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gyrA Mutations in Quinolone-Resistant Isolates of the Fish Pathogen *Aeromonas salmonicida*

HANNE OPPEGAARD* AND HENNING SØRUM

Department of Pharmacology, Microbiology, and Food Hygiene, Norwegian College of Veterinary Medicine, Oslo, Norway

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***gyrA* mutations in quinolone-resistant isolates of *Aeromonas salmonicida* have been detected by using PCR to amplify the quinolone resistance-determining region of *gyrA* and subsequent cloning and sequencing of PCR products. Comparison of nucleotide and derived amino acid sequences of PCR products from quinolone-susceptible and -resistant bacteria revealed a serine 83-to-isoleucine substitution in the gyrase A protein of resistant isolates. One of the resistant isolates differed from the other by a two- to fourfold-higher MIC of the fluoroquinolone enrofloxacin and carried an additional alanine 67-to-glycine substitution, which may contribute to the higher level of resistance.**

The antibacterial 4-quinolone oxolinic acid has been used extensively since 1988 in Norwegian aquaculture, mainly to control outbreaks of furunculosis caused by *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar* L.). From 1985 to 1991 a total of 65 metric tons of oxolinic acid was prescribed to combat furunculosis (23). The widespread use of oxolinic acid and, to a minor degree, the 4-quinolone flumequine has led to emergence of quinolone-resistant isolates of *A. salmonicida* at several geographical locations along the Norwegian coastline (17, 21) and in Japan, Scotland, and Ireland, where quinolone derivates have been used to treat furunculosis. *A. salmonicida* strains with reduced susceptibility to quinolones have been isolated (3, 16, 24). More potent fluoroquinolones like sarafloxacin and enrofloxacin have also been evaluated for treatment of furunculosis (6, 31). However, complete cross-resistance has been observed between the older 4-quinolones and the fluoroquinolones in fish-pathogenic bacteria (21).

The target for quinolone drugs is the enzyme DNA gyrase, which catalyzes ATP-dependent DNA supercoiling (13). The *Escherichia coli* DNA gyrase is an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes, respectively, and is involved in replication, transcription, and recombination (28). The enzyme is thereby essential for bacterial viability. High-level quinolone resistance in *E. coli*, *Staphylococcus* spp., *Campylobacter jejuni*, and *Mycobacterium tuberculosis* is due to single-nucleotide changes in the highly conserved 5′ end of the *gyrA* gene, leading to amino acid changes in the N-terminal region of the gyrase A protein (9, 26, 29, 30, 33, 35, 38, 40). *gyrA* mutations causing substitutions in the region between amino acids 67 and 106 in the gyrase A protein have been shown to be of special importance in quinolone resistance, and this region has been named the "quinolone resistance-determining region" (38).

Little is known about the mechanisms of quinolone resistance in *A. salmonicida*. Low-level resistance to a variety of antibacterial drugs, including 4-quinolones, has been associated with changes in outer membrane proteins (5, 14, 37). However, the role of *gyrA* mutations has yet to be established. The complete nucleotide sequence of *A. salmonicida gyrA* is unknown, although the gene recently has been cloned and is about to be sequenced in our laboratory (25). By using the fact that the 5′ ends of *gyrA* genes from different bacterial species contain corresponding areas which are highly conserved (7, 8, 10, 20, 22, 32, 35), PCR would be a suitable approach for analyzing this region of *gyrA*. In this article we describe a protocol for PCR to amplify the quinolone resistance-determining region of *A. salmonicida gyrA* and DNA sequencing of cloned PCR products to detect mutations in quinolone-resistant isolates of the bacterium.

