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

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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 intraabdominal 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).  $\beta$ -Lactam resistance is usually explained by the combination of low permeability of the outer membrane (34) and the presence of highly active  $\beta$ -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 ATPdependent DNA supercoiling (3, 7, 8, 21). Moreover, it was revealed that mutations in the GyrA quinolone resistancedetermining 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 $\alpha$  (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  $\mu$ Ci of [ $\alpha^{-32}$ P]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). Drugcontaining agar plates were inoculated with one loopful (5  $\mu$ l) of an inoculum corresponding to about 10<sup>4</sup> 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 *grrA* 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 *grrA*, the forward primer was Pr-BFGA03, 5'-ATGCTTGAACAAGACAGAATTATAA AG-3' (*grrA* positions 1 to 27) and the reverse primer was Pr-BFGA02, 5'-GA CTGTCGCCGTCTACAGAACCG-3' (324 to 346). The primers for the QRDR of the *grrB* gene were Pr-BFGB03, 5'-GACCCGCAGAAGTGTGAGTTATTC C-3' (*grrB* 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'-ATGCTT GAACAAGACAGAATTATAAAG-3' (*gyrA* positions 1 to 27), and the reverse primer was Pr-BFGA04, 5'-AGTTGTT<u>AAGCTT</u>TTGCGAAGGACAGGA-3' (2777 to 2802; *Hind*III). The primers for the *gyrB* gene were Pr-BFGB01, 5'-ATGCCAAGGAAGACAGGAATCACC-3' (*gyrB* positions 1 to 24), and Pr-BFGB02, 5'-ATTTTC<u>CTGCAAGGACCACC-3'</u> (*gyrB* positions 1 to 24), and Pr-BFGB02, 5'-ATTTTC<u>CTGCAGGCGCGCCTTC-3'</u> (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).

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181 categagagaaatgaagtcategtacattgactactcattgtegtee I E E E M K S S Y I D Y S M S V Y 271 gcccgttcaccgcagaattctctacggaatgatggaactggaaatacgf P V H R R I L Y G M M E L G N T S 361 agtacttggtaagtatcacccgcacggagactcttctgtatattttgcgg V L G K Y H P H G D S S V Y F A N 451 ggtagacggcaaggtaacttcggttctgtagacggcgacagtcctgctc V D G Q G N F G S V D G D S P A Y 541 agaaatgatgcaggaccotctacaaagagacgtagattcgagatagg I P N L L V N G A S G I A V G M Y 721 tgcctgcgaagcatatctggtacgtagacggcacggcaggacgg A C E A Y L D N K D V T V E E L N 811 tatatatggcataagcgggaaggtacgtacgaggagagg I Y G I S G V R E A Y L T G R G I 901 gacacagatagatggcgggaggtacgtacgagggagggg R I E G I S N A N D E S D R E G Y 1081 tgtggtggaacggctattcggagagggagggg R I E G I S N A N D E S D R E G Y 1081 tgtggggaacggctattcagagaggtcggaggg R I E G I S N A N D E S D R E G Y 1081 tgtggggaacgagctattcagagaggtacggaggt R I E G I S N A N D E S D R E G Y 1081 tgtggggaacgagctattcagagagctggaggt R I E G I S N A N D E S D R E G Y 1081 tgtagtgcgaacaagctctattacggagacgaggt R I E G I S N A N D E S D R E G Y 1081 tgtagtgcgaacaagctctattacggagactggaggt R I E G I S N A N D E S D