Vol. 67: 259–266, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 28

Molecular cloning of the *gyrA* gene and characterization of its mutation in clinical isolates of quinolone-resistant *Edwardsiella tarda*

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ABSTRACT: Knowing the entire sequence of the gene encoding the DNA gyrase Subunit A (*gyrA*) of *Edwardsiella tarda* could be very useful for confirming the role of *gyrA* in quinolone resistance. Degenerate primers for the amplification of *gyrA* were designed from consensus nucleotide sequences of *gyrA* from 9 different Gram-negative bacteria, including *Escherichia coli*. With these primers, DNA segments of the predicted size were amplified from the genomic DNA of *E. tarda* and then the flanking sequences were determined by cassette ligation-mediated polymerase chain reaction. The nucleotide sequence of *gyrA* was highly homologous to those of other bacterial species, in both the whole open-reading frame and the quinolone-resistance-determining region (QRDR). The 2637-bp *gyrA* gene encodes a protein of 878 amino acids, preceded by a putative promoter, ribosome binding site and inverted repeated sequences for cruciform structures of DNA. However, the nucleotide sequence of the flanking region did not show any homologies with those of other bacterial DNA gyrase Subunit B genes (*gyrB*) and suggested the gyrase genes, *gyrA* and *gyrB*, are non-continuous on the chromosome of *E. tarda.* All of the 12 quinolone-resistant isolates examined have an alteration within the QRDR, Ser^{83}→Arg, suggesting that, in *E. tarda*, resistance to quinolones is primarily related to alterations in *gyrA*. Transformation with the full sequence of *E. tarda gyrA* bearing the Ser^{83}→Arg mutation was able to complement the sequence of the *gyrA* temperature-sensitive mutation in the *E. coli* KNK453 strain and to induce increased resistance to quinolone antibiotics at 42°C.

KEY WORDS: *Edwardsiella tarda* · *gyrA* · Quinolone · Resistance · Mutation

INTRODUCTION

Most quinolone resistance in bacteria can be attributed to the genes encoding DNA gyrase or Topoisomerase IV (TOPO IV). DNA gyrase is a Type II DNA topoisomerase, and is the enzyme essential for ATP-dependent, negative supercoiling of DNA, which is required for DNA replication and gene transcription (Hooper 1998). Here, 2 A subunits and 2 B subunits, encoded by the *gyrA* and *gyrB* genes, respectively (2628 and 2415 bp in *Escherichia coli*, respectively), make up the tetrameric active enzyme, DNA gyrase (Bébéar et al. 2000). TOPO IV, a C_2E_2 tetramer encoded by the *parC* and *parE* genes, is essential for chromosome partitioning (Adams et al. 1992). The amino acid sequences of *parC* and *parE* are homologous to some degree with those of *gyrA* and *gyrB*, respectively (Peng & Marians 1993). The majority of the quinolone-resistance mutations in the *gyrA* gene are clustered in a region called the quinolone-resistance-determining region (QRDR), which includes

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the amino acid residues from 67 to 106 in the numbering system used in *E. coli*, and is supposed to be the site of interaction between the A subunit of gyrase and quinolones (Nakamura 1997, Horii et al. 2003). Especially, mutation at the amino acid codon Ser^{83} or Asp^{87} of this region has been described as the major mechanism for quinolone resistance in many different bacteria, including *E. coli* (Drlica & Zhao 1997, Capilla et al. 2004).

The *gyrA* genes have been cloned and sequenced from several different bacterial species, among them *Escherichia coli*, *Neisseria gonorrhoeae*, *Aeromonas salmonicida*, *Klebsiella pneumoniae*, *Helicobacter pylori*, *Bacillus subtilis* and *Campylobacter jejuni* (Swanberg & Wang 1987, Dimri & Das 1990, Oppegard & Sorum 1996). Substitutions at positions corresponding to Ser^{83} in *E. coli* GyrA have been detected in quinolone-resistant strains of most bacterial species. Therefore, GyrA was considered to be one of the primary targets of quinolone resistance in many bacterial species (Bébéar et al. 2000, Goni-Urriza et al. 2002).

