ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1996, p. 1126–1133 Vol. 40, No. 5
0066-4804/96/$04.00+0

Cloning and Nucleotide Sequence of the DNA Gyrase *gyrA* Gene from the Fish Pathogen *Aeromonas salmonicida*

HANNE OPPEGAARD* AND HENNING SØRUM

Department of Pharmacology, Microbiology and Food Hygiene, Norwegian College of Veterinary Medicine, 0033 Oslo, Norway

Received 17 October 1995/Returned for modification 26 December 1995/Accepted 28 February 1996

The DNA gyrase *gyrA* gene from the fish pathogen *Aeromonas salmonicida* 2148/89 was cloned, and the nucleotide sequence was determined. An open reading frame of 2,766 nucleotides was identified and was found to encode a protein of 922 amino acids with a calculated molecular mass of 101.1 kDa. The derived amino acid sequence shared a high degree of identity with other DNA gyrase A proteins, in particular, with other gram-negative GyrA sequences. When the amino acid sequence of *A. salmonicida* GyrA was compared with that of *Escherichia coli* GyrA, a number of conserved residues were present at identical coordinates, including the catalytic Tyr residue at position 122 (Tyr-122) and residues whose substitution confers quinolone resistance, notably, Ser-83, Ala-67, Gly-81, Asp-87, Ala-84, and Gln-106. An intragenic region corresponding to 48 amino acids, which is not present in *E. coli* or other bacteria, was identified in the C-terminal part of *A. salmonicida* GyrA. This intragenic region shared sequence identity with various DNA-binding proteins of both prokaryotic and eukaryotic origins.

The bacterial enzyme DNA gyrase (topoisomerase II) catalyzes the introduction of negative supercoils into covalent closed circular DNA and is thereby essential for bacterial viability (reviewed by Reece and Maxwell [26]). In *Escherichia coli*, DNA gyrase is a heterotetramer consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. A lot of attention has been paid to DNA gyrase as a target for the quinolone antimicrobial agents and its role in the development of resistance to these drugs. Despite a wealth of information on the interaction between quinolones and DNA gyrase, the exact mechanisms of action of the quinolones have yet to be identified. It is, however, known that the quinolones interrupt the DNA breakage-reunion step performed by the A subunit in the supercoiling reaction.

Nucleotide changes in both *gyrA* and *gyrB* genes have been shown to lead to amino acid substitutions which make DNA gyrase insusceptible to quinolones. The mutations responsible for high-level quinolone resistance have all been mapped in a small region of the 5′ end of *gyrA*, which has been named the quinolone resistance-determining region of *gyrA* (37). Substitution of the Ser residue at position 83 (Ser-83) in the gyrase A protein has been shown to be the single most important amino acid substitution leading to quinolone resistance in *E. coli* (22).

DNA gyrase *gyrA* genes have been cloned and sequenced from several different bacterial species, among them *E. coli* (31), *Neisseria gonorrhoeae* (4), *Klebsiella pneumoniae* (8), *Pseudomonas aeruginosa* (15), *Staphylococcus aureus* (16), *Helicobacter pylori* (18), *Bacillus subtilis* (19), *Mycobacterium tuberculosis* (32), and *Campylobacter jejuni* (35). Substitutions at positions corresponding to Ser-83 in *E. coli* GyrA have been detected in quinolone-resistant strains of all species mentioned above except *B. subtilis* and *K. pneumoniae*. In addition, several studies have identified GyrA substitutions corresponding to Ser-83 of *E. coli* by using PCR to amplify the quinolone resistance-determining region without prior knowledge of the nucleotide sequence in the corresponding native *gyrA* gene (7, 13, 21, 25, 28–30, 33).

Another primary target for the quinolones, topoisomerase IV, has also been identified (12). The subunits of topoisomerase IV are encoded by the *parC* and *parE* genes, which share high degrees of sequence homology to the *gyrA* and *gyrB* genes, respectively (4, 9, 12). An amino acid substitution in the GyrA analog ParC has been shown to occur at a position corresponding to Ser-83 (*E. coli* coordinates) in low-level quinolone-resistant isolates of *S. aureus* and *N. gonorrhoeae* (4, 9).

The gram-negative, nonmotile rod *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids, is one of the major bacterial pathogens in aquaculture. The quinolones oxolinic acid and flumequine have been widely used to control outbreaks of furunculosis. However, isolates of *A. salmonicida* resistant to quinolones were detected shortly after the introduction of these agents. Recently, we found that substitutions of Ser-83 to Ile and Ala-67 to Gly could take place in the GyrA proteins of quinolone-resistant strains of *A. salmonicida* (21).

Here we report the cloning and the complete nucleotide sequence of the *A. salmonicida gyrA* gene. The results are discussed with regard to existing nucleotide and derived amino acid sequence data for other *gyrA* genes, with emphasis on DNA gyrase as a target for the quinolones and the development of resistance to these drugs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. The *A. salmonicida* subsp. *salmonicida* type strain, strain ATCC 14174, and isolates 2148/89 and 3085/90 from Atlantic salmon with furunculosis were included in the study. The strains were grown on blood agar plates (Blood Agar Base [Difco Laboratories, Detroit, Mich.] containing 5% citrated bovine blood). Isolate 3085/90 was known to be quinolone resistant before the start of the study (20). MAX Efficiency *E. coli* DH5α competent cells, restriction enzymes, T4 DNA ligase, and DNA size markers were from Gibco BRL, Gaithersburg, Md. Plasmid pRM386 carrying the wild-type *E. coli* K-12 *gyrA* gene and plasmid pMEC5 carrying a *gyrA* gene that encodes a gyrase A protein cross-resistant to a variety of quinolones were kindly given to us by Mark Fisher, St. Georges Hospital Medical School, University of London, London, United Kingdom. Plasmids pUC18, pUC18 (*Bam*HI-BAP), and pUC18 (*Hin*dIII-BAP) were purchased from Pharmacia, Uppsala, Sweden. The Megaprime DNA labelling system, including large fragment of

* 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. Electronic mail address: hanneo@extern.uio.no.

