

Molecular Cloning of the *gyrA* and *gyrB* Genes of *Bacteroides fragilis* Encoding DNA Gyrase

YOSHIKUNI ONODERA* AND KENICHI SATO

New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd.,
Edogawa-ku, Tokyo 134-8630, Japan

Received 12 January 1999/Returned for modification 19 April 1999/Accepted 16 July 1999

The genes encoding the DNA gyrase A and B subunits of *Bacteroides fragilis* were cloned and sequenced. The *gyrA* and *gyrB* genes code for proteins of 845 and 653 amino acids, respectively. These proteins were expressed in *Escherichia coli*, and the combination of GyrA and GyrB exhibited ATP-dependent supercoiling activity. To analyze the role of DNA gyrase in quinolone resistance of *B. fragilis*, we isolated mutant strains by stepwise selection for resistance to increasing concentrations of levofloxacin. We analyzed the resistant mutants and showed that Ser-82 of GyrA, equivalent to resistance hot spot Ser-83 of GyrA in *E. coli*, was in each case replaced with Phe. These results suggest that DNA gyrase is an important target for quinolones in *B. fragilis*.

Bacteroides fragilis is an obligate anaerobic bacterium composing intestinal flora and is the major pathogen in intra-abdominal infection following a perforated appendix or surgery on the gastrointestinal tract (11). *B. fragilis* often presents a serious problem in therapy, as it is intrinsically resistant to many antibiotics, including most of the penicillins, cephalosporins, and quinolones (9). β -Lactam resistance is usually explained by the combination of low permeability of the outer membrane (34) and the presence of highly active β -lactamases of the Bush 2e and 3 classes (4). However, the molecular basis of quinolone resistance remains poorly defined (20, 27).

Studies with *Escherichia coli* have shown that quinolones act by inhibiting the activity of DNA gyrase, which catalyzes ATP-dependent DNA supercoiling (3, 7, 8, 21). Moreover, it was revealed that mutations in the GyrA quinolone resistance-determining region (QRDR), located between amino acid residues 67 and 106 (5, 10, 23, 31), were related to quinolone resistance. Recently, the type II enzyme topoisomerase IV, essential for chromosome segregation, was shown to be another target of quinolones (14). In gram-negative bacteria, such as *E. coli* and *Neisseria gonorrhoeae*, strains with low-level resistance contained *gyrA* mutations whereas those with higher levels of resistance had mutations in both *gyrA* and *parC* (1, 13, 15, 16). On the other hand, in gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, mutations in *parC* (*grlA*) conferred low-level resistance and preceded those in *gyrA* (6, 24, 25). Moreover, mutations in the B subunits of DNA gyrase and topoisomerase IV (2, 30, 33), and the appearance of efflux pumps, were shown to be related to quinolone resistance (17, 18, 22, 26, 32).

As a first step, we report here the cloning and characterization of *gyrA* and *gyrB* of *B. fragilis* and examine the role of DNA gyrase in the stepwise acquisition of levofloxacin resistance in vitro. This study complements the genetic characterization of the type II DNA topoisomerases of *B. fragilis* and reveals the molecular basis of quinolone resistance.

MATERIALS AND METHODS

Antibacterial agents. All quinolones used in this study were synthesized at Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

Bacterial strains, plasmids, and DNA manipulations. *B. fragilis* ATCC 25285 was grown in general anaerobic GAM broth (Nissui, Tokyo, Japan) at 37°C in an anaerobic box. To construct a genomic library, chromosomal DNA was extracted from *B. fragilis* ATCC 25285. The *E. coli* strains used for plasmid transformation were MC1061 and DH5 α (19). Plasmid pUC18 was used to construct libraries and to subclone DNA inserts. Plasmid pMAL-c2 (New England Biolabs) was used to construct plasmids for overexpression of the GyrA and GyrB proteins of *B. fragilis* in *E. coli*. Manipulations of DNA, including plasmid extraction, electrophoresis, Southern hybridization, and colony hybridization, were carried out by standard methods (19). For Southern and colony hybridization, DNA was radiolabeled with 50 μ Ci of [α -³²P]dCTP (300 Ci/mmol), using the Multiprime DNA labeling kit (Pharmacia-Amersham).