Edwardsiella tarda, a Gram-negative bacterium of the Enterobacteriaceae family, causes hemorrhagic septicemia (edwardsiellosis) in fish, leading to extensive losses in both freshwater and marine aquaculture. The wide host range of *E. tarda* includes commercially important fishes, such as sea bream (Baxa et al. 1985), eel (Chen & Huang 1996), flounder (Nakatsugawa 1983), tilapia (Kubota et al. 1981) and striped bass (Herman & Bullock 1986), as well as higher vertebrates, like birds, reptiles and mammals, including humans (Janda & Abott 1993).

Currently, *Edwardsiella tarda* infections in fish are treated with quinolones in many Asian countries, following the increased resistance of this bacteria to tetracyclines (Reinhardt et al. 1985, Aoki 1988, Stock & Wiedemann 2001) that have been permitted for use in humans and aquatic animals by the Food and Drug Administration (FDA). Several strains of *E. tarda* isolated from diseased fish have been found to show resistance to quinolones (Aoki 1988), but nothing has been reported on the *gyrA* gene structure, the mutation in *gyrA* responsible for quinolone resistance, or other genetic bases for the decreased susceptibility to quinolones in this bacterium.

Here, we report on the cloning, sequencing and organization of the complete *Edwardsiella tarda gyrA* gene, as well as on the *gyrA* QRDRs from *E. tarda* isolates selected in aquatic farms for resistance to nalidixic acid.

MATERIALS AND METHODS

Bacterial cultures and drug sensitivity. A total of 138 clinical isolates of fish-pathogenic *Edwardsiella tarda*, isolated from 1993 to 2003 from different areas of Korea, were cultured aerobically on tryptose soy broth (TSB) (Difco) supplemented with 1% (w/v) NaCl at 25°C for 18 h. These isolates were diluted in phosphate-buffered saline (PBS) buffer (10^5 colony-forming units per 25 µl) and plated on selective tryptose soy agar (TSA) (Difco) plates containing 60 µl ml^{-1} nalidixic acid (NA, Sigma). From the 25 NA-resistant isolates that showed >20 colonies on the selective media, 12 isolates were selected to give a wide distribution of years and areas of isolation in this study (see Table 3). These 12 NA-resistant isolates and 1 NA-sensitive isolate, *E. tarda* GE1 (wild type), were identified by an API 20E kit (BioMerieux) and by conventional laboratory methods, including Gram staining, growth on Salmonella–Shigella agar (SS) (Difco) plates and colony morphology. Minimum inhibitory concentrations (MIC) of quinolones (NA, oxolinic acid, norfloxacin and ciprofloxacin) were determined by the Mueller-Hinton broth (Difco) micro-doubling dilution method (Frech & Schwarz 2000) on 96-well plates with incubation at 25°C for 24 h. *Escherichia coli* strains and the clinical isolates of *E. tarda* were grown at 37°C and 25°C in TSB, respectively, and were stored in 20% glycerol at –72°C until use.

DNA extraction. All of the bacterial species were grown aerobically on TSB supplemented with 1% (w/v) NaCl at 25°C for 18 h. Cultured cells were harvested by centrifugation at 8000 × *g* for 10 min and lysed with 5.5% sodium dodecyl sulfate per 0.125 mg ml^{-1} proteinase K (Boehringer Mannheim) solution. Bacterial nucleic acids were extracted by a phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) mixture. The nucleic acids were ethanol precipitated and resuspended in distilled water. The DNA was stored at –20°C until further use.

Primer design and PCR. A degenerate primer set (Table 1) was designed. DGYR-1 (sense primer) and DGYR-4 (anti-sense primer) were derived from the conserved nucleotide sequence of the *gyrA* genes in 9 bacterial species (*Escherichia coli*, *Aeromonas salmonicida*, *Pectobacterium carotovorum*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Vibrio parahaemolyticus*; X57174, L47978, X80798, X16817, U08817, AE006122, L29417, U56906 and AB023569, respectively). These primers were designed 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 with a Perkin-Elmer 2400 thermal cycler (PE Applied Biosystems) in a 50 µl reaction mixture containing 100 ng of total nucleic acid isolated from 1 or 2 different bacteria, 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 µM each