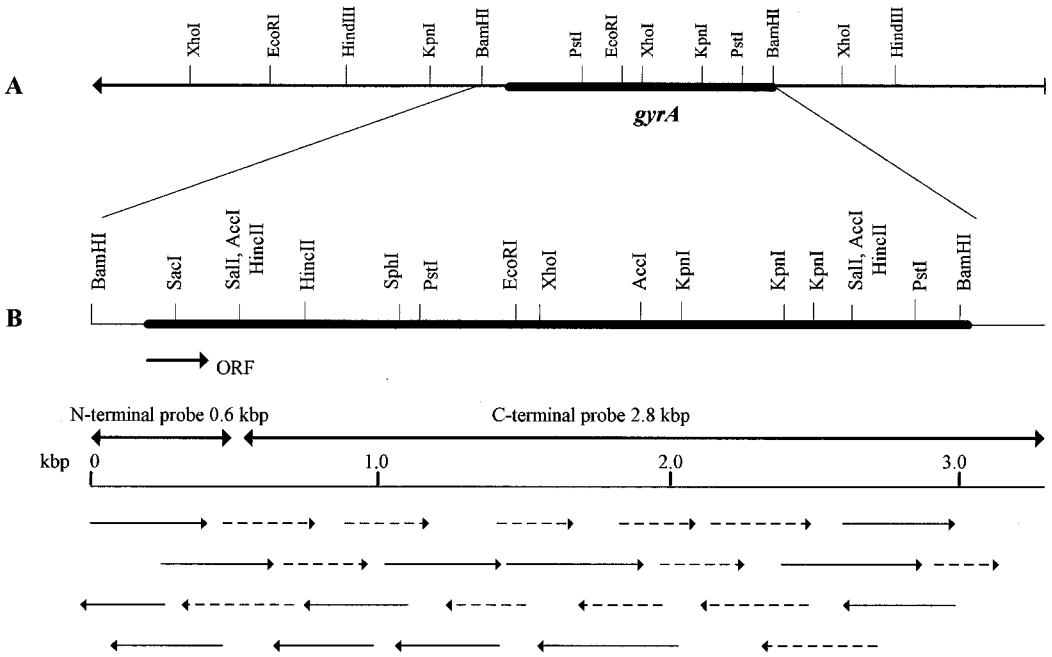

FIG. 1. (A) Low-resolution chromosomal restriction map for *A. salmonicida gyrA* and regions upstream and downstream of the gene. The *gyrA* gene is shown by the thick line. Restriction sites for *Bam*HI, *Hin*dIII, *Xho*I, *Eco*RI, *Kpn*I, and *Pst*I are indicated. (B) Detailed restriction map and sequencing strategies for pASG6 and pASG7. The open reading frame (ORF) of the *A. salmonicida gyrA* gene is shown by the thick line. Continuous arrows indicate the nucleotide sequences obtained by using standard M13/pUC forward and reverse primers. Dashed arrows indicate the sequences obtained from sequencing primers constructed on the basis of existing nucleotide sequence data and used in primer walking. Each arrow represents three to eight independent sequencing reactions.

DNA polymerase I (Klenow), [α-^{32}P]dCTP (10 μCi/μl), and Hybond-N nylon membranes, were from Amersham International, Little Chalfont, United Kingdom. Colony/Plaque screen filters were from NEN/DuPont Research Products. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), calf intestinal alkaline phosphatase, and proteinase K were from Boehringer GmbH, Mannheim, Germany. Ampicillin (Doktacillin) was from Astra Läkemedel, Södertälje, Sweden. SeaKem GTG agarose and SeaPlaque GTG low-melting-point agarose were obtained from FMC BioProducts, Rockland, Maine.

Construction of a chromosomal restriction map for the *A. salmonicida gyrA* gene. Strains ATCC 14174, 3085/90, and 2148/89 were incubated for 48 h at 15°C in Luria-Bertani (LB) broth. Chromosomal DNA was isolated from the strains as described elsewhere (2) and was digested with several different restriction enzymes and combinations of restriction enzymes. Restriction fragments were separated on 0.8% agarose gels, transferred to Hybond-N membranes, and hybridized to α-^{32}P-labelled probes representing the complete *E. coli gyrA* gene: a 600-bp *Sac*I-*Hin*dIII fragment covering the 5′ end of *gyrA* and a 2.8-kbp *Hin*dIII fragment covering the 3′ end of *gyrA*. The two fragments were obtained from plasmids pRM386 and pMEC5 (6). In addition, a 346-bp PCR-generated *A. salmonicida*-specific probe for the 5′ end of *gyrA* was used in the hybridization studies (21). Prehybridizations were done in 2× (1× SSC is 0.015 M NaCl plus 0.015 M sodium citrate) (SSC)–0.1% sodium dodecyl sulfate (SDS)–5× Denhardt solution for 2 h at 65°C. Hybridizations were carried out in 2× SSC–0.1% SDS–5× Denhardt solution with 10% dextran sulfate for 18 to 20 h at 65°C. Washings were done at 65°C in 2× SSC–0.1% SDS, 1× SSC–0.1% SDS, and 0.2× SSC–0.1% SDS, each for two times for 30 min each time; this was followed by one washing in 0.1× SSC–0.1% SDS for 15 min. The membranes were wrapped in plastic and were exposed to Amersham hyperfilm with an intensifying screen at −70°C. The different hybridization patterns obtained with the probes were used in the construction of the chromosomal restriction map.

Cloning of the *A. salmonicida gyrA* gene. Chromosomal DNA from quinolone-susceptible clinical strain 2148/89 was digested with *Bam*HI, *Hin*dIII, or *Xho*I, and DNA fragments were separated in 0.8% low-melting-point agarose. Size markers were run in parallel lanes, excised from the gel, stained with ethidium bromide, and then used to locate restriction fragments in the correct size ranges (on the basis of the chromosomal restriction map) from the unstained parts of the gel. *Bam*HI fragments in the 2- to 4-kbp range, *Hin*dIII fragments in the 5- to 7-kbp range, and *Xho*I fragments in the 4.5- to 6.5- and 1.5- to 3.5-kbp ranges were isolated from the gel with GeneClean (Bio 101, Inc., La Jolla, Calif.) and were ligated into pUC18 digested with *Bam*HI, *Hin*dIII, or *Sal*I (compatible with *Xho*I), respectively. Ligation mixtures were used to transform competent *E. coli* DH5α cells. Transformed DH5α cells were incubated on L-ampicillin plates (100 μg/ml) with X-Gal and IPTG at 37°C overnight. The colonies were replica plated, the cells were lysed, and the DNAs were denatured, neutralized, and finally fixed to the membrane discs by UV illumination. The membrane discs were then hybridized to the ^{32}P-labelled, PCR-amplified *A. salmonicida gyrA* fragment. Colony hybridization was carried out under the same high-stringency conditions described above. Strongly hybridizing colonies were picked from the master plates, and plasmid DNA was isolated from the recombinants by using the Magic Miniprep or Magic Maxiprep DNA Purification System (Promega Corp., Madison, Wis.). One of the recombinant plasmids isolated from the *Bam*HI library, named pASG6, was selected for restriction mapping and sequencing studies.