Determination of MICs. The MICs were determined by a standard agar dilution method with GAM agar (Eiken Chemical Co., Ltd., Tokyo, Japan). Drug-containing agar plates were inoculated with one loopful (5 μ l) of an inoculum corresponding to about 10⁴ CFU per spot and were incubated for 18 h at 37°C. The MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

DNA sequence analysis. DNA fragments were subcloned into plasmid pUC18 and sequenced by the chain termination method with a fluorescence sequencer (Pharmacia-Amersham). Amplification of the QRDR of the *gyrA* and *gyrB* genes from *B. fragilis* ATCC 25285 and its levofloxacin-resistant mutants was carried out by PCR with genomic DNA as a template. For the QRDR of *gyrA*, the forward primer was Pr-BFGA03, 5'-ATGCTTGAACAAGACAGAATTATAAG-3' (*gyrA* positions 1 to 27) and the reverse primer was Pr-BFGA02, 5'-GACTGTGCGCTTACAGAACCG-3' (324 to 346). The primers for the QRDR of the *gyrB* gene were Pr-BFGB03, 5'-GACCCGACAGAAGTGTGAGTTATTC-3' (*gyrB* positions 1279 to 1303) and Pr-BFGB04, 5'-TTTCAAGCGCTTTG TGATACATGGC-3' (1405 to 1429). The PCR conditions were 25 cycles of 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min. The 346-bp *gyrA* and 151-bp *gyrB* PCR products were cloned into pCRII (Invitrogen) for DNA sequence analysis.

Protein expression. GyrA and GyrB of DNA gyrase were expressed separately as fusion proteins with maltose-binding protein (MBP) by using the pMAL-c2 expression vector. Each gene was amplified by PCR and inserted into the expression vector. In the reverse primers, a *Hind*III or *Pst*I site was introduced for cloning purposes. For *gyrA*, the forward primer was Pr-BFGA03, 5'-ATGCTTGAACAAGACAGAATTATAAG-3' (*gyrA* positions 1 to 27), and the reverse primer was Pr-BFGA04, 5'-AGTTGTTAAGCTTTTGCAGAGTCAGG-3' (2777 to 2802; *Hind*III). The primers for the *gyrB* gene were Pr-BFGB01, 5'-ATGAGCGAAGAACAGAAATCCCCACC-3' (*gyrB* positions 1 to 24), and Pr-BFGB02, 5'-ATTTCTGCGAGCGCCGCGCTTC-3' (2001 to 2024; *Pst*I). PCR was carried out on genomic DNA from strain ATCC 25285 as follows: 20 cycles of 94°C for 0.5 min, 65°C for 0.5 min, and 72°C for 2 min. The PCR products were digested with restriction enzymes, ligated into expression vectors, and transformed into *E. coli* MC1061. Protein production was induced with isopropyl- β -D-thiogalactopyranoside (IPTG), and each protein was purified as described previously (29).

DNA gyrase assay. The supercoiling activity of DNA gyrase, the conversion of relaxed pBR322 DNA to the supercoiled form, was detected by the method described previously (28).

* Corresponding author. Mailing address: New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 16-13 Kitakasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan. Phone: 81-3-3680-0151, ext. 5812. Fax: 81-3-5695-8344. E-mail: onode90j@daiichipharm.co.jp.