Table 1. Primers used in this study

Name	Sequence (5′ → 3′)	Direction	Position	Object
DGYR-1	ATG(C)GGTAATTAC(T)CACCC	Sense	220–236	Screening of *gyrA*
DGYR-4	ACCAGA(T,C)TCA(G)GCA(G)ATC(T)TTC(T)TC	Anti-sense	842–825	Screening of *gyrA*
GYRS1	TGCGCATGGCGAAGATTGCTCATGAG	Sense	374–399	3′ end walking <2>[a]
GYRS2	CGGCACTGAACAGATCCCCGACG	Sense	450–472	3′ end walking <2>[a]
GYRS1R	GCAGGACGTCGGGGATCTGTTCA	Anti-sense	478–456	5′ end walking <1>[a]
GYRS2R	CTCATGAGCAATCTTCGCCATGCGCA	Anti-sense	399–374	5′ end walking <1>[a]
GYRS3	ACATCGATCCGATCATTGAGCTGATC	Sense	1166–1191	3′ end walking <3>[a]
GYRS4	TCCGGAGTGGCTGGAGCCGACAGTA	Sense	1299–1322	3′ end walking <3>[a]
SGYR-1	ATCAACTTGCCGGAGGCCA	Sense	1813–1831	Sequencing
SGYR-2	AGAACGGCTACGGCAAACGC	Sense	2246–2265	Sequencing
SGYR-3	TCGCACCACGCTCAAGGT	Sense	2860–2877	Sequencing
SGYR-4	TGTACATTGCCTCGCCG	Sense	3468–3484	Sequencing
SGYR-5	CTGGCTGTTTGAGCGCA	Sense	3850–3866	Sequencing
GYRQ	GATGTTCGGGATGGCCT	Sense	106–122	Detection of *gyrA* QRDR
GYRQR	GCCAACAGCTCATGAGCAAT	Anti-sense	407–388	Detection of *gyrA* QRDR
WGYRS	CGTGAGGCGACGGCTTCAAACTTG	Sense	(–)298 ~ (–)274	Cloning of whole *gyrA*
WGYRR	AGACGAAGTATTTCAACGTGGCGC	Anti-sense	(+)194 ~ (+)218	Cloning of whole *gyrA*
EWGS	ATCTCTTCGTGGTCTACGTTATG	Sense	(–)196 ~ (–)174	Cloning of *E. coli gyrA*
EWGR	ACAAGGAATGTGGCAATGAGTGG	Anti-sense	(+)144 ~ (+)166	Cloning of *E. coli gyrA*

[a]For cassette ligation-mediated PCR

dNTP, 1 µM each primer and 1.25 U Ampli *Taq* DNA polymerase (Applied Biosystems). 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. The results of amplification were analyzed by 1.5% agarose gel electrophoresis. The 623 bp PCR product was purified by agarose gel electrophoresis using the Prep-A-Gene DNA Purification system (Bio-Rad Laboratories) and cloned into the TOPO-TA vector (Invitrogen) following the instructions of the manufacturer. The cloned fragment was sequenced using the Big Dye Terminator Cycle DNA Sequencing Kit (ABI PRISM, PE Applied Biosystems) and an automatic sequencer (Applied Biosystems). Nucleotide sequences were compared based upon gene alignment using the MACAW program for *gyrA* gene sequences of different bacterial species.

Cloning of the *Edwardsiella tarda gyrA* gene. A commercially available, *in vitro* cloning system (Takara Shuzo, Shiga, Japan) was used to amplify the previously unknown region of genomic DNA flanking the *gyrA* gene, based on PCR using a cassette (a double-stranded synthetic oligonucleotide with a 5′-end dephosphorylated restriction site) and cassette primers after a slight modification of the method in the manufacturer's instructions.

Preparation of the cassette-ligated bacterial DNA mixture: *Edwardsiella tarda* DNA (2.5 µg) was isolated and was completely digested by 30 U of the appropriate restriction enzymes. The digested bacterial nucleic acids were precipitated with cold ethanol and resuspended in 5 µl of sterile, distilled water. The nucleic acids were mixed with the ligation components (Takara Shuzo); 15 µl of Ligation Solution I, 7.5 µl of Ligation Solution II and a cassette corresponding to the restriction enzyme were used to digest the bacterial DNA. After incubation at 16°C for 30 min, the ligated nucleic acids were precipitated with cold ethanol and resuspended in 200 µl of sterile, distilled water. After heating at 94°C for 10 min, the nucleic acids were use as a template for first PCR in cassette ligation-mediated PCR described below. We used *Eco*RI for the 5′ gene extension, and then *Pst*I and *Sal*I sequentially for the 3′ extension.