R E G Y 1081 tgtagtgcgaacaagctctgtagtgttccgtagaacaagacggt R I E G I S N A N D E S D R E G Y 1081 tgtagtgcgaacaagctctattaaatgcaacgacgagtcagacgagt R I E G L I I A S D N I I 1081 tgtagtgcgaacaagctctgaatgaggtcgaatatagacacgat L N L R D L I V Y F V E H R H D Y 1041 tctgagcaagtcagactgatggaaggtctgaatacgagaggtattagaacagag D A I S G L M E R F N L S E I Q Y 1441 tctgatgcaagatcagctcaatgctgaatacgaggaggtatgaaggcgaattacga C A H I L E G L I I A S D N I I 1531 ccgtaaagtaatcaagagcgactgaatacgagaggtatgaagacgattagag C M Q D Q L H A E Y E E V M K Q S 1531 ccgtaaagtaatcaagagagattgcgaatacgagaggtatgaagagctaaatatggtc R K V I K D E L L E V R A K Y G I 1621 caatccggaagaactttatgcggagtaggaggaggatggaggatggaacgatatacaactcaac N P E D F Y A D D Q M I I T I S H 1711 tgctcaaaaccgggggaggaggatgggaggaggaggaggatggaacggtaggaacgatat A O N R G G V G S K G T F T P D	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$
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811 tatatatggcataaggggtacgtagagcctatcttacgggacggaga I Y G I S G V R E A Y L T G R G I 901 gacacatgataagatcgtcgttacagagattcctacaacgtgaataag T H D K I V V T E I P Y N V N K J 991 aagaatagaaggcatatcaaatgccaacgagagtccgacgtgaaggt R I E G I S N A N D E S D R E G P 1061 tgtagtgctgaacaagctctataaaatgccagccttgcagacgtcatca V V L N K L Y K M T A L Q T S F C 1171 gctgaatttacgcgacttgattgtttacttcgtagaacatagacacgat L N L R D L I V Y F V E H R H D V 1261 agaacgtgcaacatctcggaaggtcgatctcaacctgagcgaattcag D A I S G L M E R F N L S E I Q J 1441 tctgatgcagaatcaagacgaattgctgatatcagagaggtatagaacataggtc R K V I K D E L L E V R A K Y G I 1531 ccgtaaagtatcaaagacgaattgctgaagagagatatatat	atggaatatgtaaaagcgcccgacttccctacaggaggata M E Y V K A P D F P T G G Y
901gacacatgataagatcgtcgttacagagattccctacaacgtgaataag T H D K I V V T E I P Y N V N K $J$ 991aagaatagaaggcatatcaaatgccaacgagagtccgacgtgaaggt R I E G I S N A N D E S D R E G P1061tgtagtgctgaacaagctctataaatgccagcagtcgaaggtcgacgtcatc V V L N K L Y K M T A L Q T S F C1171gctgaatttacgcgacttgattgtttacttcgtagaacatagacacagat L N L R D L I V Y F V E H R H D V1261agaacgtgcacacatcttggaaggtcgattacggtcgatattacg E R A H I L E G L I I A S D N I I1351cgatgcaatctccggactgatggaacgctcaacatcgagaggtctgatacagatgaggtcgatagagggggagaggggggaggaggaggaggaggagtagaatagggt L M Q D Q L H A E Y E V M K Q D D1441tctgatgcaagatcaagacgattgtggagagaggagtagaatataggtc R K V I K D E L L E V R A K Y G I1531ccgtaaagtaatcaagaggagtttatggagatagagtatacacatataggt R K V I K D E L L E V R A K Y G I1621caatccggaagacttttatgcggagtagtagatacacatatcaga R K V I K D E L L E V R A K Y G I1621tgctcaaaaccggggggggagtaggagtaggagtaggatacacatcaca N P E D F Y A D D Q M I I T I S F1711tgctcaaaaccgggggggggggggggggggggggggggg	cgcgtggttatgcgcgcgaaagcagaaatcgaatccggaca R V V M R A K A E I E S G O
