [27].

The two complete ORFs for *parC* and *parE* (separated by 185 bp), exhibited 73.6% and 80.0% identities to those of the corresponding genes for topoisomerase IV of *E. coli* [23] but only 38.3% and 37.3% identities to those of *S. pneumoniae*, respectively [37] (Table 2). A residue known to be crucial to quinolone resistance in *E. coli* and *S. aureus*, Ser80 in ParC, was detected as Ser84 in the conserved sequence HPHGDS in *E. tarda* ParC. The catalytic Tyr124 of ParC engaged in DNA breakage-reunion was also identified by alignment of the conserved sequence AAMRYTE with the catalytic Tyr122 of *E. coli* [21]. In the C-terminal region of ParE, there were the highly conserved motifs VEGDSAGG and PL(R/K)GK (positions 418 to 425 and

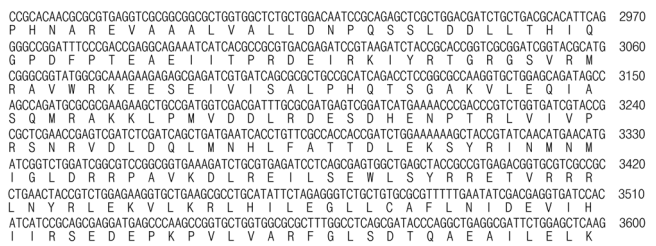

Fig. 3. Continued.

440 to 444, respectively) [29]. A glycine-rich ATP-binding site in the N terminus (positions 1 to 150, approximately) similar to that of *E. coli* was also conserved [8] (Fig. 3).

Mutations in *gyrA*, *gyrB*, *parC*, and *parE* in Quinolone-Resistant Strains of *E. tarda*

All 12 quinolone-resistant *E. tarda* isolates from 1994 to 2003 have been demonstrated to contain GyrA (Ser83→Arg) substitutions [38]. The additional 6 new isolates obtained in the current study also contain substitutions within the QRDR of GyrA (Ser83→Arg). According to the nucleotide sequence determination, no mutations in the 18 total isolates were observed within the QRDRs of GyrB, ParC, or ParE (data not shown). Thus, quinolone-resistant mutants were selected stepwise *in vitro* to further evaluate the role of the three genes cloned in this study in the development of drug resistance in *E. tarda*. Two first-, second-, third-, and fourth-step mutants were obtained (PC1-2/1-4, PC2-3/2-5, PC3-1/3-3, and PC4-4/4-6, respectively) and their susceptibilities to six quinolones and the status of their *gyrA*, *gyrB*, *parC*, and *parE* QRDRs were characterized (Table 3). All 8 mutants resistant to quinolones carried a GyrA (Asp87→Gly) substitution compared with the 18 isolates from the natural environment, which contained a GyrA (Ser83→Arg) substitution. No change was found at position 83 of the QRDR in GyrA. The *gyrA*, *gyrB*, *parC*,

Table 3. Mutations detected in the *gyrA*, *gyrB*, *parC*, and *parE* sequences of mutants of *E. tarda* GE1 selected for resistance by stepwise exposure to CIP *in vitro*.

Mutant	Parent	MIC (μg/ml)						Mutation in							
								gyrA		*gyrB*		*parC*		*parE*	
		NA	OA	NOR	CIP	OF	PEF	AA[b]	NA[c]	AA[b]	NA[c]	AA[b]	NA[c]	AA[b]	NA[c]
1st step	GE1[a]	2	0.13	0.03	0.02	<0.02	<0.02	Asp^{87}	G**A**C	Ser^{464}	T**C**G	Ser^{84}	A**G**C	Phe^{416}	TTC
PC1-2		64	1	0.13	<0.02	0.25	4	Gly	G**G**C	Ser	T**C**G	Ser	A**G**C	Phe	TTC
PC1-4		64	1	0.13	<0.02	0.25	4	Gly	G**G**C	Ser	T**C**G	Ser	A**G**C	Phe	TTC
2nd step	PC1-4														
PC2-3		128	2	0.5	0.13	0.5	8	Gly	G**G**C	Ser	T**C**G	Ser	A**G**C	Phe	TTC
PC2-5		128	2	0.5	0.13	0.5	8	Gly	G**G**C	Ser	T**C**G	Ser	A**G**C	Phe	TTC
3rd step	PC2-5														
PC3-1		128	2	2	0.5	2	8	Gly	G**G**C	Leu	T**T**G	Ser	A**G**C	Phe	TTC
PC3-3		128	2	2	0.5	2	8	Gly	G**G**C	Leu	T**T**G	Ser	A**G**C	Phe	TTC
4th step	PC3-3														
PC4-4		512	16	≥16	8	≥16	≥16	Gly	G**G**C	Leu	T**T**G	Ile	A**T**C	Phe	TTC
PC4-6		512	16	≥16	8	≥16	≥16	Gly	G**G**C	Leu	T**T**G	Ile	A**T**C	Phe	TTC