DNA sequencing. By using the restriction map of pASG6, various subclones were constructed by digestion with restriction enzymes having sites both in the area corresponding to the pUC18 polylinker region and within the 3.0-kbp *Bam*HI fragment; this was followed by religation, transformation, and isolation of recombinants. A second plasmid, pASG7, containing the 3.0-kbp *Bam*HI fragment in pUC18 in an inverted orientation, was also constructed by digesting pASG6 with *Bam*HI; this was followed by ligation. Subclones originating from pASG7 were constructed in a similar manner. The DNA sequences of the subclones were determined for both complementary strands by using T7 DNA polymerase and fluorescent primers corresponding to standard forward and reverse primers for M13/pUC vectors (14). In addition, 12 primers flanking areas which were not covered by sequencing from both ends of the subclones were made on the basis of existing sequence data. These unlabelled primers were used in primer walking, with T7 DNA polymerase and fluorescein-15-*dATP used as internal labels (34). Sequence analysis was performed on an automated fluorescent DNA sequencer (1). Primer synthesis and sequence analysis were carried out at the Biotechnology Centre of Oslo, University of Oslo, Oslo, Norway.

Preliminary nucleotide sequence data for the *Bam*HI fragment revealed that a few nucleotides, corresponding to the 3′ end of the *gyrA* gene, were missing. Because of difficulties with isolating overlapping clones from the *Hin*dIII and *Xho*I libraries, ligation-mediated PCR was chosen as an approach for obtaining the missing nucleotide sequence. The *Xho*I ligation mixture corresponding to *Xho*I fragments in the size range of 1.5 to 3.5 kbp, which represented the 3′ end of the gene and the regions downstream, was subjected to PCR by using one 21-mer primer located near the 3′ end of the *Bam*HI fragment in pASG6 and the M13/pUC forward primer. PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using the GeneAmp PCR reagent kit (Perkin-Elmer Cetus), the *Xho*I ligation mixture with an amount of DNA corresponding to approximately 50 ng of the pUC vector, and 100 pmol of each primer; otherwise, we followed the manufacturer's instructions. Thirty cycles were used, with the following temperature profile; 94°C for 2 min, 56°C for 2 min, and 72°C for 2.5 min. The PCR product of approximately 900 bp that was obtained was isolated from 0.8% low-melting-point agarose by using GeneClean