991aagaatagaaggcatatcaaatgccaacgacgagtccgaccgtgaaggtr R I E G I S N A N D E S D R E G N1081tgtagtgctgaacaagctctataaaatgccagccttgcagacgtcattc V V L N K L Y K M T A L Q T S F G1171gctgaatttacgcgacttgattgtttacttcgtagaacatagacacgat L N L R D L I V Y F V E H R H D V1261agaacgtgcacacatcttggaaggtctgattacggtcgattatcggtgaaggtcgattacggacgtcgattgatgtgaaggtcgattacggtggaaggtcgattacgg D A I S G L M E R F N L S E I Q D1441tctgatgcaagatcaagacgaatgctgaatggaaggtcgaaggtctgaagaggtaagaggtaagaggtaagaggtaagaggtaagaggtaagaggtaagaggtaagaggtaagaggta R K V I K D E L L E V R A K Y G I1531ccgtaagagacttttatgcggaggagtaggatggataccacatctcag R K V I K D E L L E V R A K Y G I1621caatccggaagacttttatgcggagtagtagaggtattaccactcac N P E D F Y A D D Q M I I T I S F1711tgctcaaaaccgcggtggagtaggtaggtcgaaggtacgaacccgtgat A O N R G G V G S K G T F T D D D	- gcagaattgattaaagcaattgctgatcttgtcaatgaaaa A E L I K A I A D L V N E K
1081 tgtagtgetgaacaagetetataaaatgacageettgeagaegtetate V V L N K L Y K M T A L Q T S F G 1171 getgaatttaegegaettgattgttaettegtagaacatagaeaegate L N L R D L I V Y F V E H R H D V 1261 agaaegtgeaeaeatettggaaggtetgattaeeggaagatetegaeatteeggaetgatgaegeetteaaeetgggagaatteegg D A I S G L M E R F N L S E I Q V 1441 tetgatgeaagategaeteeatgetgataeagaggaggtatgaagaeget L M Q D Q L H A E Y E E V M K Q T 1531 cegtaaagtaateaaagaegaattgetgaagaeggtaagaegetaaatatege R K V I K D E L L E V R A K Y G I 1621 caateeggaagaettttatgeggatgateagaegataeaeatateget N P E D F Y A D D Q M I I T I S F 1711 tgeteaaaeeegggtggagtaggeteggaaggteeggaaceegtgat	atgcgcatcgttattgatatcaaacgggatgcaaatgcaag M R I V I D I K R D A N A S
1171 gctgaatttacgcgacttgattgtttacttcgtagaacatagacacgat L N L R D L I V Y F V E H R H D V 1261 agaacgtgcacacatcttggaaggtcgattatcgctcggataatatt E R A H I L E G L I I A S D N I I 1351 cgatgcaatctccggactgatggaacgcttcaacctgggcgaaattcag D A I S G L M E R F N L S E I Q V 1441 tctgatgcaagatcagctccatgctgaatacgaggaggttatgaagcag L M Q D Q L H A E Y E E V M K Q S 1531 ccgtaaagtaatcaaagacgaattgctggaagtaagagctaaatatgtg R K V I K D E L L E V R A K Y G I 1621 caatccggaagacttttatgcggatgatcagatgagagtattacacatctcaa N P E D F Y A D D Q M I I T I S F 1711 tgctcaaaaccgcggtggagtaggaggtacgaaggtacggaaccgtgat	ggtgtaaataacgttgcactggtcaacggacgccctaaaat G V N N V A L V N G R P K M
1261 agaacgtgoacaacatottggaaggtotgattatcgottoggataatatto E R A H I L E G L I I A S D N I I 1351 cgatgoaatotcoggactgatggaacgottcaacotggaggaaattoag D A I S G L M E R F N L S E I Q D 1441 totgatgoaagatcagotcoatgotgaatacgaggaggtatgaagagca L M Q D Q L H A E Y E E V M K Q D 1531 cogtaaagtaatcaaagacgaattgotggaagtaagagctaaatatggto R K V I K D E L L E V R A K Y G I 1621 caatocggaagaotttatgoggatgatacgatgatacoactocad N P E D F Y A D D Q M I I T I S F 1711 tgotcaaaacogggtggagtagggtacggaaggcocggaat	gtggtaattegtegtaeteaatttgaeetgegtaaggeeaa V V I R R T Q F D L R K A K
<ul> <li>1351 cgatgcaatctccggactgatggaacgcttcaacctgagcgaaattcagg D A I S G L M E R F N L S E I Q J</li> <li>1441 tctgatgcaagatcagctccatgctgaatacgagggggttatgaagagg L M Q D Q L H A E Y E E V M K Q 1</li> <li>1531 ccgtaaagtaatcaaagacgaattgctggaagtaagagctaaatatggt R K V I K D E L L E V R A K Y G I</li> <li>1621 caatccggaagacttttatgcggatgatcagatgattacaccatccac N P E D F Y A D D Q M I I T I S F</li> <li>1711 tgctcaaaccgcggtggagtagggtagggtacggaagccgaagctgatgatcgaagccggagt</li> </ul>	gacgaagtaattegtateateegegeegeeaaaaeaeeaaa D E V I R I I R A A K T P N