Cassette ligation-mediated PCR: Primers GYRS1R and GYRS2R for 5′ end walking were derived from the sequence of 623 bp PCR products sequenced in the above experiment and corresponded to Bases 478 to 456 and Bases 399 to 374 from the 5′-end of the amplified *gyrA* region. Cassette Primers C1 (5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3′) and C2 (5′-CGTTAGAACGCGTAAT-ACGACTCACTATAGGGAGA-3′) (Takara Shuzo) were derived from the common nucleotide sequence of the cassettes designed for various restriction enzymes. The first PCR amplification was carried out with a Perkin-Elmer 2400 thermal cycler in a 50 µl reaction containing 34.5 µl of *Edwardsiella tarda* nucleic acids (*Eco*RI cassette-ligated *E. tarda* DNA mixture), 1× Takara LA buffer, 2.5 U Takara LA *Taq* polymerase, 400 µM of each dNTP mixture, 10 pmol C1 primer and GYRS1R primer. After 10 min of heating at 94°C, amplification consisted of 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 4 min in 0.2 ml, thin-walled tubes. Using the same conditions, a second PCR was performed using the Primers C2 and GYRS2R and 1 µl of the product of the first PCR

amplification as a template. The PCR products were cloned and sequenced as described above. The 3′-end gene extensions obtained from other cassette-ligated *E. tarda* DNA mixtures (prepared sequentially with *Pst*I, *Sal*I treatments and their corresponding cassettes), and newly designed primers (GYRS1 and GYRS2, GYRS3 and GYRS4 for 3′-end walking) derived from the sequence of cloned fragments. With this method, 3 sequential cloning steps by cassette ligation-mediated PCR with *Eco*RI, *Pst*I and *Sal*I cassette-ligated bacterial DNA were performed to obtain the complete *E. tarda gyrA* gene and flanking regions (Fig. 1).

Cloning of the full-length *Edwardsiella tarda gyrA* gene. The full-length *gyrA* gene and the contiguously extended regions (3153 bp) were amplified from the chromosomal DNA of *E. tarda* GE1 (wild type) and RED7 (mutant type) by PCR with the primer set WGYRS/WGYRR (Table 1). Chromosomal DNA of *Escherichia coli* K-12 HB101 was also used to amplify the full-length *E. coli gyrA* gene (2980 bp) (Swanberg & Wang 1987) in PCR with the primer set EWGS/EWGR (Table 1). PCR amplification was carried out as described above. The resulting amplicons were cloned into the TOPO-TA vector following the instructions of the manufacturer and were introduced into *E. coli* TOPO by transformation. Three different plasmids, pGE1, pRED7 and pHB101 extracted from *E. coli* TOPO transformants by the method of Kado & Liu (1981), were used to transform a temperature-sensitive *gyrA* mutant, *E. coli* KNK453 (Kreuzer & Cozzarelli 1979) and designated *E. coli* KNK453 (pGE1), *E. coli* KNK453 (pRED7) and *E. coli* KNK453 (pHB101), respectively (see Table 4).

RESULTS

Cloning of the *Edwardsiella tarda gyrA* gene

The 4505-bp nucleotide sequence of the *Edwardsiella tarda gyrA* gene and flanking regions has been deposited in GenBank under Accession Number DQ019315. The organization of the *E. tarda gyrA* gene locus is shown in Fig. 1, with the restriction enzyme recognition sites and positions of primers used for each extension in the cassette ligation-mediated PCR shown. Sequence positions are given as negative numbers, indicating the distance upstream from the *gyrA* gene translation start codon, and as positive numbers, indicating the distance downstream from the *gyrA* gene translation termination codon.

To confirm that this ORF is the *gyrA* gene of *Edwardsiella tarda*, the nucleotide and deduced amino acid sequences were compared with those of other bacterial species and very high identities, especially to those of Gram-negative bacteria, using the BioEdit program (Version 5.0.9, Dept of Microbiology, North Carolina State University, Raleigh, NC, USA) (Table 2). The higher nucleotide sequence (deduced amino acid sequence) identities between the region suspected as a QRDR in this ORF, from 199 to 318 (67 to 106), agreed with the characteristics of QRDR known to be a conserved region in *gyrA* of other bacteria. Additionally, this ORF exhibited relatively high identity with *parC* sequences of other bacteria, but less than those found in the comparison with *gyrA* genes (Table 2). All of these results indicate that the ORF that contained the amplicon of 623 bp was the *E. tarda gyrA* gene.