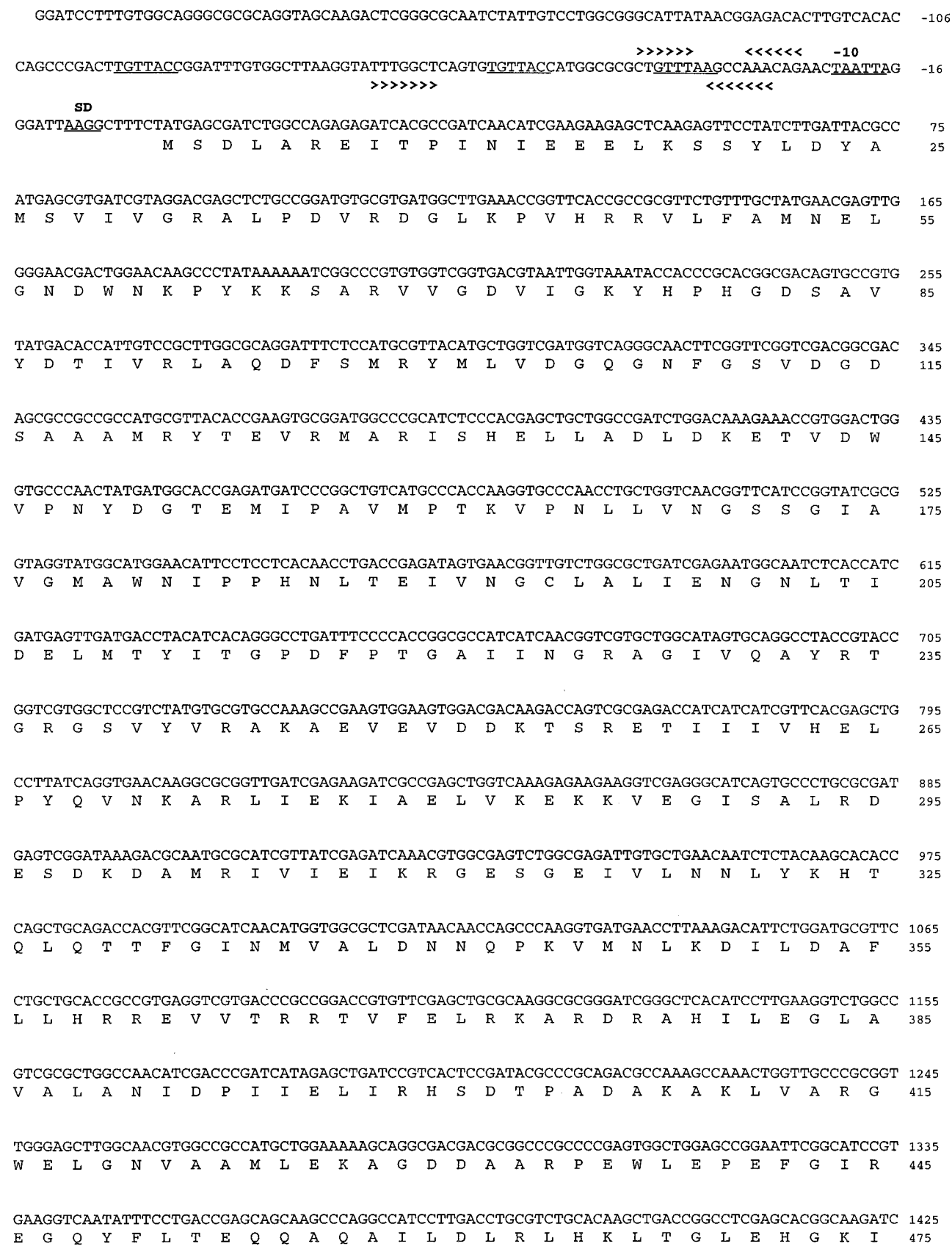

FIG. 2. Nucleotide and deduced amino acid sequences of the *A. salmonicida gyrA* gene from strain 2148/89. Underlining indicates a putative ribosome-binding site (Shine-Dalgarno sequence [SD]), the −10 region, and possible *gyrA* promoter region motifs (see text). The intragenic region between nucleotide positions 2134 and 2277 is also shown. The symbols > and < indicate two inverted repeats in the promoter region and an inverted repeat downstream of the TAA stop codon.

```
CTGGAAGAGTATCAATCTCTGCTGGATCTGATTGCCGAGCTGCTGTTCATCCTGGCAAGCCCGGAACGGTTGATGGAAGTGATCCGCGAT 1515
L  E  E  Y  Q  S  L  L  D  L  I  A  E  L  L  F  I  L  A  S  P  E  R  L  M  E  V  I  R  D   505

GAACTGCTGGCCGTGCGCGAGCAGTATGGCGACGAACGTCCACCCGAGATCAGCGCCTCCAGCGCCGAGATCAACATCGAAGATCTGATC 1605
E  L  L  A  V  R  E  Q  Y  G  D  E  R  P  P  E  I  S  A  S  S  A  E  I  N  I  E  D  L  I   535

ACGCCGGAAGACGTGGTGGTTACCCTGTCTCACCAGGGTTACGTGAAATACCAGCCGATCACGGACTACGAGGCCCAGCGCCGCGGTGGT 1695
T  P  E  D  V  V  V  T  L  S  H  Q  G  Y  V  K  Y  Q  P  I  T  D  Y  E  A  Q  R  R  G  G   565

CGTGGCAAGTCGGCCACCCGGATCAAGGAAGAAAACTTTGTGGAACGTCTGCTGGTGGCCAACACCCATGACACCATTCTGTGCTTCTCC 1785
R  G  K  S  A  T  R  I  K  E  E  N  F  V  E  R  L  L  V  A  N  T  H  D  T  I  L  C  F  S   595

ACCCGTGGCAAGGTCTACTGGCTCAAGGTGTATCAGCTGCCGGAGGCGTCCCGTGGTGCCCGTGGTCGTCCGATCATCAACTTGCTGCCG 1875
T  R  G  K  V  Y  W  L  K  V  Y  Q  L  P  E  A  S  R  G  A  R  G  R  P  I  I  N  L  L  P   625

CTGGAAGAGGGTGAACGTATCACCGCCATCCTGCCGGTCAAAGAGTATGCCGACGACAAGTACGTCTTCTTCGCTACCGCTGATGGTACC 1965
L  E  E  G  E  R  I  T  A  I  L  P  V  K  E  Y  A  D  D  K  Y  V  F  F  A  T  A  D  G  T   655

GTGAAGAAAACCAGCCTGTCTGCGTTCAGCCGTCCGCTCTCCTCCGGTATTCGTGCCATCAACCTCAAAGAGGGTGATGAGCTGATCGGG 2055
V  K  K  T  S  L  S  A  F  S  R  P  L  S  S  G  I  R  A  I  N  L  K  E  G  D  E  L  I  G   685

GTGGACATCACAGATGGCAGCAACGAGATCATGCTGTTCTCCGATGCGGGCAAGGTGGTTCGCTTCAATGAAGGTAGCGCCGCGCCGATG 2145
V  D  I  T  D  G  S  N  E  I  M  L  F  S  D  A  G  K  V  V  R  F  N  E  G  S  A  A  P  M   715

CAGCCGATGCTGATGTCAAGCGATGATGTCGACGGGGATGACGAGAGTGTCATTGACGCTGGCAACGACGATGATGGCAGCGACAACGGC 2235
Q  P  M  L  M  S  S  D  D  V  D  G  D  D  E  S  V  I  D  A  G  N  D  D  D  G  S  D  N  G   745

GAAGGCAGCGAGAGTACCGAAAGCAAGGGTACCTTCAAGGGCGTGCGTCCCATGGGCCGTACCGCCGGTGGGGTACGCGGTATCCGCCTG 2325
E  G  S  E  S  T  E  S  K  G  T  F  K  G  V  R  P  M  G  R  T  A  G  G  V  R  G  I  R  L   775

CTCAACGGCGACAAGGTGGTTTCCCTGATCGTCCCCCGTGGCGAGGGTGCCATCCTGACCGCGACCGAGAACGGTTACGGCAAGGGTACC 2415
L  N  G  D  K  V  V  S  L  I  V  P  R  G  E  G  A  I  L  T  A  T  E  N  G  Y  G  K  G  T   805

GCGCTGACCGAGTACCCGACCAAGAGCCGTGGTACTCAGGGCGTTCGTTCCATCAAGGTGGACGAGGACGGCAAGGTTTCGATCGACCAG 2505
A  L  T  E  Y  P  T  K  S  R  G  T  Q  G  V  R  S  I  K  V  D  E  D  G  K  V  S  I  D  Q   835

GTCGACGATACCGACCAGATCATGCTTATCACCAATGGCGGCACCCTGGTACGTACTCGTGTCTCCGAAGTGAGCATCATAGGCCGCAAC 2595
V  D  D  T  D  Q  I  M  L  I  T  N  G  G  T  L  V  R  T  R  V  S  E  V  S  I  I  G  R  N   865

ACCGGCGGTGTTCGTCTGATCCGTACCGGTGAAGATGAAACCGTGGTTGGCCTGCAGCGCATTGCCGAGAGCTATGAGGAAGAGAACGAC 2685
T  G  G  V  R  L  I  R  T  G  E  D  E  T  V  V  G  L  Q  R  I  A  E  S  Y  E  E  E  N  D   895

GTCATGGCCATTGACGGTGAGGTGTCTGAAGGCACCGATACTGCGCCAGACGCCGGCTCTGCCGCCGCRGATCCCGAAGAATAAGTTTTC 2775
V  M  A  I  D  G  E  V  S  E  G  T  D  T  A  P  D  A  G  S  A  A  A  D  P  E  E  -*-        922

TTTTCACAGCTTCAGCTTTGGGGTTCTAGTCATAGAACGTACT
                  >>>>>>     <<<<<<
```

FIG. 2—*Continued.*

(Bio 101). By using the TA cloning kit (Invitrogen Corp., San Diego, Calif.), the PCR product was ligated to the pCRII plasmid vector and was used to transform competent *E. coli* (INVαF′) cells. The nucleotide sequence of the cloned PCR product was determined by the dideoxy chain termination method with T7 DNA polymerase and fluorescent M13/pUC forward and reverse primers as described above.