1 tgggtgtcacagacctaaagaataagggtagggcactcttacaaaaagcaaaaaactttcttctgtaaagttttcgtatatcaaggaaaa
91 gtagtactcttagcgtggtttttgataaacccgcaaatgtataataatcttttaaatgcttgaacaagacagaattataaagattaa
M L E Q D R I I K I N
181 catcaggaggaaatgaagtcacgtactgactactccatgctcggtcatcgtttcccgctccctcccgatgtagagatggatttaa
I E E E M K S S Y I D Y S M S V I V S R A L P D V R D G F X
271 gccgttcaccgcagaattctctacggaatgatggaactgggaaatcgtcagacaaaccctataagaatcagccagaatcgtaggtga
P V H R R I L Y G M M E L G N T S D K P Y K K S A R I T V G G E
361 agtacttgtaagtatcacccgcaogagactctctgtatattttgcgatggtacgtatggtcaggaatgggcaatgctgatcogct
V L G K Y H P H G D S S V Y F A M V R M A Q E W A M R Y P L
451 ggtagcgggcaaggtaacttcggttctgtagacggcgacagctcctgctgccatgcttacactgaagcagctcgaacaaattaggtga
V D G Q G N F G S V D G D S P A A M R Y T E A R L N K L G E
541 agaaatgatgcaggacctctacaaagagactgtagatttcgaaacctactcgataatcgtgatggaacccaaagtgatgcgcagacg
E M M Q D L Y K E T V D F E P N F D N T L M E P K V M P T R
631 tattccgaatttgctggtaaacggtgctccggattgtgtaggtatggcaaccaatcgcgccccaatactgtctgaagtcacgca
I P N L L V N G A S G I A V G M A T N M P P H N L S E V I D
721 tgctgcgaagcatatcttgacaataaagatgtgacogtagaggaactgatggaatgatgaaagcggcgaacttccctacagaggata
A C E A Y L D N K D V T V E E L M E Y V K A P D F A P R I T V G G Y
811 tataatggcataagcggcgtacgtgaagcctatctacgggacggcagcgtggttatgcgcgcaagcagaatcgaatccggaca
I Y G I S G V R E A Y L T G R G R V V M R A K A E I E S G Q
901 gacacatgataagatcgtcgttacagagattccctacacgtgaataaggcagaatgattaagcaatgctgatctgtcaatgaaaa
T H D K I V V T E I P Y N V N K A E L I K A I A D L V N E K
991 aagaatagaaggcatatcaaatgccaacgacgagtcggaccgtgaaggtatgcgcacogttattgatatacaacgggatgcaaatgcaag
R I E G I S N A N D E S D R E G M R I V I D I K R D A N A S
1081 ttagtgctgaacaagctctataaaatgacagccttcgacagctcattcgggtgataaaacgctgcaactggtcaacggcgcctcaaat
V V L N K L Y K M T A L Q T S F G V N N V A L V N G R P K M
1171 gctgaatttaccgacttgattgtttacttcgtagaacatagacacogtggtaattcgtcgtactcaatttgacctcgtgaaggccaa
L N L R D L I V Y F V E H R H D V V I R R T Q F D L R K A K
1261 agaacgtgcacacatcttggaaggtctgattatcgttcggataatattgacgaagtaattcgtatcatccgcccgcacaaacacccaaa
E R A H I L E G L I I A S D N I D E V I R I I R A A K T P N
1351 cgatgcaactcctcggactgatggaacgcttcaacctgagcgaatcaggcaccgcccactgtaaatgcgcctgcgccaatcaacagg
D A I S G L M E R F N L S E I Q A R A I V E M R L R Q L T G
1441 tctgatcaagatcagctccatcgtgaatcagagggttatgaagcagatagcatatttgaaagtatcctggccgatgatgaagtatg