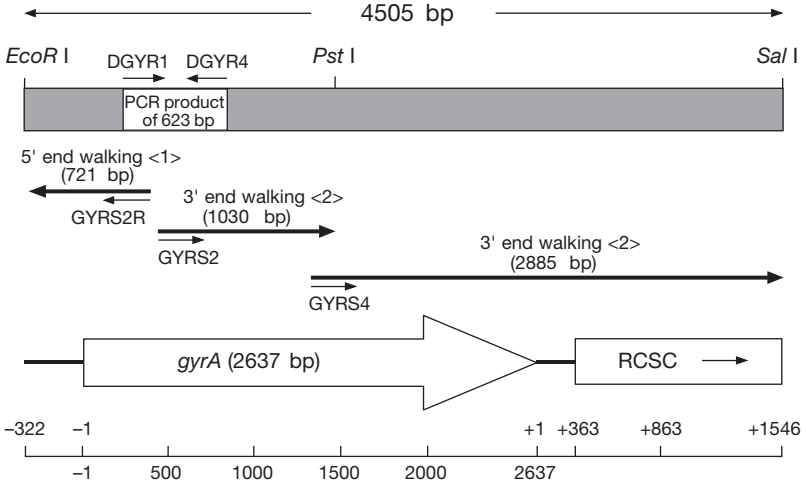

Fig. 1. *Edwardsiella tarda.* Restriction map and organization of the *gyrA* locus. Thin arrows indicate positions of the primers used for cloning of the *gyrA* gene. Thick arrows indicate the DNA fragments produced in cassette ligation-mediated PCR by the indicated restriction enzymes and walking directions. Complete nucleotide sequences are given in GenBank (Accession Number: DQ019315) (RCSC, capsular synthesis regulator component)

Sequence analysis of the *Edwardsiella tarda gyrA* gene

The *gyrA* gene of *Edwardsiellla tarda* was larger than that of *Escherichia coli*, containing 3 more amino acids. In the analysis of the nucleotide sequence, we found the presence of a ribosome binding site (–16 from the ATG codon), a putative promoter region (–25 from the ATG codon) and 3 pairs of inverted repeat sequences involved in the regulation of transcription (–203/–86 and –199/–44 from the ATG codon and +20/+37 from the termination codon). In addition, residues, the substitution of which confers quinolone resistance in *E. coli*, notably, Tyr^{122}, Ala^{67}, Gly^{81}, Ser^{83}, Asp^{87} and Gln^{106}, were conserved in *E. tarda gyrA* at identical coordinates

Table 2. Identity of the nucleotide and deduced amino acid sequences of *gyrA* gene in *Edwardsiella tarda* compared with those of other bacterial strains. QRDR: quinolone-resistance-determining region

	Organism	Accession no[a]	Nucleotide sequence (%)		Deduced amino acid sequence (%)	
			Whole *GyrA*	QRDR	Whole *GyrA*	QRDR
Gram(–)	*Escherichia coli*	X57174	78(49)[b]	84	85(30)	100
	Aeromonas salmonicida	L47978	71	83	71	93
	Pectobacterium carotovorum	X80798	78	83	85	97
	Klebsiella pneumoniae	X16817	80	84	86	97
	Neisseria gonorrhoeae	U08817	59(49)	77	51(29)	85
	Pasteurella multocida	AE006122	64	88	72	95
	Pseudomonas aeruginosa	L29417	68(52)	78	63(32)	97
	Serratia marcescens	U56906	83(53)	91	86(31)	100
	Vibrio parahaemolyticus	AB023569	70(50)	85	77(31)	100
Gram(+)	*Streptococcus pneumoniae*	AY157689	52(49)	65	46(34)	68
	Campylobacter jejuni	LO4566	48	63	45	70
	Mycoplasma hominis	U59880	45	65	39	75

[a]The nucleotide sequence accession number in the GenBank database
[b]The numbers in parentheses indicate the identity to *parC* gene