<ul> <li>1441 totgatgcaagatcagctcaatgctgaatacgaggaggttatgaagcag; L M Q D Q L H A E Y E E V M K Q 1</li> <li>1531 ccgtaaagtaatcaaagacgaattgctggaagtaagagctaaatatggtg R K V I K D E L L E V R A K Y G I</li> <li>1621 caatccggaagacttttatgcggatgatcagatgattatcaccatctca N P E D F Y A D D Q M I I T I S F</li> <li>1711 tgctcaaaaccgcggtggggtaggctagggtagggtactgaagccgtgat A O N R G G V G S K G T E T P D T</li> </ul>	gcacgcgccatcgttgaaatgcgcctgcgccaattaacagg A R A I V E M R L R Q L T G
<ul> <li>1531 ccgtaaagtaatcaaagacgaattgctggaagtaagagctaaatatggtg R K V I K D E L L E V R A K Y G I</li> <li>1621 caatccggaagacttttatgcggatgatcagatgattatcaccatctcaa N P E D F Y A D D Q M I I T I S H</li> <li>1711 tgctcaaaaccgcggtggagtaggtcggaagggtacgaaggtactgaaacccgtgatg</li> <li>A O N R G G V G S K G T E T P D D</li> </ul>	atagcatatttggaaagtatcctggccgatgatgaagtatg I A Y L E S I L A D D E V C
1621 caatcoggaagacttttatgoggatgatcagatgattatcaccatctcac N P E D F Y A D D Q M I I T I S H 1711 tgotcaaaacogoggtggagtaggotcgaagggtactgaaaccogtgatc A O N R G G V G S K G T E T P D	gacgaacgccgttctgaaatcgtttattcatcagaagaatt D E R R S E I V Y S S E E F
1711 tgctcaaaaccgcggtggagtaggctcgaagggtactgaaaccgtgatg A O N R G G V G S K G M R M P D D	cacatgggatatatcaaacgtacaccattgacagaattccg H M G Y I K R T P L T E F R
	gaagactttgttgagcacatctacccggcaacaatgcacaa E D F V E H I Y P A T M H N
1801 cacgatgatgttetttaeteaaaagggtaaatgttaetggetgaaggta T M M F F T Q K G K C Y W L K V Y	tatgaaatacctgaaggaacaaagaactctaagggccgtgc Y E I P E G T K N S K G R A
1891 tatccagaacttcctgaacattgactcggacgatgctgttaatgcatat I Q N F L N I D S D D A V N A Y D	ttgcgtgtgaagagtttgaatgaccaggaatatattaacag L R V K S L N D Q E Y I N S
1981 tcattatgtactgttctgtaccaagaatggcgttataaagaaaacatct H Y V L F C T K N G V I K K T S 1	ttggaacaatactcacgcccgcgccagaatggtgtcaatgc L E Q Y S R P R Q N G V N A
2071 aattactatacgtgaagacgacgagtaatagaagtgcgtatgaccaacg I T I R E D D R V I E V R M T N O	ggaaacaacgaaatcatcatagccaaccgtaacggacgcgc G N N E I I A N R N G R A
2161 aatacgtttccatgaagcagcagttcgcgtaatgggccgtacagctacc I R F H E A A V R V M G R T A T C	ggagttcgtggtatcacactggatgacgacggacaggatga G V R G I T L D D D G Q D E
2251 agtaataggcatgatttgcattaaggatctcgagacagagtccgtaatg V I G M I C I K D L E T E S V M V	gttgtctccgaacaaggctatggtaaacgttctgatattga V V S E Q G Y G K R S D I E
2341 agattatcgtaaaacaaaccgtggcggcaaaggtgtgaagaccatgaat DYRKTNRGGKGVKTMN	attaccgaaaaaaacaggtaaactggttacaatcaagtctgt I T E K T G K L V T I K S V
2431 aacagacgaaaacgacctgatgatcattaataaatcgggtattacaatt T D E N D L M I I N K S G I T I 1	cgtctgaaagtagctgatgtccgcatcatggggcgtgcaac R L K V A D V R I M G R A T
2521 tcaaggagtccgtctgatcaatcttgaaaaacgtaacgaccagatcggt Q G V R L I N L E K R N D Q I G	tctgtatgtaaagttacatccgaaagcctggaagatgaagt S V C X V T S E S L E D E V
2611 tccggaagaagaaagaaggaaatattccaagcgatccggaaacgaat P E E R E G N I P S D P E T N '	acaccggtaaatgacacagaagaatagacataatattaata T P V N D T E E *