L M Q D Q L H A E Y E E V M K Q I A Y L E S I L A D D E V C
1531 ccgtaaaatcaaacgcaaatgctgggaagtaagagctaaatattggtgacgaacccggtctgtaaatcgtttattcatcagaagaatt
R K V I K D E L L E V R A K Y G D E R R S E I V Y S S E E F
1621 caatccggaagacttttatgcggatgatcagatgattatccacctctcacacatgggatatacaaacgtacaccattgacagaattccg
N P E D F Y A D D Q M I I T I S H M G Y I K R T Q F D L R K A K
1711 tgctcaaaacccggtggagttagctcgaagggtactgaaaccctgatgaagactttgttgacacatctaccggcacaacatgacaaa
A Q N R G G V G S K G T E T R D E D F V E H I Y P A T M H N
1801 cacgatgatgttcttactcaaaagggttaattgactggctgaaggtatataaatacctgaaggaacaaagaactcaagggcggctgc
T M M F F T Q K G K C Y W L K V Y E I P E G T K N S K G R A
1891 tatccagaactcctgaacattgactcggacgatgctgtaattgcatatttgcgtggaaggtttgaaatgaccaggaatataaataacag
I Q N F L N I D S D D A V N A Y L R V K S L N D Q E Y I N S
1981 tcatatgtactgttctgtaccaagaatggcgttataaagaaacatcttggaaacactcagcccgcgcgaatggtgtcaatgc
H Y V L F C T K N G V I K K T S L E Q Y S R P R Q N G V N A
2071 aattactatcgtgaagcagcagcagtaataagaagtcggtatgaccaacggaaacaaacgaatcatcatagccaacgttaacggcagcgc
I T I R E D D R V I E V R M T N G N N E I I I A N R N G R A
2161 aatacgtttccatgaagcagcagcttcgctaatggcggctacagctaccggagttcgtggtatcacactggatgacgacggacaggtga
I R F H E A A V R V M G R T A T G V R G I T L D D D G Q D E
2251 agtaataggcatgatttgcattaaagatctcgcagacagagtcgtaattggttctccgaacaaggctatggttaaacgttctgatattga
V I G M I C I K D L E T E S V M V V S E Q G Y G K R S D I E
2341 agattatcgtaaaacaaacccgtggcggcaaggtgtgaagaccatgaatattaccgaaaaacaggtaaacgtggttacaatcaagctgt
D Y R K T N R G G K G V K T M N I T E K T G K L V T I K S V
2431 aacagcgaacaaacacgtgatcattataaactcgggtattacaattcgtctgaaagttagctggtcgcgatggggcgtgcaac
T D E N D L M I I N K S G I T I R L K V A D V R I M G R A T
2521 tcaaggagtcctgatcaatcttgaaaaacgtaacgaccagatcgttctgtagtaagttacatcgaagacgtggaagatgaagt
Q G V R L I N L E K R N D Q I G S V C K V T S E S L E D E V
2611 tccggaagaagaagagaagaaatattccaagcagatccggaacgaatacaccggttaaatgacacagaagaatagacataatataata
P E E E R E G N I P S D P E T N T P V N D T E E *
2701 attaatcaaacacaaatcatgaaaagagtattatttcaatggtttactgatggcagtaagttttgcatcgcctcaggagaaaaaatgt
2791 aaaagaagcgaacgacttggcggagaagtaaaacctgacttcgcaaaagctgaaacactgattaacggagcattaaactaacctgaaac
2881 aaaggataatcggcaacttggcagtagcaggttatattcagaaaagaatcaacgaaaaggagatggaataatgcttctgagaaaacc
2971 ttatgatcattgaaagtatacaatagcgtactgaaatgtacaattattatgtaaatgtgacgaaactggcacagatttccatgaaa
3061 agggtaaaataaaacaaatacagagggccactcaaaaacaaattctggcagaacgtcctaact