For these studies we used a total of 10 *A. salmonicida* subsp. *salmonicida* strains, including the type strain ATCC 14174, 7 fish isolates from Atlantic salmon (*Salmo salar* L.), 1 laboratory-derived quinolone-resistant clone, and its parent quinolone-susceptible isolate (Table 1). Six of the isolates were known to be quinolone resistant according to disc diffusion tests and MIC determinations (17, 21; also, this study) and by the criteria of Tsoumas et al. (34), which define strains for which the MIC of oxolinic acid is less than 1.0 μg/ml as susceptible and strains for which the MIC of oxolinic acid is higher than 1.0 μg/ml as resistant. MICs of oxolinic acid (Sigma Chemical Co., St. Louis, Mo.), flumequine (Sigma), sarafloxacin (Abbott Laboratories, North Chicago, Ill.), and enrofloxacin (Bayer AG, Leverkusen, Germany) were determined by a standard microdilution method in modified Mueller-Hinton broth supplemented with 1% NaCl as described by Martinsen et al. (21). The inoculum size was approximately 2.5×10^5 CFU/ml in a total volume of 100 μl. The microtiter trays were incubated for 5 days at 15°C. MICs were determined by visual inspection and are listed in Table 1.

PCR was carried out by using the GeneAmp PCR reagent kit with *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). All oligonucleotides used for PCR as described below are listed in Table 2 and were synthesized at the Biotechnology Centre of Oslo, University of Oslo, Oslo, Norway. In addition, two oligonucleotides described by Oram and Fisher (26) for amplification of an *E. coli gyrA* fragment were evaluated. High-molecular-weight DNAs were isolated from the *A. salmonicida* subsp. *salmonicida* strains (4). DNA isolated from the type strain ATCC 14174 was at first subjected to nested PCR by using two pairs of 21-mer oligonucleotide primers based on the *E. coli gyrA* sequence (1A and B and 2A and B) and covering the quinolone resistance-determining region (38). Genomic DNA from *E. coli* HB101 was included as a positive control. The PCR was performed in a DNA Thermal

* Corresponding author. Mailing address: Department of Pharmacology, Microbiology, and Food Hygiene, Norwegian College of Veterinary Medicine, P.O. Box 8146 Dep., 0033 Oslo, Norway. Phone: 47 22 96 48 30. Fax: 47 22 96 48 50.

TABLE 1. Quinolone resistance mutations in the *gyrA* gene of *A. salmonicida* isolates used in this study and corresponding MICs of oxolinic acid, flumequine, sarafloxacin, and enrofloxacin

Strain or isolate	Year of isolation	MIC (μg/ml)[a]				*gyrA* mutation(s)
		OA	F	S	E	
ATCC 14174		0.02	0.04	0.02	0.005	None (wild type)
1693	1988	0.02	0.04	0.02	0.01	None (wild type)
4046	1989	3.20	3.20	2.56	0.64	Ser-83 (AGT) to Ilc (ATT), Ala-67 (GCC) to Gly (GGC)
3708	1990	0.02	0.08	0.04	0.01	None (wild type)
3708*[b]		3.20	3.20	1.28	0.32	Ser-83 (AGT) to Ilc (ATT)
3475	1990	2.56	1.28	2.56	0.32	Ser-83 (AGT) to Ilc (ATT)
3704	1990	2.56	1.28	2.56	0.32	Ser-83 (AGT) to Ilc (ATT)
7106	1991	3.20	1.60	1.28	0.16	Ser-83 (AGT) to Ilc (ATT)
7093	1991	3.20	1.60	2.56	0.32	Ser-83 (AGT) to Ilc (ATT)
7098	1991	3.20	1.60	1.28	0.16	Ser-83 (AGT) to Ilc (ATT)

[a] OA, oxolinic acid; F, flumequine; S, sarafloxacin; E, enrofloxacin.
[b] Laboratory-derived quinolone-resistant clone of isolate 3708, selected with sarafloxacin.