2701 attaatcaaacaacaatcatgaaaagagtattattttcaatgqttttac	
2791 aaaagaagcgaaaagcattgccggagaagtaaaacctgacttcgcaaaa	tgatggcagtaagttttgcattcgctcaggagaaaaaatgt
2881 aaaggataatacggcaacttgggacgtagcaggttatattcagaaaaga	tgatggcagtaagttttgcattcgctcaggagaaaaaatgt gctgaacaactgattaacggagcattaactaaccctgaaac
2971 ulaugatacauugaaagtatacaatagcgtactgaatatgtacaattat 3061 agggtaaaattaaaacaaatacagaggcgccactcaaaaacaattctoo	tgatggcagtaagttttgcattcgctcaggagaaaaaatgt gctgaacaactgattaacggagcattaactaaccctgaaac atcaacgaaaaggagatggaaatgcttatctgagaaacc tatettaatatgagagagagagagagaga

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 91	gactctata aatattagaagccqgacttttcaqttcgqctttttatgctttcaattacccggtcgggtttatttttaattcttcttttt tcactcttctttctttttttt
181	cccaccaataacgggtcttattcagcagatagtatccaagtattggaaggacttgaagcagttagaaaacgccctgcgatgtacattggt 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	gacatcagcgtaaagggacttcatcatcgtgtatatga aattgtcgacaactctatcgacgaagcattggccggttattgcgaccatatc $D$ 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	gaagtaactatcaacgaagacaactctatcaccgtacaggataatggacgtggtattccggtagatttccacgaaaaagagcagaaatct 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	gccctcgaagttgccatgaccgtactgcatgccggaggtaagttcgtaaaagttcgtacaaagtatccggaggtcttcacggtggtagt 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	atgteetgtgtgaatgeattgtetaeeaeatgaetaeeeaggtatteegeaaeggtaaaatetateageaggaatatgaaateggtaaa 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 G K
631	ccgctttatcccgttaaagaagtaggaatagcggaccacacaggaaccaaacagcaattctggcccgatgacagtatctttaccgaaacc 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	atttatgattataagatt ctqcqttacqttacqtgaattggcttatctgaatqccgqtctgcqcatctcgctgacagatcgtcgcgta 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	gtgaatgaggacggcagtttcaacacgaaactttctattcggaaqagggtttaagagaatttgtacgtttcatcgaatcgtcacggaa 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	cactigatiaacgatgtgattatctaaacacagagaaacaaaaca
991	aatatocattegtaegteaataacattaataetatagaaggtggtaegeatetggeaggttteegeegegeeetgaeeegaeaetgaag 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 T L K
1081	aaatatgcagaagacagcaaaatgctggagaaagttaaagtagaatcccggcgatgactttcgtgaaggtctgacagctgtgatct 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	gtaaaagtagctgaaccocaattigaaggacagactaaaactaagtigggaaacaacgaagttaatggggtggtgctgccgatcaggcggtat 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 R Y
1261	ggcgaagtactaaactattatCtggaagaacacccgaaagaggctaaagcaattgtagacaaagtgatttggctgctactggaagaacagccac G E V L N Y Y L E E H P X E A X A I V D K V I L A A T A R H
1351	gccgcccgcaaagcgcgtagatggtacagcgtaatctcctatgtcaggtggcgtcttccgggtaaactggccgactgctccgacaaa
1441	gaccegeagaagtgtgagttatleetegtegaggagaetetgeeggegtaeagetaageaagtegtaeegtaeegteat 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	cttccactacgcggtaagattctgaacgtagaagaagccatgtatcacaaagcgctgaaagcgaagaaatacgcaatatatacccggca 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	ctyggtgtcactatoggaacggaagaagacagcaaagctgccaatattgataagctgcgctatcataaaatcattatcatgaccgatgcc 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	gacgtcgatggatcacacatcgacaccatgatcatgactttttttt