(Horowitz & Wang 1987, Swanberg & Wang 1987, Oppegard & Sorum 1996). However, the nucleotide sequences of the 5′ and 3′ end flanking regions of the *E. tarda gyrA* gene did not show any homologies to those of other bacterial *gyrB* genes and this suggests that the gyrase genes *gyrB*/*gyrA* are non-contiguous in the genome of *E. tarda*. We also determined the partial nucleotide sequences of another putative ORF cloned with the *gyrA* gene by 3′ end DNA walking and found it to show 49% nucleotide sequence identity with that of the RCSC in *E. coli* (Fig. 1).

Characterization of *gyrA* mutations in quinolone-resistant clinical isolates of *Edwardsiella tarda*

In order to examine the role of *gyrA* in quinolone-resistant *Edwardsiella tarda*, 12 strains were selected from 25 clinical isolates showing a MIC of >60 µg ml^{-1} of NA. These quinolone-resistant *E. tarda* strains were also resistant to various quinolone antibiotics (Table 3).

A 302-bp *gyrA* region spanning Nucleotides 106 to 407 was amplified by single-step PCR with specific primers, GYRQ/GYRQR, from quinolone-resistant *Edwardsiella tarda* isolates. This region encompasses the sequence equivalent to the QRDR of *Escherichia coli gyrA* (Residues 201 to 318) and flanking regions. The amplified sequences were ligated into the TOPO-TA vector, and the inserts were sequenced and compared. Table 3 presents the nucleotide and amino acid changes in the QRDR of the *gyrA* genes from clinical isolates. All 12 clinical isolates resistant to quinolone carried a single nucleotide change compared to the quinolone-susceptible *E. tarda* GE1. One AGC to CGC and 11 AGC to AGA alterations were found at Codon 83 in 12 quinolone-resistance *E. tarda* isolates, all of which would result in a Ser to Arg substitution in *gyrA*. Apart from this substitution, the nucleotide sequence of the QRDR in all quinolone-resistant isolates was identical to that of the susceptible strain. However, for each of the quinolone-resistant isolates, there were significant variations in the MICs of OA, NOR and CIP (Table 3). It cannot be excluded that other mechanisms may also contribute to change the ultimate MICs of quinolone antibacterial agents in quinolone-resistant *E. tarda*.

Expression of *Edwardsiella tarda gyrA* in *Escherichia coli gyrA* mutants

The PCR products which were amplified using DNA extracted from *Edwardsiella tarda* GE1 and RED7 isolates as templates with WGYRS/WGYRR as primers (Table 1) were cloned into the TOPO-TA vector. These plasmids contained the full-length *E. tarda gyrA* gene and the contiguously extended regions that have the potential to regulate the expression of the structural *gyrA* gene. *E. coli* KNK453 transformed with pGE1 (bearing the wild-type *gyrA*) exhibited growth on TSA plates at both the permissive temperature of 30°C and the non-permissive temperature of 42°C (Table 4). Transformation with pRED7 (bearing a *gyrA* gene with Arg^{83}) increased the resistance of *E. coli* KNK453 to NA and OA at 42°C. The level of resistance was much lower than that of the *E. tarda* RED7 isolate, but significantly higher than that of *E. coli* KNK453 transformed with pGE1 at 42°C.

Table 3. Susceptibility to quinolones and mutations in the QRDR of *gyrA* genes of *Edwardsiella tarda* isolates (MIC, minimum inhibitory concentrations; NA, nalidixic acid; OA, oxolinic acid; NOR, norfloxacin; CIP, ciprofloxacin)