Cycler (Perkin-Elmer Cetus) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–200 μM each deoxynucleoside triphosphate–1 to 1.5 U of *Taq* DNA polymerase–10 ng each of oligonucleotide primers 1A and B–10 ng of bacterial DNA in a final volume of 50 μl. After PCR with the first set of primers (1A and B), 5 μl from this reaction mixture was used for PCR with primers 2A and B under otherwise unchanged reaction conditions. The number of cycles used for each reaction was 30, with the following temperature profile: 94°C for 2 min, 45°C for 1 min, and 72°C for 2.5 min. The 442-bp *gyrA* PCR product from ATCC 14174 was isolated, cloned, and sequenced as described below. A new pair of PCR primers (3A and B) specific for *A. salmonicida gyrA* were then constructed, still covering the quinolone resistance-determining region. This allowed an increase in the PCR annealing temperature. DNAs from the isolates listed in Table 1 were subjected to PCR using these primers, reaction conditions described above, and a temperature profile of 94°C for 2 min, 69°C for 1 min, and 72°C for 2.5 min. To confirm that the resulting PCR products were part of *gyrA*, hybridizations of Southern blots were carried out with a ^{32}P-labelled 600-bp *Sac*I-*Hin*dIII fragment from the 5′ end of the *E. coli gyrA* gene, harbored in plasmid pMEC5 (9). In addition, PCR products were ^{32}P labelled and hybridized to *A. salmonicida* and *E. coli* genomic DNAs to decide if PCR products were *A. salmonicida* specific. All hybridizations and washings were carried out at high stringency. Hybridizations were done in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–5× Denhardt's solution with 10% dextran sulfate for 18 h at 65°C. Washings were done in 2× SSC–0.1% SDS, 1× SSC–0.1% SDS and 0.2× SSC–0.1% SDS twice each for 30 min each time at 65°C and then once in 0.1× SSC–0.1% SDS for 15 min at 65°C. Hybond-N nylon membranes, the Megaprime DNA labelling system, and [α-^{32}P] dCTP (5 μCi/μl) were obtained from Amersham International.

TABLE 2. Oligonucleotide primers used to amplify *A. salmonicida gyrA* fragments by PCR

Primer[a]	Sequence (5′ to 3′)	Positions[b]
1A	TCCTATCTGGATTATGCGATG	58–78
1B	GGGATGTGTTCCATCAGCCCT	618–638
2A	GCGCTGCCAGATGTCCGAGAT	97–117
2B	TTGCCATACCTACGGCGATAC	518–538
3A	TCTGTTTGCTATGAACGAGTT	144–164
3B	CACCTTGGTGGGCATGACAGC	470–490

[a] Oligonucleotides 1B, 2B, and 3B are noncoding sequences; oligonucleotides 3A and 3B are *A. salmonicida* specific (see the text).
[b] Corresponding to the *E. coli gyrA* sequence determined by Swanberg and Wang (32).

PCR products originating from each strain listed in Table 1 were then isolated from 1% low-melting-point agarose by Geneclean (Bio 101, Inc., La Jolla, Calif.). By using the TA cloning kit (Invitrogen Corp., San Diego, Calif.), PCR products were ligated to the pCRII plasmid vector and used to transform *E. coli* (INVαF′) competent cells. Isolation of plasmid DNA from white, ampicillin-resistant colonies was done by using the Magic Miniprep or Magic Maxiprep kit (Promega Corp., Madison, Wis.). Plasmids containing *gyrA* PCR products were identified by digestion with suitable restriction enzymes (Boehringer GmbH, Mannheim, Germany) followed by high-stringency hybridizations of Southern blots with the *E. coli* and *A. salmonicida gyrA* probes as described above. To reveal possible misreadings by *Taq* polymerase, two independent recombinant clones for each strain were selected for sequencing. These clones originated from different PCR and cloning experiments. The DNA sequences of cloned PCR products were determined by the dideoxy chain termination method using T7 DNA polymerase and fluorescent primers (1, 2, 19). Standard universal and reverse primers for M13 were used. Two or more parallel sequencing reactions were performed with each sequencing primer. Sequence analysis was performed on an automated fluorescent DNA sequencer at the Biotechnology Centre of Oslo. Nucleotide and derived amino acid sequences were analyzed by using the GCG sequence analysis software package, version 7 (Genetics Computer Group, Madison, Wis.).