[a]Wild-type strain.
[b]Amino acid residue.
[c]Base change of nucleic acid.
Abbreviations: NA (nalidixic acid), OA (oxolinic acid), NOR (norfloxacin), CIP (ciprofloxacin), OF (ofloxacin), PEF (pefloxacin).

and *parE* QRDRs of the second-step mutants did not differ from those of the parental strain (PC1-4), and were not associated with an increase in the MICs of fluoroquinolones. In the third-step mutants, PC3-3, there was a substitution in GyrB (Ser464→Leu) in addition to GyrA (Asp87→Gly), but it was also not associated with an increase in the MICs of fluoroquinolones. However, two fourth-step mutants from parental strain PC3-3 were selected, and both had acquired an additional ParC (Ser84→Ile) substitution and showed a significant increase, more than 4-fold, in the MICs of fluoroquinolones. In contrast to the changes in *gyrA*, *gyrB*, and *parC* in both isolates and mutants selected *in vitro*, the sequence of the QRDR of *parE* in all quinolone-resistant isolates was identical to that of the wild-type strain, *E. tarda* GE1 (Table 3).

Discussion

In a variety of quinolone-resistant bacteria, alterations in GyrA and ParC, subunits of DNA gyrase and topoisomerase IV along with GyrB and ParE, respectively, are critical resistance mechanisms [32]. We cloned and determined the full-length sequences of *gyrB*, *parC*, and *parE* in *E. tarda* isolates obtained from aquatic farms in Korea. The three cloned genes contain specific regions involved in the regulation of transcription, including putative promoter regions consisting of -10/-35 elements as well as inverted repeats at the 3' end that may induce binding of various DNA-binding proteins through the formation of cruciform structures. We were also able to detect ribosome-binding sites for translation in *gyrB* and *parC* (Fig. 2 and 3). The GyrB, ParC, and ParE protein sequences deduced from the nucleotide sequences of the three cloned ORFs exhibited marked homology with the corresponding proteins of a variety of Gram-negative bacteria.

The assigned proteins, GyrB (804 residues), ParC (758 residues), and ParE (631 residues), showed very high homology to GyrB of *E. coli* (87.6%), ParC of *K. pneumoniae* (78.8%), and ParE of *S.* Typhimurium (89.5%), respectively (Table 2) [39]. Like other GyrBs of Gram-negative bacteria, compared with the *S. pneumoniae* proteins [37], *E. tarda* GyrB also contains an extra segment of approximately 160 residues in the C-terminal region. Additionally, the glycine-rich ATP binding site at the N-terminus in *gyrB*, *parC*, and *parE* and the catalytic Tyr124, equivalent to Tyr122 of *E. coli* and involved in DNA breakage and reunion in ParC [6, 8], were also detected (Fig. 3).

The two gyrase genes (*gyrA* and *gyrB*) are unlinked on the *E. coli* chromosome; however, they are linked in *S. aureus*, *Mycoplasma pneumoniae*, and other Gram-positive bacteria [10, 20, 31], as are the two topoisomerase genes (*parC* and *parE*) in *S. pneumoniae* [37]. In analyzing the location of the genes on the map of *E. tarda*, we failed to find any homology between the upstream and downstream regions of *gyrB* and those of *gyrA* in other bacterial species [27, 38]. In contrast, the contiguity of *parC*/*parE* on the chromosome of *E. tarda* was confirmed by the extended

nucleotide sequence of the 5' end flanking region of *parC* as in *S. pneumoniae*.

Sequence changes in QRDRs have been recognized as hotspots for fluoroquinolone resistance; thus, we analyzed QRDR alterations in quinolone-resistant isolates in order to further evaluate the potential functions of DNA gyrase and topoisomerase IV in the development of resistance in *E. tarda*. In a previous study characterizing *gyrA* in *E. tarda*, all 12 of the quinolone-resistant isolates examined had an alteration within the QRDR, Ser83→Arg, and it was suggested that resistance to quinolones in this bacterium is primarily related to alterations in GyrA [38]. Evaluation of an additional 6 quinolone-resistant clinical *E. tarda* isolates in the present study showed the same amino acid substitution within the QRDR. No alterations in the QRDRs of GyrB, ParC, and ParE were detected in the isolates in either study (data not shown). Therefore, mutations in the gyrase gene (*gyrB*) and topoisomerase IV genes (*parC* and *parE*) should be considered relatively rare in *E. tarda* isolates acquired from the environment, as has been demonstrated in salmonellae [11, 26].