Strain	Isolation	MIC (μg ml^{-1})				Mutation in *gyrA*	
		NA	OA	NOR	CIP	Amino acid	Base change
GE1	1994, Kujedo	2	0.13	0.03	0.008	83(Ser)	AGC
RED7	1994, Yosu	≥512	2	0.31	0.06	83(SER → Arg)	AGA
RE1	1995, Kwangju	64	0.5	0.08	0.03	83(SER → Arg)	CGC
RE8	1995, Pohang	≥512	4	0.31	0.06	83(SER → Arg)	AGA
LE2	1995, Namhae	≥512	2	0.31	0.13	83(SER → Arg)	AGA
JH4	1996, Tongyoung	≥512	1	0.16	0.06	83(SER → Arg)	AGA
KFE	1997, Gampo	64	2	0.13	0.06	83(SER → Arg)	AGA
JH9	1999, Tongyoung	≥512	2	0.16	0.06	83(SER → Arg)	AGA
JDE2	2000, Yosu	256	1	0.63	0.06	83(SER → Arg)	AGA
JDE30	2001, Namhae	256	1	1.25	0.06	83(SER → Arg)	AGA
JDE37	2002, Kuryongpo	≥512	4	1.25	1.25	83(SER → Arg)	AGA
CJE13	2003, Jejudo	≥512	2	0.5	0.25	83(SER → Arg)	AGA
JDE45	2003, Kujedo	≥512	4	0.5	0.13	83(SER → Arg)	AGA

Table 4. MICs of quinolones for *Escherichia coli* strains K-12 HB101, KNK453, and KNK453 with different transforming plasmids. MICs were determined at 42°C, except for KNK453 without plasmid (30°C) (NA, nalidixic acid; OA, oxolinic acid; NOR, norfloxacin; CIP, ciprofloxacin)

Strain	MIC (μg ml^{-1}) at 30°C				MIC (μg ml^{-1}) at 42°C			
	NA	OA	NOR	CIP	NA	OA	NOR	CIP
K-12 HB101	4	0.13	0.03	0.008	4	0.13	0.03	0.008
KNK453	2	0.06	0.015	0.015				
KNK (pHB101)	4	0.13	0.03	0.008	2	0.06	0.015	0.015
KNK (pGE1)	4	0.06	0.03	0.015	2	0.06	0.015	0.008
KNK (pRED7)	1	0.06	0.015	0.008	32	0.5	0.15	0.015

DISCUSSION

The approach used to determine the nucleotide sequence of the *Edwardsiella tarda gyrA* gene is based on conservation of the QRDR and its flanking regions and thus would be applicable to sequence determination of the *gyrA* or *parC* homologues in many bacterial species (Peng & Marians 1993).

Sequence analysis of the *Edwardsiella tarda gyrA* gene revealed a ribosome-binding site with homology to the *Escherichia coli* Shine-Dalgarno consensus sequence (GAGGG) located 16 bp upstream from the ATG translation initiation codon. At 9 bp upstream of the Shine–Dalgarno sequence, there was a region (TAATTA) that is the same as the –10 *Aeromonas salmonicida* promoter and similar to the –10 *E. coli* promoter consensus sequences (TATAAT). Further upstream, although it was not apparent, a putative –35 region (TGTATT) similar to that of *Helicobacter pylori* (TTGATT) (Moore et al. 1995) was observed. However, the sequences found in the –35 *gyrA* promoter regions in other bacteria, such as the conserved form (GTTTACC) in *E. coli* (Swanberg & Wang 1987), *Klebsiella pneumoniae* (Dimri & Das 1990) and *Bacillus subtilis* (Moriya et al. 1985), or as other forms ATTTTCC, GTTTGCC, GTTTCCC and GTTTAAG in *Serratia marcescens* (Kim et al. 1998), *Pseudomonas aeruginosa* (Kureishi et al. 1994), *Erwinia carotovora* (Rosanas et al. 1995) and *A. salmonicida* (Oppegard & Sorum 1996), respectively, were not found.

Two pairs of inverted repeat sequences that may form cruciform structures involved in the regulation of transcription were identified in the upstream region of the *Edwardsiella tarda gyrA* gene. These are the AAAGAC/GTCTTT pair, located at –203/–86 and the ACCCTC/GAGGGT pair, located at –199/–44 from the start codon, and could form a cruciform structures with unpaired loops of 111 and 149 nucleotides, respectively. The presence of these inverted repeat sequences indicated that supercoiling-dependent regulation of *gyrA* transcription may work in *E. tarda*, as suggested for *Escherichia coli* (Horowitz & Loeb 1988). Inverted repeat sequences have also been identified in *gyr*A promoters, in *Aeromonas salmonicida* (Oppegard & Sorum 1996), *Pseudomonas aeruginosa* (Kureishi et al. 1994) and *Klebsiella pneumoniae* (Dimri & Das 1990). Another inverted repeat sequence, AAGGGC/GCCCTT, located +20/+37 from the translational stop codon TAA, may act as a transcriptional termination signal by the formation of a loop structure with 11 unpaired nucleotides (Oppegard & Sorum 1996).