Sequence analysis. Nucleotide and derived amino acid sequences were analyzed by using the GCG Sequence Analysis Software Package, version 8, from Genetics Computer Group, Madison, Wis. Protein and nucleotide sequence comparisons were carried out by searching the GenBank/EMBL databases.

Nucleotide sequence accession number. The nucleotide sequence has been assigned accession number L47978 in the GenBank/EMBL database.

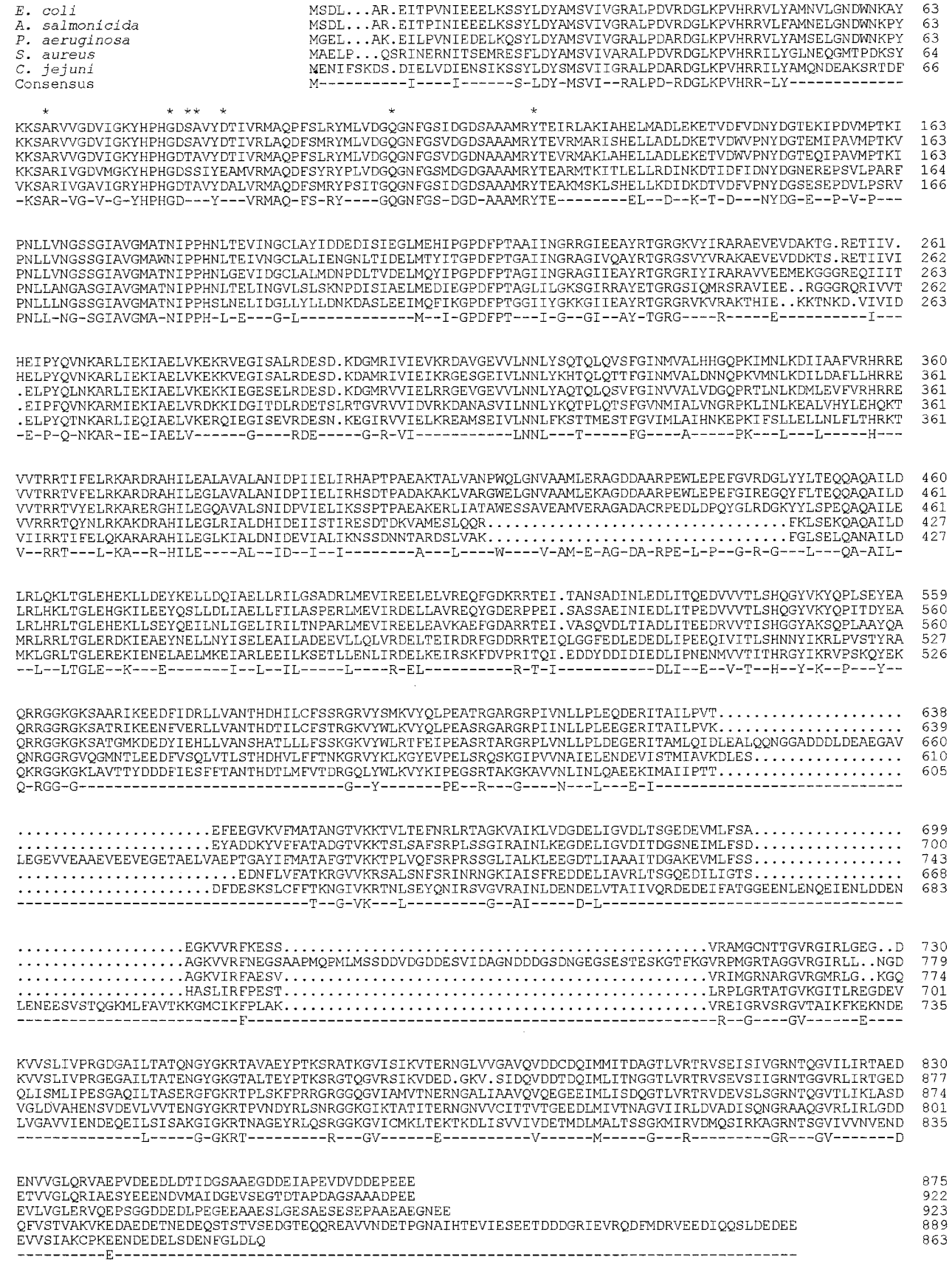

FIG. 3. Comparison of deduced amino acid sequences of the gyrase A subunits from *E. coli* (31), *A. salmonicida* (Fig. 2), *P. aeruginosa* (15), *S. aureus* (16), and *C. jejuni* (35). Identical amino acids in all five GyrA protein sequences are listed in the lower line (consensus sequence). Asterisks indicate the active Tyr-122 and amino acids whose substitution will confer quinolone resistance in *E. coli* (26).

RESULTS

Cloning and restriction mapping of the *A. salmonicida gyrA* gene. Figure 1A shows the low-resolution chromosomal restriction map for the *A. salmonicida gyrA* gene and the regions upstream and downstream of the gene. The heterologous *gyrA* probes originating from *E. coli* produced identical hybridization patterns in strains ATCC 14174, 2148/89, and 3085/90, i.e., no restriction site polymorphism was detected. The results obtained with the PCR-generated *A. salmonicida* probe were in agreement with those obtained with the 600-bp *Sac*I-*Hin*dIII probe from *E. coli* (data not shown). To be certain that the complete *gyrA* sequence would be represented on a single restriction fragment, both the 600-bp *Sac*I-*Hin*dIII and the 2.8-kbp *Hin*dIII *gyrA* probes needed to hybridize to the same fragment. This seemed to be the case for a *Bam*HI fragment of approximately 3.0 kbp.

When *Bam*HI-digested chromosomal DNA in the 2- to 4-kbp size range was ligated into pUC18 and was used to transform *E. coli* DH5α, the result was a size-selected plasmid library which contained approximately 500 white, ampicillin-resistant colonies. Four independent positive clones were obtained from this library when the ^{32}P-labelled PCR-generated probe for *A. salmonicida gyrA* was used. The positive clones all contained plasmids with identical 3.0-kbp *Bam*HI inserts which hybridized strongly to all of the *gyrA* probes. No positive clones could be detected from the *Hin*dIII or *Xho*I libraries by using the *E. coli gyrA* probes, the *A. salmonicida* PCR probe, or the entire *Bam*HI fragment from pASG6 as a probe. Figure 1B shows the detailed restriction map for the pASG6 clone.