The nucleotide and derived amino acid sequences of the 442-bp nested *gyrA* PCR product from *A. salmonicida* subsp. *salmonicida* ATCC 14174 are shown in Fig. 1. The DNA sequences of the 346-bp PCR products from all *A. salmonicida* isolates were highly homologous to the type strain sequence. In fact, the quinolone-susceptible isolates 1693 and 3708 had 100% DNA identity with the corresponding region of *gyrA* from ATCC 14174. Before cloning and sequencing, all PCR products were confirmed to be part of *gyrA* by hybridization with the *E. coli gyrA* gene probe. They were also shown to be specific for *A. salmonicida* by hybridization with *A. salmonicida* and *E. coli* genomic DNAs (data not shown).

gyrA fragments from quinolone-resistant *A. salmonicida* isolates 3475, 3704, 7106, 7093, and 7098 differed at one single nucleotide position from the susceptible strains; all carried a

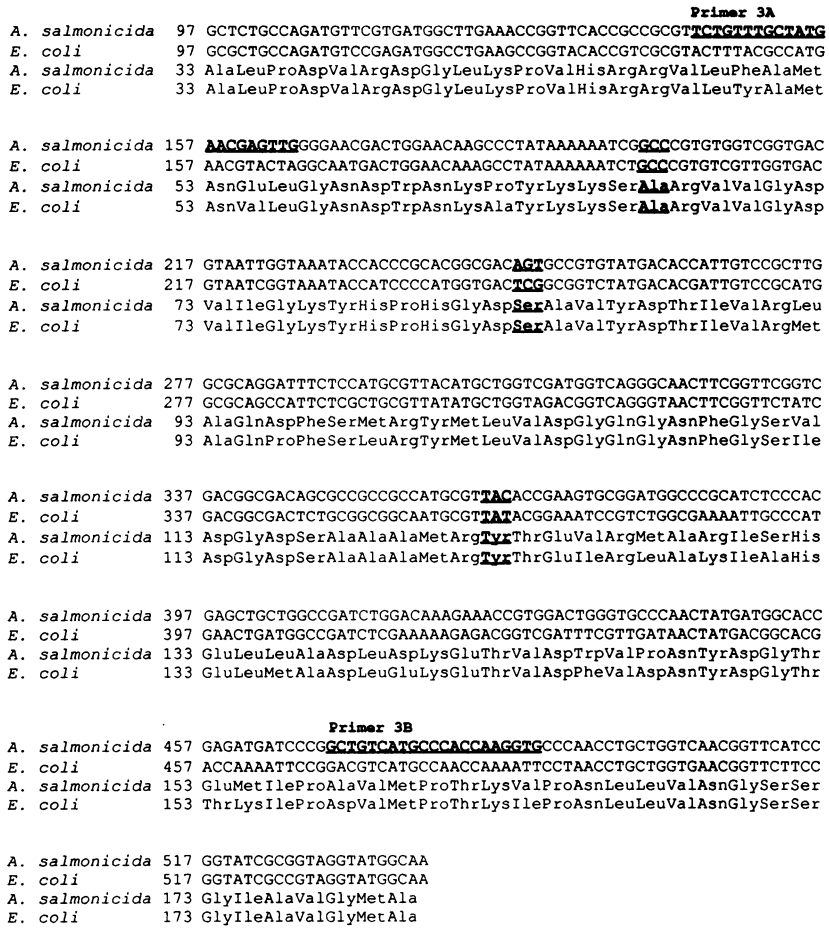

FIG. 1. Comparison of nucleotide and deduced amino acid sequences of the 442-bp *gyrA* PCR fragment obtained from amplification of genomic DNA from the quinolone-susceptible *A. salmonicida* subsp. *salmonicida* type strain ATCC 14174 and the corresponding region of *E. coli*. The nucleotides and amino acids are numbered as they appear in the native *A. salmonicida* and *E. coli* gyrase genes and proteins (25, 32). The serine 83 and alanine 67 positions, where substitutions result in a quinolone-resistant gyrase A protein (see the text), the catalytic tyrosine 122, and the PCR primers (3A and B) used to amplify *gyrA* fragments from *A. salmonicida* isolates are underlined.