In the alignment of the deduced amino acid sequence, we could not find the intragenic stretches present in the 3′-end of *gyrA* genes from *Campylobacter jejuni*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (Wang et al. 1993, Kureishi et al. 1994, Moore et al. 1995). The catalytic Tyr^{122} involved in DNA breakage and reunion was also present, as in *Escherichia coli* and other bacteria (Horowitz & Wang 1987). Additional residues, the substitution of which confers quinolone resistance in *E. coli*, notably, Ala^{67}, Gly^{81}, Ser^{83}, Asp^{87} and Gln^{106}, were also conserved in *Edwardsiella tarda gyrA*.

However, upstream and downstream regions of the *Edwardsiella tarda gyrA* gene did not show any homologies to the *gyrB* gene of other bacterial species and failed to reveal if the *gyrA* and *gyrB* genes are contiguous. Non-contiguous gyrase genes have been

found to be common in many Gram-negative bacteria (Dimri & Das 1990, Kureishi et al. 1994, Wang et al. 1993). On the other hand, contiguous *gyrB/gyrA* gyrase genes have been identified in *Mycoplasma pneumoniae* and other Gram-positive bacteria (Colman et al. 1990, Moriya et al. 1985).

Amino acid residues, Ser^{83} and Glu^{87}, of *Escherichia coli gyrA* have been shown to be hot spots for quinolone resistance (Vila et al. 1994, Yolanda et al. 2003). We examined the similarities among *gyrA* genes from *Edwardsiella tarda* isolates for quinolone resistance and found only a single substitution in all quinolone-resistant *E. tarda* isolates of this study: Ser^{83}→Arg. The Ser^{83}→Arg substitution has also been observed at a high frequency in quinolone-resistant clinical isolates of *Yersinia ruckeri* (Gibello et al. 2004) and as one of the mutations in quinolone-resistant clinical isolates of *Enterococcus faecalis* and *Aeromonas salmonicida*. (Korten et al. 1994, Tankovic et al. 1996). Additional studies of the alteration of *gyrA* using *E. tarda* mutants obtained in *in vitro* conditions are ongoing in our laboratory.

To examine the complementation of the *Escherichia coli gyrA* mutant, the full length the *gyrA* gene of *Edwardsiella tarda* was cloned and used to transform *E. coli* KNK453, a temperature-sensitive *gyrA* mutant. Transformation with the plasmid pRED7 (mutant type) was able to restore growth and induce resistance to NA and OA in *E. coli* KNK453 at 42°C. The level of resistance was significantly higher than that of *E. coli* KNK453 transformed with pGE1 (wild type) at 42°C, but did not reach the levels of the *E. tarda* RED7 isolate. These results indicated that: (1) the complete *gyrA* gene was successfully cloned, (2) the promoter region of the *E. tarda gyrA* gene was active in the *E. coli* system, (3) an *E. tarda gyrA*–*E. coli gyrB* holoenzyme complex is functionally active and (4) the variable MICs of quinolones for the quinolone-resistant *E. tarda* isolates might be explained by additional mechanisms, including mutations in other DNA gyrase (or TOPO IV) genes, *gyrB*, *parC*, *parE*, or those that can cause decreased drug permeability or increased drug efflux (Nakamura 1997). Further study is required to determine between these possibilities. However, all quinolone-resistant isolates had a mutation within the QRDR, suggesting that the primary target for resistance is an alteration in the *gyrA* gene rather than in other potential genes.

Additional characterization of the *Edwardsiella tarda gyrA* gene reported here should facilitate further understanding of this important fish pathogenic bacteria and its quinolone resistance. However, the use of quinolones in Asian aquaculture should be avoided as much as possible, to prevent the emergence and spread of quinolone-resistant strains of *E. tarda* in the aquatic environment.

Acknowledgements. This work was supported by a grant from the Ministry of Maritime Affairs and Fisheries of Korea (KSGP of 2003).

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Editorial responsibility: Carey Cunningham, Aberdeen, UK

Submitted: January 20, 2005; Accepted: June 21, 2005
Proofs received from author(s): November 2, 2005