FIG. 1. Nucleotide and deduced amino acid sequence of a 3,124-bp fragment which contains the *gyrA* gene of *B. fragilis* ATCC 25285. The methionine initiation codon is underlined. An asterisk indicates the stop codon.

```

1  gactctata aatattagaagccggaacttttcagttcggctttttatgcttcaattaccocggctcggtttattttaattctctttt
91  tcaactcttcaattctctcaactcagatattttcactatatttgcctctgtattataaattaggaatcaaatatagagcgaagacaagaat
      M S E E Q N
181  cccaccaataacgggtctttatcagcagatagatccaagtattggaaggacttgaagcagttgaaaacgcoctgcgatgtacattggt
      P T N N G S Y S A D S I Q V L E G L E A V R K R P A M Y I G
271  gacatcagcgtaaaggacttcaactcttggtatataaaattgctgacaactctatcgacgaagcattggccggttattgcgcatatc
      D I S V K G L H H L V Y E I V D N S I D E A L A G Y C D H I
361  gaagtaactatcaacgaagacaactctatcaccgtacaggataatggaogtggattccggttagatttccagaaaagagcagaatct
      E V T I N E D N S I T V Q D N G R G I P V D F H E K E Q K S
451  gccctgaaagtggccatgaccgtactgcatgccggaggtaagttcgataaaaggttcgtacaaagatccggaggtcttccaggttaggt
      A L E V A M T V L H A G G K F D K G S Y K V S G G L H G V G
541  atgtcgtgtgtaagtcattgtctacacacatgactaccocggattccgcaacggtaaaatctatcagcaggaatgaaatcggttaa
      M S C V N A L S T H M T T Q V F R N G K I Y Q Q E Y E I R G K
631  ccgctttatcccgttaaagaagtaggaatagcggaccacaggaacaaacagcaattctggccgatgacagttatctttaccgaaacc
      P L Y P V K E V G I A D H T G T K Q Q F W P D D S I F T E T
721  atttatgattataagattctggcttcaactctcagttacgtgaattgcttctgtaagccggtctgcgcatctcgtcagacatcgctcgt
      I Y D Y K I L A S R L R E L A Y L N A G L R I S L T D R R V
811  gtgaatgaggaacggcagtttcaaacacgaaactttctattcggaaagggttaagagaatttgtagctttcagcaatcgctcagcgaa
      V N E D G S F K H E T F Y S E E G L R E F V R F I E S S R E
901  cacttgattaaagctgatttatctaaacacagaaacaaacatcccatcgaggtggctatcatgtacaaatccggattttcagaa
      H L I N D V I Y L N T E K Q N I P I E V A I M Y N T G F S E
991  aataccattcgtcagcaataaacattactatagaaggtggtacgcattcggcaggttccgcccgcocctgaccocgtacactgaag
      N I H S Y V N N I N T I E G G T H L A G F R R A L T R L K
1081  aaatagcagaagcagcaaaaatgctgggaaagttaaagtagaattctccggcagactttcgtgaaaggtctgacagctgtgatctct
      K Y A E D S K M L E K V K V E I S G D D F R E G L T A V I S
1171  gtaaaagtacgtgaacccaatttgaaggacagactaaaactaagttgggaaacacgaagttatggggtgctgccatcaggcggtat
      V K V A E P Q F E G Q T K T K L G N N E V N G V L P I R D A
1261  ggcaagtaactaaactattatctggaagaacacccgaagagctaaagcaattgtagacaaagtgattttggctgctactgcaacccac
      G E V L N Y Y L E E H P K E A K A I V D K V I L A A T A R H
1351  gcccccgaagcgcgtgagatggtacagcgtaaatctcctatgctcaggtggcggcttccgggtaaactggccagctgctcgacaaa
      A A R K A R E M V Q R K S P M S G G G L P G K L A D C S D K
1441  gaccgcagaagtgtagttatctcctcgtcagggagactctgccggcgtacagctaaagcaggtcgtaaccgtgactttcaggtctatt
      D P Q K C E L F L V E G D S A G G T A K Q G R N R A F Q A I
1531  cttccaactacgggttaagattctgaactgagaaagccatgtatcacaagcgttgaagcgaagaataacgcaatataacacggca
      L P L R G K I L N V E K A M Y H K A L E S E E I R N I Y T A
1621  ctgggtgtaactatcggaacggaagaacagcagaagctgccaatattgataagctgcgctatcataaatcattatcatcgcatgccc
      L G V T I G T E E D S K A A N I D K L R Y H K I I I M T D A
1711  gacgtcgatggatcacacatcgacacactgatcactttttctccgctatatagccacagatccagaatggctatctgtacatt
      D V D G S H I D T L I M T F F F R Y M P Q I I Q N G Y L Y I
1801  gccactcccccgctcactcttgcgaaaggaagaaatagaagagattgctggaagatgccaacgccaagaagttatcgacacttat
      A T P P L Y L C K K G K I E E Y C W T D A Q R Q K F I D T Y
1891  ggtggcgttcggaaatgcaatccatcacacagcgtacaaaaggttggggtgagatgaatgccacagcagttgggnaacgactatggat
      G G S E N A I H T Q R Y K G L G E M N A Q Q L W E T M D
1981  ccgaaaaccgtatgctgaaacaggttataatcgacaacgacgagaagccgactatatacttccatgttgatgggtgaagcagctaggt
      F E N R M L K Q V N I D N A A E A D Y I F S M L M G E D V G
2071  ccacgcgcgagttcattgaagaaatgcaacgtatgcaaatatogatgcataatcgtaatataaacccaacctcacatcttacaacg
      P R R E F I E E N A T Y A N I D A *
2161  aagaagcgcgcgctgaaagaaatccttcggaccggcgttttttgaattc
    
```