1801	gccactcccccgctctaccttgcaaaaaagagaaaatagaagagtattgctggacagatgcgcaacgcagaagttatcgacacttat 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	ggtggcggttcggaaaatgcaatccatacacagcgctacaaaggtttgggtgagatgaatgcacagcagttgtgggaaacgactatggat G 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 T M D
1981	ccggaaaaccgtatgctgaaacaggttaatatcgacaacgcagcagaagcgactatatcttctccatgttgatgggtgaagacgtaggt P 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	ccacgocgogagttcattgaagaaaatgcaacgtatgcaaatatcgatgcataattcgtaatataaacaccaacctcacatcttacaacg P R R E F I E E N A T Y A N I D A *
6 I U I	aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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 *Sau*3AI-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

A

B.fragilis	1:MLEQDRIIKINIEEEMKSSYIDYSMSVIVSRALPDVRDGFKPVHRRILYGMMELGNTS	58	
E.coli	1:MSDLARE-ITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDW	59	
S.aureus	1:MAELPQSRINERNITSEMRESFLDYAMSVIVARALPDVRDGLKPVHRRILYGLNEQGMTP	60	
	* * * ** * * *** ***** ****** ***** **		
B.fragilis	59:DKPYKKSARIVGEVLGKYHPHGD <u>s</u> SVYFAMVRMAQEWAMRYPLVDGQGNFGSVDGDSPAA	118	
E.coli	60:NKAYKKSARVVGDVIGKYHPHGD <u>S</u> AVYDTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAA	119	
S.aureus	61:DKSYKKSARIVGDVMGKYHPHGD <u>\$</u> SIYEAMVRMAQDFSYRYPLVDGQGNFGSMDGDGAAA	120	
	* ****** ** * ******** * ***** ** ******		
B.fragilis	119:MRYTEARLNKLGEEMMQDLYKETVDFEPNFDNTLMEPKVMPTRIPNLLVNGASGIAVGMA	178	
E.coli	120:MRYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMA	179	
S.aureus	121:MTTEARMTKITLELLRDINKDTIDFIDNYDGNEREPSVLPARFPNLLANGASGIAVGMA	180	
B.fragilis	179; TNMPPHNLSEVIDACEAYLDNKDVTVEELMEYVKAPDFPTGGYIYGISGVREAYLTGRGR	238	
E.coli	180 : TNIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGK	239	
S.aureus	181: TNIPPHNLTELINGVISISKNPDISIAELMEDIEGPDEPTAGLILGKSGIRRAVETGRGS	240	
	** **** * * *** ***** * * *****		
B.fragilis	239: VVMRAKAEI-ESGOTHDKIVVTEIPYNVNKAELIKAIADLVNEKRIEGISNANDESD-	294	
E.coli	240: VYTRARAEV-EVDAKTGRETI I VHEI PYOVNKARI. TEKTAFI. VKEKRUEGI SALRDESD-	297	
S.aureus	241: IOMRSRAVIEEBGGGBORIVVTEIPFOVNKARMIEKIAELVROKKIDGITDI.RDETSI.	298	
		200	
B.fragilis	295 REGNELVIDIKEDANASVVINKLYKMTALOTSFOUNNVALVNORPKMINI. DDI. LVVFVFH	354	
F coli	298 K REMERT VIEW REAL CONTRACT AND A COMPACT AND A CONTRACT AND A COMPACT	357	
S auraug	290, RUGHRIVIEVRRDAVGEVVERNEISUTED OF STREVERNEISUTED UND THAT VER	359	
b.ddiedb		550	
B fracilic	355 PHDUVIDETOEDLEKAKEDAUTLECL_LL-A-SDNI	301	
E coli	358. DEFUVTERTIFELEVADDAUTIENI AVALANIDETTELIDUADTEAVAALVANDUOL	417	
S aurous	250.0VTUUDDDTOVNIDVAVDDAUTIE CI DI A LDUI DE II	305	
5.duleus	555;QKIVVKKIQINGKKAKDKAHILEGE-KI-K-IDHIBEII	727	
B fragilie		440	
E coli	418 CONVANTEDACODA ADDEWLEDECUDDCL VVLTECOACA LLOL DI CALTCU PREMI LOR	440	
S auroug	410; ONVARMEERAGDDAARPEWLEPERGVRDGLIILLEVOROATLEDEREQREIGEEREALEDE	4//	
5.001605		444	