G-to-T transition at the position corresponding to nucleotide 152 in the type strain PCR product. When aligned with *E. coli gyrA* sequence (Fig. 1), this position corresponds to nucleotide 248, the second nucleotide in the codon for serine 83 (32). A serine residue was also found at this position in *A. salmonicida* gyrase A, and the nucleotide change would result in a serine (codon AGT)-to-isoleucine (codon ATT) substitution at the amino acid level. An identical nucleotide change was found in the laboratory-derived quinolone-resistant clone 3708*. Isolate 4046 carried an additional mutation which would also lead to amino acid substitution; a C-to-G transition was found at position 104 in the type strain *gyrA* PCR sequence. This corresponds to the second nucleotide position in the codon for alanine 67 in the *E. coli* gyrase A protein. As in *E. coli* (32), an alanine residue was also found at this position in *A. salmonicida*, and the mutation would give rise to a substitution from alanine (codon GCC) to glycine (codon GGC). These results are presented in Table 1.

Although the complete *A. salmonicida gyrA* nucleotide sequence is not yet known, the whole *gyrA* gene has recently been cloned in our laboratory and about 1,500-bp has been sequenced (25). This has made us able to position the PCR products relative to the native *A. salmonicida gyrA* gene and thereby locate the sites of mutation. When the PCR products were aligned to the *E. coli gyrA* sequence (32), the numbers of nucleotides from the first ATG to the start of the PCR oligonucleotide sequence 2A were exactly the same for the two species. Therefore, the serine-to-isoleucine and the alanine-to-glycine substitutions correspond to amino acid positions 83 and 67, respectively, in the *A. salmonicida* gyrase A protein. The alignment showed that the catalytic tyrosine 122 in *E. coli* (18) also was present in *A. salmonicida* gyrase A.

DNA sequence identities between the *A. salmonicida* gyrA PCR fragment and corresponding sequences from other known *gyrA* genes were 78, 80, 65, 69, and 40% for *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, and *C. jejuni*, respectively (7, 10, 20, 22, 32, 35); at the amino acid level, the identities were 88, 88, 69, 74, and 69%, respectively. Several nucleotide differences were detected when the *gyrA* fragment of *A. salmonicida* was compared with the corresponding region of *E. coli gyrA* (Fig. 1). However, most of the nucleotide differences were silent. When a single PCR was performed with primers 1A and B, only the *E. coli* PCR product of 580 bp was detected by agarose gel electrophoresis. The nested PCR strategy revealed amplification of the 442-bp PCR product from both *E. coli* and the type strain ATCC 14174. The oligonucleotides used by Oram and Fisher (26) to amplify a *gyrA* fragment from *E. coli* were also tried out,

but no PCR product was detected from *A. salmonicida* (data not shown). The GC content of the amplified fragments was 58%.

Our results show that serine 83-to-isoleucine and alanine 67-to-glycine substitutions are found in the deduced amino acid sequence of the gyrase A proteins of quinolone-resistant *A. salmonicida* strains. The serine 83-to-isoleucine substitution occurs in fish isolates and in the laboratory-derived resistant clone 3708*. Thus, the substitution is probably associated with both clinical and in vitro-selected resistance. These results are in agreement with all previous reports on *gyrA* mutations and quinolone resistance. In *E. coli*, substitutions of several amino acids in the gyrase A protein have been shown to be of importance with regard to quinolone resistance. The most frequently occurring substitutions replace serine 83. Substitution of serine 83 with leucine or tryptophan has been shown to confer high-level quinolone resistance both in clinical isolates and in laboratory-derived resistant mutants of *E. coli* (9, 26, 38, 40). A genetically engineered serine 83-to-alanine *gyrA* codon change confers low-level quinolone resistance in *E. coli* (15). Correspondingly, a serine 84-to-leucine substitution in *S. aureus* and a serine 84-to-phenylalanine substitution in *Staphylococcus epidermidis* confer high-level quinolone resistance in these species (29, 30). In *C. jejuni*, in which threonine 86 is analogous to amino acid 83 in *E. coli* gyrase A, threonine 86-to-isoleucine substitution has been detected in quinolone-resistant isolates of the bacterium (35). Although quinolone resistance mutations, to our knowledge, have not yet been identified for *K. pneumoniae*, a threonine residue is found at position 83 in the gyrase A protein of a quinolone-susceptible strain (10). However, both *K. pneumoniae* and *C. jejuni* are at least 10-fold more resistant to quinolones than other enteric pathogens, like *E. coli*. Since both *C. jejuni* and *K. pneumoniae* contain threonines at positions 86 and 83, respectively whereas *E. coli* contains a serine at position 83, it has been suggested that threonine at this position is associated with intrinsic quinolone resistance (35).