Nucleotide sequencing of the *A. salmonicida gyrA* gene. The sequencing strategy for the 3.0-kbp *Bam*HI fragment is shown in Fig. 1B. Subclones originating from pASG6 or pASG7 were constructed by digestion with *Sal*I, *Acc*I, *Hin*cII, *Pst*I, *Sph*I, *Eco*RI, and *Kpn*I; this was followed by religation. The ligation-mediated PCR and sequencing of the 900-bp PCR product revealed six additional nucleotides before the stop codon.

Sequence analysis of the *A. salmonicida gyrA* gene. Starting at 194 nucleotides downstream of the 5′ end of the *Bam*HI fragment in pASG6, an open reading frame of 2,766 nucleotides, coding for a polypeptide of 922 amino acids, was identified. The last six nucleotides of the open reading frame were identified by sequencing the PCR product obtained in the ligation-mediated PCR. Figure 2 shows the nucleotide and the derived amino acid sequences for the *A. salmonicida gyrA* gene. The calculated molecular mass of *A. salmonicida* GyrA was 101.1 kDa.

Upstream of the ATG translation initiation codon, a putative ribosome-binding site (Shine-Dalgarno sequence) (AAGG) was revealed. Further upstream of the Shine-Dalgarno sequence, the sequence TAATTA, which is similar to the −10 *E. coli* promoter consensus sequence TATAAT, was observed. A −35 region resembling the *E. coli* consensus sequence TTGACA was not found. However, spaced by 18 nucleotides to the −10 region, the upstream sequence GTT TAAG was present (Fig. 2). This sequence resembles the conserved sequence GTTTACC found in the *E. coli*, *K. pneumoniae*, and *B. subtilis gyrA* promoter regions and the sequence GTTTGCC found in the *P. aeruginosa gyrA* promoter region (8, 15, 19, 31). Located between 51 and 57 nucleotides upstream of the ATG translation initiation codon, the sequence TGTTACC was identified. This sequence was present once more between 89 and 95 nucleotides upstream of the initiation codon. Such repetitive sequences have previously been identified in the *E. coli gyrA* promoter region (17, 31). The relaxation-stimulated transcription sequence (CTGTGTTATA ATTTGCGACC) identified in the *E. coli* and *K. pneumoniae gyrA* promoters (8, 17), which includes the nucleotides surrounding the −10 region (underlined), was not found.

Two cruciform structures may theoretically form from inverted sequences in the promoter region of the *A. salmonicida gyrA* gene (Fig. 2). First, the 6-bp sequence AAACAG located just upstream of the −10 region could adopt a cruciform structure with the inverted sequence CTGTTT, forming an unpaired loop of five nucleotides. Second, a similar structure may form from the inverted 7-bp sequence AGCCAAA, which partly overlaps the 6-bp sequence mentioned above, and the sequence TTTGGCT. This inverted repeat could form a loop of 27 nucleotides. Inverted repeats have been identified also in other *gyrA* promoters (8, 15) and may be an indication of supercoiling-dependent regulation of *gyrA* transcription (11). Located 29 nucleotides downstream of the translational stop codon TAA was a 6-bp inverted repeat spaced by 4 bp, which may act as a transcription termination signal.

Starting at nucleotide position 2134 in the *A. salmonicida gyrA* gene was an intragenic region of 144 bp, corresponding to 48 amino acids, which is not present in *E. coli* or the *gyrA* sequence of any other organism. Asp (D) residues were predominant in the deduced amino acid sequence of this region. While the percentage of Asp residues in *A. salmonicida* GyrA as a whole was 7.5%, the corresponding figure for this stretch was 20.8%. In addition, the Asp residues were clustered in the central region of the stretch. Intragenic stretches, corresponding to 35, 43, and 45 amino acids, have been found also in the 3′ ends of *gyrA* genes from *C. jejuni*, *P. aeruginosa*, and *H. pylori*, respectively (15, 18, 35). Glu (E) residues are found to be predominant in the derived amino acid sequences of the intragenic regions of *P. aeruginosa* and *C. jejuni* GyrA, making up 27.9 and 25.7% of the total number of amino acids, respectively. When the internal GyrA stretches of *A. salmonicida*, *P. aeruginosa*, *C. jejuni*, and *H. pylori* were compared, they shared a relatively low degree of overall sequence homology. By searching protein databases, the deduced amino acid sequence of the intragenic region of *A. salmonicida gyrA* was shown to share some degree of identity with DNA-binding proteins of diverse origin. Common Asp residues were in most cases solely responsible for this observed amino acid identity.

A region of 34 amino acids, starting at position 415 in the *A. salmonicida* gyrase A protein, bears a high degree of identity to corresponding sequences in *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (8, 15, 31). In *C. jejuni*, *H. pylori*, and gram-positive bacteria, this stretch is absent (16, 18, 19, 35).

When the codon usage for the *A. salmonicida gyrA* gene was compared with those for other *A. salmonicida* genes that have been sequenced, a marked similarity with the serine protease gene (*aspA*) data was observed (36). The G+C content of the *A. salmonicida gyrA* gene was 58%, which is in perfect agreement with the overall G+C content of this bacterium (57 to 59%) (24).

Figure 3 compares GyrA amino acid sequences for *A. salmonicida*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. jejuni* (15, 16, 31, 35). Sequence alignment showed that the catalytic Tyr-122, which is involved in DNA breakage and reunion by gyrase, was present in *A. salmonicida* GyrA, as in *E. coli* and other bacterial species (10). In addition, residues whose substitution confers quinolone resistance in *E. coli*, notably, Ser-83, Ala-67, Gly-81, Asp-87, Ala-84, and Gln-106, were conserved in *A. salmonicida* GyrA at identical coordinates (31). Figure 4 compares the derived amino acid sequence of the quinolone resistance-determining region of the *A. salmonicida* gyrase A protein with those of the corresponding regions of 14 other GyrA sequences (4, 5, 8, 13, 15, 16, 18, 19, 20, 29, 31–33, 35).