FIG. 2. Nucleotide and deduced amino acid sequence of a 2,215-bp fragment which contains the *gyrB* gene of *B. fragilis* ATCC 25285. The symbols are defined in the legend to Fig. 1.

Nucleotide sequence accession numbers. The DNA sequences corresponding to the *gyrA* and *gyrB* genes have been assigned GenBank accession no. AB017712 and AB017713, respectively.

RESULTS

Cloning and sequencing the *gyrA* and *gyrB* genes of *B. fragilis*. Southern blot hybridization analysis of genomic DNA from *B. fragilis* ATCC 25285 revealed that a 1.5-kb *EcoRI* fragment and a 4-kb *SphI* fragment hybridized to the *E. coli gyrA* and *gyrB* probes, respectively (data not shown). These fragments were isolated by colony hybridization of a size-selected *B. fragilis* ATCC 25285 *EcoRI* fragment library and an *SphI* fragment library. DNA sequence analysis of both clones indicated that the sequences showed high homology with *gyrA* and *gyrB* of *E. coli*. To obtain full-length *gyrA* and *gyrB* genes,

a partially *Sau3AI*-digested genomic library was screened with the two genes as probes. Fragments of 1.8 and 3 kb were screened by the *gyrA* probe, and a 0.6-kb fragment was screened by the *gyrB* probe. Analysis of the nucleotide sequences revealed two open reading frames for GyrA and GyrB. The *gyrA* and *gyrB* genes encoded 845- and 653-residue proteins with predicted molecular masses of 95.7 and 70.9 kDa (Fig. 1 and 2). The deduced products of *gyrA* and *gyrB* exhibited 48 and 52% identity, respectively, to GyrA and GyrB of *E. coli*. The homology of the GyrA QRDR between *B. fragilis* and *E. coli* was particularly high (70%), suggesting that this region of *B. fragilis* is also related to quinolone resistance (Fig. 3).

Purification of GyrA and GyrB in *E. coli*. To identify the proteins encoded by *gyrA* and *gyrB*, we overexpressed the proteins and examined their enzymatic properties. The putative



FIG. 3. Alignment of *B. fragilis* GyrA (A) and GyrB (B) protein sequence with their counterparts in *E. coli* and *S. aureus*. An asterisk indicates identity among all three proteins. The numbers indicate amino acid residues. Residue Ser-82 (S) in *B. fragilis* GyrA and the position of the catalytic tyrosine (Y) residue involved in DNA breakage reunion (12) are in boldface and underlined.

GyrA and GyrB proteins were expressed as MBP fusion proteins and purified separately. The bands for each protein on a sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie brilliant blue were about 95 and 70 kDa for GyrA and GyrB, respectively (Fig. 4). Neither protein alone had supercoiling activity, but the reconstituted proteins showed ATP-dependent enzymatic activity (Fig. 4). These results demonstrate that the 95- and 70-kDa proteins of *B. fragilis* are GyrA and GyrB, respectively.

Sequence analysis of stepwise-selected levofloxacin-resistant mutants of *B. fragilis*. In order to examine the role of DNA gyrase in quinolone-resistant *B. fragilis*, we developed mutants of susceptible strain ATCC 25285 by stepwise exposure to levofloxacin. In the first round of selection, isolate ATCC 25285 (approximately 10⁸ CFU) was plated on GAM agar plates containing increasing concentrations of levofloxacin in multiples of the MIC. More than 100 colonies (first-step

mutants) grew on the plate containing 0.78 µg of levofloxacin/ml, and no growth was seen at higher drug concentrations. Two first-step mutants (L1-1 and L1-2) were selected for *gyrA* sequence analysis. Mutant L1-1 was exposed to increased drug levels on plates. At a concentration of 3.13 µg/ml, more than 100 colonies (second-step mutants) were able to grow. Third- and fourth-step mutants, which grew in the presence of 12.5 and 25 µg of levofloxacin per ml, respectively, were generated similarly. Mutant strains were also cross-resistant to other quinolones: sitafloxacin, ciprofloxacin, and sparfloxacin (Table 1).

A 346-bp *gyrA* fragment spanning codons 1 to 115 was amplified by PCR from levofloxacin-resistant mutants. This region encompasses sequence equivalent to the quinolone resistance-determining region of *E. coli* GyrA (residues 67 to 106). PCR products were ligated into plasmid pCRII, and the inserts were sequenced. The nucleotide sequences of the PCR products

TABLE 1. Properties of mutants of *B. fragilis* ATCC 25285 selected for resistance by stepwise exposure in vitro to levofloxacin

Strain ^a	MIC ($\mu\text{g/ml}$) ^b				Mutation	
	LVFX	STFX	CPFX	SPFX	<i>gyrA</i>	<i>gyrB</i>
ATCC 25285	0.78	0.025	1.56	0.78		
L1	3.13	0.10	12.5	1.56	None	None
L2	12.5	0.78	25	6.25	Ser-82 (TCT)→Phe (TTT)	None
L3	50	1.56	50	25	Ser-82 (TCT)→Phe (TTT)	None
L4	50	1.56	50	50	Ser-82 (TCT)→Phe (TTT)	None

^a Two clones were analyzed for each strain.