B fragilie	AA1 - VEEUMKOTAVI ESTI ADDEVODKUTKDELI EVDAKVODEDDCETUVCC- FEENDEDEVAD	400	
E coli	479. VKELLDOLAFILIDIL GSADDI MEVIDERI EL UDEOFGDEREDETTANSAD. INI EDI ITO	536	
S aurous	4/5. VNELINVISELEATLADERVIIOLVDDELTEIDDECDEDDTEIDLCCEEDIEDENIG	504	
Diddrodb	* * * * * ** ** ** ** ** ** ** **	504	
B.fragilis	500 · DOMI ITTI SHMGYIKETELTEEBAONEGGUGSKGTETEDEEPEVEHIVEATMANTETOK	550	
E.coli	537 · FDVVVTLSHOGYVKVODLSEVFAORDCCKGKSAADIKEEDEIDBLLVANTHDHILCESSE	596	
S. aureus	505 • FOTVITISHNYIKRIPUSTYRAONRORDAMTIFEDEUSOLUTISTHDHULFFINK	564	
braaroab	* ** * * * ** *** * **** * *** * *	504	
B.fragilis	560 · CKCYWLKVYFI DECTKNSKCDATONFI NI DSDDAVNAVI DVKSI NDOEVI NSHVVI FOTK	619	
E.coli		618	
S. altrells	565 * GRVYKLKGYEVDELSBOSKGI DVVNA I FLONDEVI STMI AVKDL-FSED-NFLVFATK	620	
Diadicab	* * * * * * * * *	020	
B.fragilis	620 · NG_VIKKTSLEOVSBDBONGUNAITTIDEDDDVIEVDMTNGNNEIIIANDNGDAIDEHEAA	678	
E-coli	639 + EEE-FGVKVEMATANGTVKKTVLTEENDL PTAGKV	672	
S-aureus	621 · BG_VVKRSALSNESBINDNCKIAISEBEDDELIAVBLTSGOEDILIGTSHASLIEEDEST	679	
Bradrodb	* * *	075	
B.fradilis	679, VRVMGRTATGVRGITI.DDDGODEVIGNICIKDI.ETESVMVVSEOGVGKRSDIEDVDKTND	738	
E.coli	673: ALKL_VDCDFLIC_VDLTSGFDFVMLFSAFCKVVDFKFSSVPAMC_CNTTCVPGLP_LCF	728	
S auroug	680. I DDI COMANCIAL DEC DEVICI DUALANCUDEVI UVERICACAREDUNDADI SND	727	
bruureab		, 51	
B.fragilis	739 GCKGVKTMNITEKTCKI.VTIKSVTDENDIMIINKSCITIBI.KVADVPIMCPATOCVPIIN	798	
E coli	729 CORVEST LUDGOCALL TATONCYCKDTAUAEVDTKSDATKCVLSLKVTEDNCLVVCAVO	788	
S-aureus	738 + GGKGIKTATITERNGNUUCITTUTGEEDI MIUTNAGUIIDI NAADI COMOBAACCUDIID	707	
2.441945	* * * * * *	, , ,	
B.fragilis	799 IFKBNDOIGSVCKVTSFSI_FDFVDFFFDFCNIBSD_D_FT_NTDUNDT_	844	
E.coli	789, UDDCDOIMMITDACTLUPTRUSEISIUGBNTOQUILIPAEDENUUCIOPUATE-UDET	846	
S aurous	798+LGD_DOFUSTUAKUKEDADEUNEDEOSTUSEDGTE_OODEAUUNDETEGMATUTEUTDED	855	
5.441645	*	0.00	
B.fragilis	845FF	845	
F coli	847.DI_DTIDGSAAFGD_DFIAPEVDVDDEPEFF	875	
5 surous	856 · ENDEDGELEVENDENDEVENDOSSDEDEE	886	
aureus 856:ENDEDGRIEVRQDFMDRVEEDIQQSSDEDEE 886			

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 ATPdependent 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 10<sup>8</sup> 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  $\mu$ g 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  $\mu$ g/ml, more than 100 colonies (second-step mutants) were able to grow. Thirdand fourth-step mutants, which grew in the presence of 12.5 and 25  $\mu$ g 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

В	B.fragilis	1:MSEE-QNPTNNGSYSADSIQVLEGLEAVRKRPAMYIG-DISVKGLHHLVYEIVDNSIDEA	58
-	E.coli	1:MSNSYDSSSIKVLKGLDAVRKRPGMYIGDTDDGTGLHHMVFEVVDNAIDEA	51
	S.aureus	1:MVTALSDVNNTDNYGAGQIQVLEGLEAVRKRPGMYIG-STSERGLHHLVWEIVDNSIDEA	59
		* * * * ** ** ***** **** **** * ***	
	R fragilie	50. I ACYCOUIFUTINEDNCITUADNOBOIDUDEEDOYCAI SUAMUU UACCYEDYCCYYU	, 1 0
	E.coli	52 · LACHCKEI LVTIHADNSVSVODDCRCIDTCIHDEECUSALEVAMIVLAGGEPDNSVEV	111
	5 aurous	52: LAGHCKEITVIIHADNSVSVQDDGRGIPIGIHFEEGVSAAEVIMIVLAAGGKFDDNSIKV 60: LAGVANOIFVUIFKDNWIKUTDNGDGIDVDIOFKMGDDAVEVIIJU HAGGKFGGGGYKU	110