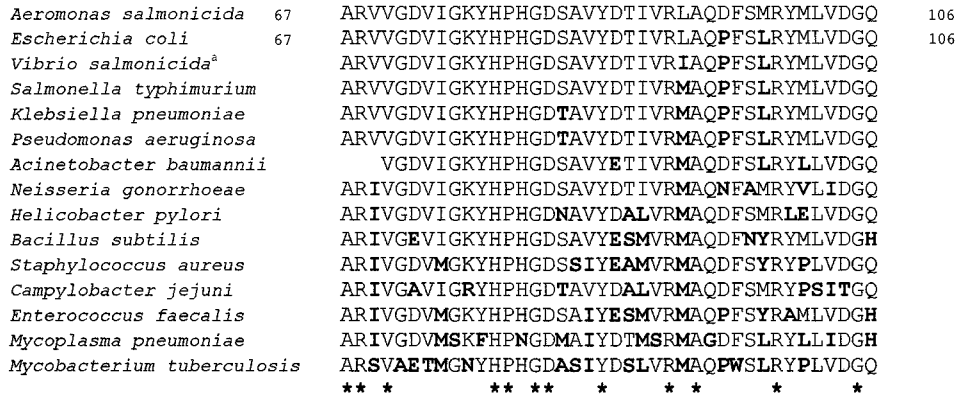

FIG. 4. Comparison of the deduced amino acid sequences of the quinolone resistance-determining regions of 15 *gyrA* genes. Amino acids which differ from those in the *A. salmonicida* GyrA sequence are shown in boldface type. The various quinolone resistance-determining regions are ranged with respect to their amino acid identities with the *A. salmonicida* quinolone resistance-determining region. Perfectly conserved positions are indicated by asterisks. The *E. coli* sequence represents amino acids 67 to 106 of the gyrase A protein (31). a, unpublished data (GenBank accession number L42449 [20]).

The *A. salmonicida* gyrase A protein exhibited 68.9, 68.5, 58.4, 54.7, 43.0, 40.2, 36.4, 34.3, and 33.1% overall amino acid sequence identity with the corresponding sequences of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *N. gonorrhoeae*, *B. subtilis*, *H. pylori*, *C. jejuni*, *S. aureus*, and *M. tuberculosis*, respectively (4, 8, 15, 16, 18, 19, 31, 32, 35). The highest degree of homology was found in the N-terminal end, and homology became progressively less toward the C terminus. Alignment of *A. salmonicida* GyrA to *E. coli* topoisomerase IV subunit ParC (12) showed 45.5% sequence identity in an overlap of 420 amino acids.

DISCUSSION

In *E. coli*, the N-terminal region of GyrA forms a 64-kDa trypsin-resistant domain that includes the catalytic Tyr-122 and residues responsible for susceptibility to quinolones (26). When aligning the GyrA sequences of *A. salmonicida*, *E. coli*, *Vibrio salmonicida*, *Salmonella typhimurium*, and *N. gonorrhoeae*, these residues match perfectly (7, 20, 29, 31). In a previous study, we found that substitutions of Ser-83 to Ile and Ala-67 to Gly took place in quinolone-resistant isolates of *A. salmonicida* (21). The Ser-83 substitution seems to be the far most important, since eight of nine quinolone-resistant isolates investigated so far exclusively have the Ser-83 to Ile substitution (20, 21). In *E. coli*, substitution of Ser-83 is also the most frequently encountered change in GyrA associated with high-level resistance to quinolones (22, 37).

Only the *P. aeruginosa gyrA* gene has been shown to be larger than the *A. salmonicida gyrA* gene, containing three more nucleotides (15). The large size is mainly due to the intragenic stretch of 144 nucleotides in the 3′ end of the gene. The finding that this stretch shares sequence homology with various DNA-binding proteins is consistent with the fact that the tryptic 33-kDa C terminus of *E. coli* DNA gyrase A has been identified as a DNA-binding protein essential for gyrase-DNA complex stability (27). An increasing accumulation of data shows that the presence of short stretches of unique DNA are common features of *gyrA* genes. Similar DNA regions with no homology to other *gyrA* genes have been identified in *P. aeruginosa*, *C. jejuni*, and *H. pylori* (15, 18, 35). Further studies are needed to determine if such stretches have any biological significance.

Analysis of the nucleotide sequence upstream of the *A. salmonicida gyrA* gene does not reveal if the *gyrA* and *gyrB* genes are contiguous. Noncontiguous gyrase genes are found to be common in gram-negative bacteria (3, 8, 15, 23, 35). On the other hand, contiguous gyrase genes (*gyrB-gyrA*) have been identified in *M. tuberculosis*, *Mycoplasma pneumoniae*, and other gram-positive organisms (5, 16, 19, 32). The special construction of *gyrA* promoters, such as the absence of a consensus −35 region and the presence of inverted repeats that may form hairpin structures, is believed to be responsible for supercoiling-dependent transcription (11, 17).

We find it noteworthy that we were not able to isolate the entire *gyrA* gene and its surroundings on the 6.0-kbp *Hin*dIII fragment or the 2.0-kbp *Xho*I fragment representing the C-terminal end of the protein and areas downstream. Similar problems were observed in a study in which the *C. jejuni gyrA* gene was cloned (35). The authors concluded that cloning and/or expression of the *C. jejuni* GyrA C terminus was lethal to *E. coli* cells. In the present study, no growth inhibition of *E. coli* cells was observed when they were transformed with plasmid pASG6 or subclones corresponding to the C-terminal end of GyrA. It seems unlikely that the two "missing" amino acids at the C terminus of the corresponding *Bam*HI fragment in pASG6 would be of any significance in this respect. It could be tempting to speculate if there may be sequences downstream of the *A. salmonicida gyrA* gene that caused the difficulties in cloning the *Xho*I and *Hin*dIII fragments in *E. coli*. Also, the observation that *E. coli* cells transformed with the ligation-mediated PCR clone showed relatively slow growth could support this hypothesis.

The sequence analysis presented here may facilitate further studies of the *A. salmonicida gyrA* gene and its gene product. Possible quinolone-resistant *gyrA* mutations outside the quinolone resistance-determining region can now be easily detected. Additionally, the high degree of sequence similarity between *gyrA* and *parC* genes should make it possible to analyze *A. salmonicida parC* in detail. The chromosomal restriction map of the regions surrounding *A. salmonicida gyrA* may also be a basis for the further analysis of the areas upstream and downstream of the gene.

ACKNOWLEDGMENTS

This work was supported by a grant from the Norwegian Fisheries Research Council.

We are grateful to Mark Fisher for providing plasmids pRM386 and pMEC5. We also thank the National Veterinary Institute, Oslo, for supplying bacterial strains and the Biotechnology Centre of Oslo for performing nucleotide sequence analysis and primer synthesis.