We isolated a series of CIP-resistant *E. tarda* mutants *via* stepwise selection on plates containing increasing drug concentrations. Interestingly, for each of the first- and second-step mutants selected *in vitro*, only the GyrA substitution Asp87→Gly was associated with a significant increase in the MIC of CIP (Table 3). A similar level of resistance was found in the isolates containing the GyrA substitution Ser83→Arg, suggesting that substitution at these two residues has a similar influence on potential quinolone resistance in *E. tarda*. However, it should be noted that, in this study, the clinically resistant isolates showed the GyrA substitution Ser83→Arg, whereas the *in vitro* mutants showed Asp87→Gly. As suggested previously by Björkman *et al*. [7] and Mclver *et al*. [30], differences in environments and conditions *in vitro* on artificial plates versus *in vivo* in fish in aquatic farms may induce different QRDR-mediated changes for the development of resistance. Moreover, mutations for potential quinolone resistance in *E. tarda via* double mutation (positions 83 and 87) in GyrA were not detected, a result that is consistent with the findings of studies concerning quinolone-resistant isolates of *Campylobacter jejuni* and *Mycoplasma hominis* [3, 4]. Mutations in the QRDR of GyrA in various bacteria, such as salmonellae (Ser83→Phe; Ser83→Tyr; Asp87→Gly, Asn or Tyr), *K. pneumoniae* (Ser83→Phe; Asp87→Gly), and *N. gonorrhoeae* (Ser91→Phe), have been previously reported [8, 25, 27, 33].

Relative to the dominant types of mutation demonstrated in *gyrA* in highly fluoroquinolone-resistant strains of various Gram-negative bacteria, mutations in *gyrB* and *parE* are observed less frequently and are generally detected in isolates at low quinolone concentrations [28]. However, mutations responsible for high levels of resistance have also been reported in GyrB, in particular, Asp426→Asn and Lys447→Glu in *E. coli* [43], and Ser463→Tyr in salmonellae [15]. In this study, we were able to detect a mutation in GyrB (Ser464→Leu) in the third-step mutant (PC3-3), but it was not associated with an increase in the MICs of fluoroquinolones compared with that of the parental strain (PC2-5). It is likely that this mutation may not be directly responsible for high-level resistance or increased resistance levels in mutants already possessing the GyrA mutation (Asp87→Gly) (Table 3).

The presence of mutations in *parE* in quinolone-resistant *E. coli* (Leu445→His) and the viridans group of streptococci (Pro424→Gln) seems to suggest that the region spanning positions 424 to 460 in ParE confers quinolone resistance [17, 30]. However, no mutations in the corresponding region of *parE* were detected in either quinolone-resistant *E. tarda* isolates or mutants selected *in vitro* (Table 3). Additionally, the ParE (Leu446→Phe) substitution occurring in all quinolone-resistant mutants of *M. hominis* was also not detected [3]. The fourth-step mutants, PC4-4, had a substitution in ParC (Ser84→Ile) in addition to GyrA (Asp87→Gly), which was associated with a more than 16-fold increase in the MIC of CIP.

Studies conducted on GyrA have demonstrated that the mutations Ser83→Leu/Trp and Asp87→Val/Gly induce highly elevated levels of quinolone resistance in *E. coli* [35, 36]. Other studies regarding mutations in ParC in highly quinolone-resistant *E. coli* have demonstrated that serine 80 and glutamate 84 have a tendency to be substituted with hydrophobic and positively charged amino acids, respectively [19]. In *E. tarda* mutants selected *in vitro* in this study, the mutation of Asp87 of GyrA was associated with moderate-level resistance; the additional ParC mutation of Ser84 to a hydrophobic amino acid (Ile) was associated with high-level resistance, similar to the pattern of CIP resistance observed in *H. influenzae* and *N. gonorrhoeae* [12, 16]. Thus, as is the case with other Gram-negative bacteria [5, 19], gyrase appears to be the primary target in *E. tarda*; *parC*-mediated resistance was found only when *gyrA* was mutated and when the concentration of fluoroquinolone was high [22]. On the other hand, *parC,* rather than *gyrA*, has been considered to be the primary target of CIP in *S. aureus* [14, 32]. Additionally, the results of a study by Trees *et al*. [42] indicated that double mutations in *parC* may explain, to some degree, the high level of resistance noted in CIP-resistant *N. gonorrhoeae*. However, none of our *E. tarda* strains, whether *in vitro* mutants or environmental isolates, had double mutations in *parC*.

In summary, *gyrB*, *parC*, and *parE* of *E. tarda*, potentially involved in resistance to quinolones, were cloned and their full nucleotide sequences were determined. A high level of

resistance to quinolones in *E. tarda* mutants was associated with alternating changes in DNA gyrase (*gyrA*) and topoisomerase IV (*parC*).

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