^b The MIC is the lowest drug concentration at which no bacterial growth on GAM agar plates was observed after anaerobic incubation overnight at 37°C. LVFX, levofloxacin; STFX, sitafloxacin; CPFX, ciprofloxacin; SPFX, sparfloxacin.

tance for the two species are likely identical, and the mutation is related to quinolone resistance. Mutations in *gyrB* are also related to quinolone resistance (33), but no mutation was detected in our strains. Although *gyrB* mutations were not involved in quinolone resistance in this study, mutations in *gyrB* may, in general, be related to quinolone resistance in *B. fragilis*. Since no other mutation was detected in the GyrA and GyrB QRDRs of the highly quinolone-resistant strains L3 and L4, mutations in other regions may occur. Mutations in *parC* or *parE* are possible explanations. No mutation was detected in the first-step mutants (L1). As the level of resistance is modest, it is conceivable that an efflux pump or outer membrane permeability is related to quinolone resistance in first-step mutants (20). In this study, no mutation besides Ser-82 was observed in the QRDR of *gyrA* in the quinolone-resistant mutants, but alteration of Phe-86, which is equivalent to

Asp-87 of GyrA in *E. coli* (5, 10, 23), or other alterations of GyrA may also confer quinolone resistance in *B. fragilis*.

In the gram-negative species *E. coli* and *N. gonorrhoeae*, quinolone resistance arises initially from a mutation in *gyrA*, and additional mutation of *parC* leads to highly resistant isolates (1, 13, 15). Thus, DNA gyrase appears to be the primary target in these bacteria, with topoisomerase IV acting as a secondary target. Although the *parC* gene of *B. fragilis* is not yet cloned and analyzed, the observation of GyrA mutations in quinolone-resistant mutants indicates that DNA gyrase is an important target for quinolones in *B. fragilis*.

For further study of quinolone resistance in *B. fragilis*, analysis of the topoisomerase IV gene and efflux pumps is needed. Additional characterization of the *B. fragilis gyrA* and *gyrB* genes reported here should facilitate further understanding of this important anaerobic pathogen.

REFERENCES

- Belland, R. J., S. G. Morrison, C. Ison, and W. H. Huang. 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Mol. Microbiol.* **14**:371–380.
- Breins, D. M., S. Ouadhbesselam, E. Y. Ng, J. Tankovic, S. Shah, C. J. Soussy, and D. C. Hooper. 1997. Quinolone resistance locus *nfxD* of *Escherichia coli* is a mutant allele of the *parE* gene encoding a subunit of topoisomerase IV. *Antimicrob. Agents Chemother.* **41**:175–179.
- Brown, P. O., and N. R. Cozzarelli. 1980. A sign inversion mechanism for enzymatic supercoiling of DNA. *Science* **206**:1081–1083.
- Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* **39**:1211–1233.
- 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.
- Ferrero, L., B. Cameron, B. Manse, D. Lagneux, J. Crouzet, A. Famechon, and F. Blanche. 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target for fluoroquinolones. *Mol. Microbiol.* **13**:641–653.
- Gallert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. I. Tomizawa. 1977. Nalidixic acid resistance: a second character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. USA* **74**:4772–4776.
- Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash. 1977. DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proc. Natl. Acad. Sci. USA* **73**:3872–3876.
- Hecht, D. W., and H. M. Wexler. 1996. In vitro susceptibility of anaerobes to quinolones in the United States. *Clin. Infect. Dis.* **23**:S2–S8.
- Heisig, P., H. Schedletzky, and H. Falkenstein-Paul. 1993. Mutations in the *gyrA* gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli*. *Antimicrob. Agents Chemother.* **37**:696–701.
- Hill, G. B. 1992. Introduction to the anaerobic bacteria: non-spore-forming anaerobes, p. 621–635. *In* W. K. Joklik, H. P. Willett, D. B. Amos, and C. M. Willett (ed.), *Zinsser microbiology*, 20th ed. Appleton & Lange, Norwalk, Conn.
- Horowitz, D. S., and J. C. Wang. 1987. Mapping the activity site tyrosine of *Escherichia coli* DNA gyrase. *J. Biol. Chem.* **269**:5339–5344.
- Hoshino, K., A. Akasaka, I. Morrissey, K. Sato, J. Kato, and H. Ikeda. 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. *Antimicrob. Agents Chemother.* **38**:2623–2627.
- Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Higara, and H. Suzuki.