Only one *A. salmonicida* isolate, 4046, carried the additional alanine 67-to-glycine substitution. Substitution of alanine with serine at this position in *E. coli* has previously been associated with quinolone resistance (38, 40). Isolate 4046 differed from the other isolates with respect to the MIC of the fluoroquinolone enrofloxacin, which was two to four times higher than those for the other resistant isolates. The additional mutation may account for the higher level of resistance. The MIC of flumequine for isolate 4046 was also found to be somewhat higher than those for the other resistant isolates.

The frequent occurrence of the *gyrA* mutation at the codon for serine 83 also in *A. salmonicida* further emphasizes the importance of this serine residue in quinolone action and resistance. Willmott and Maxwell recently reported that the serine 83-to-tryptophan substitution in the *E. coli* gyrase A protein greatly reduced binding of fluoroquinolones to the gyrase-DNA complex (36). Most *gyrA* mutations conferring quinolone resistance are situated close to the tyrosine at position 122 of the A subunit that is covalently bound to DNA when the enzyme breaks the phosphodiester bonds of DNA (18). However, the exact mechanism of action and resistance against quinolones is not fully understood, mainly because the sites of interaction between quinolones, the DNA gyrase A subunit, and DNA remain unidentified.

Our study also shows the high degree of amino acid conservation in the N-terminal region of the gyrase A protein when *A. salmonicida* is compared with other bacterial species, particularly the other gram-negative species, *E. coli* and *K. pneumoniae* (10, 32). As other authors have emphasized, this conservation underlines the key role of this portion of the gyrase A subunit in the catalytic activity of the enzyme. A large number of nucleotide differences were found when the *gyrA* fragment of *A. salmonicida* was compared with the corresponding region of *E. coli gyrA*, but most of the nucleotide differences were silent. The higher degree of difference at the DNA level could explain why a nested PCR strategy was necessary to amplify the 442-bp *A. salmonicida gyrA* fragment when primers based on *E. coli* sequences were used. It may also explain why the oligonucleotides used by Oram and Fisher to amplify an *E. coli gyrA* fragment (26) do not work on *A. salmonicida* DNA. These primers have also been reported not to work on *Salmonella typhimurium* DNA (27).

There was a remarkable degree of nucleotide sequence identity when *gyrA* fragments from the different *A. salmonicida* strains were compared, considering the fact that they were isolated at distant geographical locations and in different years. No sense or nonsense mutations, except those conferring quinolone resistance, were detected. The high degree of conservation indicates that the PCR and sequencing techniques will be suitable for detecting quinolone resistance mutations in this region of *A. salmonicida gyrA*. However, when a large number of strains are screened for *gyrA* mutations, this method may be time-consuming and expensive. Other techniques for detecting *gyrA* mutations like restriction fragment length polymorphism analysis and hybridization with different oligonucleotides have previously been tried out on *E. coli* and *Staphylococcus* spp. (11, 12, 29, 30) and may be evaluated.

We cannot exclude the possibility that other mutations in the *gyrA* gene or other mechanisms of resistance (e.g., changes in outer membrane proteins or *gyrB* mutations) contribute to high-level quinolone resistance in *A. salmonicida*. Unpublished results from our laboratory indicate that changes in outer membrane protein contents are rarely the mechanism of resistance in clinical isolates. However, there is no reason to believe that quinolone resistance mutations in the *gyrB* gene should be less common in *A. salmonicida* than in *E. coli* (39). Further analyses of the *A. salmonicida* gyrase genes and proteins need to be carried out to clarify this point.

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