REFERENCES

1. **Ansorge, W., B. S. Sproat, J. Stegeman, C. Schwager, and M. Zenke.** 1987. Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis. Nucleic Acids Res. **15:**4593–4602.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology, vol. 1, suppl. 9, p. 2.4.1. John Wiley & Sons, Inc., New York.
3. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of the *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44:**1–56.
4. **Belland, R. J., S. G. Morrison, C. Ison, and W. M. Huang.** 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Mol. Microbiol. **14:**371–380.
5. **Colman, S. D., P.-C. Hu, and K. F. Bott.** 1990. *Mycoplasma pneumoniae* DNA gyrase genes. Mol. Microbiol. **4:**1129–1134.
6. **Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fisher.** 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrob. Agents Chemother. **33:**886–894.
7. **Deguchi, T., M. Yasuda, M. Asano, K. Tada, H. Iwata, H. Komeda, T. Ezaki, I. Saito, and Y. Kawada.** 1995. DNA gyrase mutations in quinolone-resistant clinical isolates of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **39:**561–563.
8. **Dimri, G. P., and H. K. Das.** 1990. Cloning and sequence analysis of *gyrA* gene of *Klebsiella pneumoniae*. Nucleic Acids Res. **18:**151–156.
9. **Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13:**641–653.
10. **Horowitz, D. S., and J. C. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **262:**5339–5344.
11. **Horowitz, M. S. Z., and L. A. Loeb.** 1988. An *E. coli* promoter that regulates transcription by DNA superhelix-induced cruciform extrusion. Science **241:**703–705.
12. **Kato, J., H. Suzuki, and H. Ikeda.** 1992. Purification and characterization of DNA topoisomerase IV in *Escherichia coli*. J. Biol. Chem. **267:**25676–25684.
13. **Korten, V., W. M. Huang, and B. E. Murray.** 1994. Analysis by PCR and direct DNA sequencing of *gyrA* mutations associated with fluoroquinolone resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **38:**2091–2094.
14. **Kristensen, T., H. Voss, C. Schwager, J. Stegeman, B. Sproat, and W. Ansorge.** 1988. T7 polymerase in automated dideoxy sequencing. Nucleic Acids Res. **16:**3487–3496.
15. **Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan.** 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **38:**1944–1952.
16. **Margerrison, E. E. C., R. Hopewell, and L. M. Fisher.** 1992. Nucleotide sequence of the *Staphylococcus aureus gyrB-gyrA* locus encoding the DNA gyrase A and B proteins. J. Bacteriol. **174:**1596–1603.
17. **Menzel, R., and M. Gellert.** 1987. Modulation of transcription by DNA supercoiling: a deletion analysis of the *Escherichia coli gyrA* and *gyrB* promoters. Proc. Natl. Acad. Sci. USA **84:**4185–4189.
18. **Moore, R., B. Beckthold, S. Wong, A. Kureishi, and L. E. Bryan.** 1995. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. Antimicrob. Agents Chemother. **39:**107–111.
19. **Moriya, S., N. Ogasawara, and H. Yoshikawa.** 1985. Structure and function of the region of the replication origin of the *Bacillus subtilis* chromosome. III. Nucleotide sequence of some 10,000 basepairs in the origin region. Nucleic Acids Res. **13:**2251–2262.
20. **Oppegaard, H.** 1995. Unpublished data.
21. **Oppegaard, H., and H. Sørum.** 1994. *gyrA* mutations in quinolone-resistant isolates of the fish pathogen *Aeromonas salmonicida*. Antimicrob. Agents Chemother. **38:**2460–2464.
22. **Oram, M., and L. M. Fisher.** 1991. 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrob. Agents Chemother. **35:**387–389.
23. **Parales, R. E., and C. S. Harwood.** 1990. Nucleotide sequence of the *gyrB* gene of *Pseudomonas putida*. Nucleic Acids Res. **18:**5880.
24. **Popoff, M.** 1984. Genus *Aeromonas*, p. 547–548. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
25. **Rahman, M., G. Mauff, J. Levy, M. Couturier, G. Pulverer, N. Glasdorff, and J. P. Butzler.** 1994. Detection of 4-quinolone resistance mutation in *gyrA* gene of *Shigella dysenteriae* type 1 by PCR. Antimicrob. Agents Chemother. **38:**2488–2491.
26. **Reece, R. J., and A. Maxwell.** 1991. DNA gyrase: structure and function. Crit. Rev. Biochem. Mol. Biol. **26:**335–375.
27. **Reece, R. J., and A. Maxwell.** 1991. The C-terminal domain of the *Escherichia coli* DNA gyrase A subunit is a DNA-binding protein. Nucleic Acids Res. **19:**1399–1405.
28. **Revel, V., E. Cambau, V. Jarlier, and W. Sougakoff.** 1994. Characterization of mutations in *Mycobacterium smegmatis* involved in resistance to fluoroquinolones. Antimicrob. Agents Chemother. **38:**1991–1996.
29. **Reyna, F., M. Huesca, V. Gonzàlez, and L. Y. Fuchs.** 1995. *Salmonella typhimurium gyrA* mutations associated with fluoroquinolone resistance. Antimicrob. Agents Chemother. **39:**1621–1623.
30. **Sreedharan, S., L. R. Peterson, and L. M. Fisher.** 1991. Ciprofloxacin resistance in coagulase-positive and -negative staphylococci: role of mutations at serine 84 in the DNA gyrase A protein of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **35:**2151–2154.
31. **Swanberg, S. L., and J. C. Wang.** 1987. Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. J. Mol. Biol. **197:**729–736.
32. **Takiff, H., L. Salazar, C. Guerrero, W. Philipp, W. M. Huang, B. Kreiswirth, S. T. Cole, W. R. Jacobs, Jr., and A. Telenti.** 1994. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob. Agents Chemother. **38:**773–780.
33. **Vila, J., J. Ruiz, P. Goni, A. Marcos, and T. J. De Anta.** 1995. Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **39:**1201–1203.
34. **Voss, H., S. Wiemann, U. Wirkner, C. Schwager, J. Zimmermann, J. Stegemann, H. Erfle, N. A. Hewitt, T. Rupp, and W. Ansorge.** 1992. Automated DNA sequencing system resolving 1,000 bases with fluorescein-15-*dATP as internal label. Methods Mol. Cell. Biol. **3:**153–155.
35. **Wang, Y., W. M. Huang, and D. E. Taylor.** 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob. Agents Chemother. **37:**457–463.
36. **Whitby, P. W., M. Landon, and G. Coleman.** 1992. The cloning and nucleotide sequence of the serine protease gene (*aspA*) of *Aeromonas salmonicida* ssp. *salmonicida*. FEMS Microbiol. Lett. **99:**65–72.
37. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34:**1271–1272.