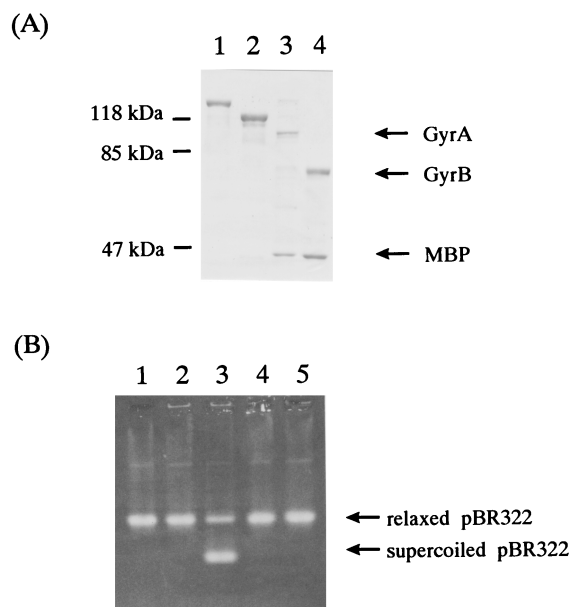


FIG. 4. Purification of *B. fragilis* GyrA and GyrB proteins. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified *B. fragilis* GyrA and GyrB proteins. The proteins were electrophoresed in a 10% polyacrylamide gel and stained with Coomassie brilliant blue. The masses of the protein markers are indicated in kilodaltons on the left. Lane 1, MBP-GyrA fusion protein; lane 2, MBP-GyrB fusion protein; lane 3, MBP-GyrA fusion protein after factor Xa cleavage; lane 4, MBP-GyrB fusion protein after factor Xa cleavage. (B) Supercoiling activity of purified GyrA and GyrB proteins. Lane 1, purified GyrA (1 U); lane 2, purified GyrB (1 U); lane 3, purified GyrA (1 U) and GyrB (1 U); lane 4, purified GyrA (1 U) and GyrB (1 U) without ATP; lane 5, no addition. The source of DNA is pBR322.

1990. New topoisomerase essential for chromosome segregation in *E. coli*. *Cell* **63**:393–404.
15. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli.** 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**:11801–11805.
16. **Kumagai, Y., J. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda.** 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. *Antimicrob. Agents Chemother.* **40**:710–714.
17. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. *Trends Biochem. Sci.* **19**:119–123.
18. **Li, X.-Z., D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob. Agents Chemother.* **38**:1732–1741.
19. **Maniatis, T., R. F. Fritsch, and J. Sambrook.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Miyamae, S., H. Nikaido, Y. Tanaka, and F. Yoshimura.** 1998. Active efflux of norfloxacin by *Bacteroides fragilis*. *Antimicrob. Agents Chemother.* **42**:2119–2121.
21. **Mizuuchi, K., L. M. Fisher, M. H. O'Dea, and M. Gallert.** 1980. DNA gyrase action involves the introduction of transient double-strand DNA breaks into DNA. *Proc. Natl. Acad. Sci. USA* **77**:1847–1851.
22. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. *J. Bacteriol.* **178**:5853–5859.
23. **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.
24. **Pan, X. S., and L. M. Fisher.** 1996. Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: role in fluoroquinolone resistance. *J. Bacteriol.* **178**:4060–4069.
25. **Pan, X. S., J. Ambler, S. Mehtar, and L. M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **40**:2321–2326.
26. **Piddock, L. J. V.** 1995. Mechanism of resistance to fluoroquinolones: state-of-the-art 1992–1994. *Drugs* **49**:29–35.
27. **Rasmussen, B., K. Bush, and F. P. Tally.** 1997. Antimicrobial resistance in anaerobes. *Clin. Infect. Dis.* **24**:S110–S120.
28. **Tanaka, M., K. Sato, Y. Kimura, I. Hayakawa, Y. Osada, and T. Nishino.** 1991. Inhibition by quinolones of DNA gyrase from *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **35**:1489–1491.
29. **Tanaka, M., Y. Onodera, Y. Uchida, K. Sato, and I. Hayakawa.** 1997. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV purified from *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **41**:2362–2366.
30. **Tanaka, M., Y. Onodera, Y. Uchida, and K. Sato.** 1998. Quinolone resistance mutation in the GrlB protein of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **42**:3044–3046.
31. **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.
32. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Kanno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus* *norA* gene, which confers resistance to quinolones. *J. Bacteriol.* **172**:6942–6949.
33. **Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura.** 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* **35**:1647–1650.
34. **Yotsuji, A., J. Mituyama, R. Hori, T. Yasuda, I. Saikawa, M. Inoue, and S. Mitsuhashi.** 1988. Outer membrane permeation of *B. fragilis* by cephalosporins. *Antimicrob. Agents Chemother.* **32**:1097–1099.