	STUITCUD	*** * * * * * * * ***** * * * ********	119
	B.fragilis	119:SGGLHGVGMSCVNALSTHMTTQVFRNGKIYQQEYEIGKPLYPVKEVGIADHTGTKQQFWP	178
	E.COll	112: SGGLHGVGVSVVNALSQKLELVIQREGKIHRQIYEHGVPQAPLAVTGETEKTGTMVRFWP	171
	S.aureus	******** * ***** * * * * * * * * * * *	1/9
	B.fragilis	1/9:DDSIFTE-TIYDYKILASRLRELAYLNAGLRISLTDRRVVNEDGSFKHETFYSEEGLREF	237
	E.COLL	<pre>1 MSEE-OMPTINGSYSADSIGVLEGLEAVERKPAMYIG-DISVKGLHULVTEIVDNSTDEA 51 1.MVTALSDVNYEDNYGAGGIGVLEGLEAVERKPAGYIG-STSERGLHULVTEIVDNSTDEA 53 1.MVTALSDVNYEDNYGAGGIGVLEGLEAVERKPGWYIG-STSERGLHULVTEIVDNSTDEA 53 59.LAGYCDHIEVTIHEDNSITYQDDGKGIFYDFHEKEGKSALEVAMTVLHAGGKFDGGSYKV 118 52.LAGYCDHIEVTIHEDNSITYQDFKGKIYQUEGGLEAVERKPGWYIG-STSERGLHULVTUHAGGKFDGGSYKV 118 52.LAGYCDHIEVTIHEDNSITYQDFKGKIYQUEGGLEAVERKPGWYIL-VLHAGGKFDGGSYKV 118 52.LAGYCDHIEVTIHEDNSITYQDFKGKIYQUEGGLEAVERRPACHILVUHAGGKFDGGGYKV 119 119.SGGLBGVGMSCVNALSTHMTTQVFKNGKIYQGEYEIGKPLYPVKEVGIADHTGTKOGGVFW 178 119.SGGLBGVGMSVVNLSQLELVIQREX HAQIYENGVPAPLAYTGETETGGMVRWP 171 120.SGGLBGVGSVVNLSQLEVIQHKKIYQEYEIGKPLYPVKEVGIADHTGTKOGTWP 171 120.SGGLBGVGSVVNLSQLEVIQHKKIYKNGNGYENKKRD-C-AEDEFYSGGIKAF 255 180.DGEIFTETVIYEFILAKURGLESHASGVSIKLMKRD-C-AEDEFNGEGGYKX 1236 ************************************</pre>	
	5.duleus	** * * * * * *** ** * * * * * * * *	230
	B.fragilis	238: VRFIESSREHLINDVIYLNTEKQNIPIEVAIMYNTGFSENIHSYVNNINTIEGGTHLAGF	297
	E.COll	226:VEYLNKNKTP1HPN1FYFSTEKDGIGVEVALQWNDGFQEN1YCFTNN1PQRDGGTHLAGF	285
	S.aureus	237:VELLNENKEPIHDEPIYIHQSKDDIEVEIAIQYNSGYATNLLTYANNIHTYEGGTHEDGF * * * * * * * * * * * * * * * * * * *	296
	B.fragilis	298: RRALTRTLKKYAEDSKMLEKVKVEISGDDFREGLTAVISVKVAEPQFEGQTKTKLGNNEV	357
	E.coli	286: RAAMTRTLNAYMDKEGYSKKAKVSATGDDAREGLIAVVSVKVPDPKFSSQTKDKLVSSEV	345
	S.aureus	297: KRALTRVLNSYGLSSKIMKEEKDRLSGEDTREGMTAIISIKHGDPQFEGQTKTKLGNSEV	356
		*** * * * * * * * * * * * * * * * *	
	B.fragilis	358:NGVLPIRRYGEVLNYYLEEHPKEAKAIVDKVILAATARHAARKAREMVQRKSPMSGGGLP	417
	E.coli	346:KSAVE-QQMNELLAEYLLENPTDAKIVVGKIIDAARAREAARRAREMTRRKGALDLAGLP	404
	S.aureus	357: RQVVD-KLFSEHFERFLYENPQVARTVVEKGIMAARARVAAKKAREVTRRKSALDVASLP	415
		* * * * * * * * * * * * * * * * *	
	B.fragilis	418: GKLADCSDKDPOKCELFLVEGDSAGGTAKOGRNRAFOAILPLRGKILNVEKAMYHKALES	477
	E.coli	405: GKLADCQERDPALSELYLVEGDSAGGSAKOGRNRKNOAILPLKGKILNVEKARFDKMLSS	464
	S.aureus	416:GKLADCSSKSPEECEIFLVEGDSAGGSTKSGRDSRTQAILPLRGKILNVEKARLDRILNN	475
		****** * * ******* * ** ******	
	B.fragilis	478: EEIRNIYTALGVTIGTEEDSKAANIDKLRYHKIIIMTDADVDGSHIDTLIMTFFFRYMPO	537
	E.coli	465 + OEVATLITALGCGIGRD-EYN-PDKLRYHSIIIMTDADVDGSHIRTLLLTFFYROMPE	520
	S.aureus	476:NEIRQMITAFGTGIGGDFDLAKARYHKIVIMTDADVDGAHIRTLLLTFFYRFMRP	530
		* ** * ** * ********* ** ** *** *	
	B.fragilis	538: LIONGVLVIATPPLVLCKKGKIERYCWTDAOROKFIDTVGGGSENAIHTORVKGL-GE-M	595
	E.coli	521: IVERGHVYIAOPPLYKVKKGKOEOYIKDDEAMDOYOISIALDGATLHTNASAPALAGEAL	580
	S.aureus	531:LIEAGYVYIAQPPLYKLTQGKQKYYVYNDRELDKLKSELNPTPKWSI-A-RYKGL-GE-M	586
		* *** **** * * * * * *	
	B fragilie	596 • _N_3001 WFT_TM_DD_ENDMIKOUNTON33F3DVIFSMIMCEDUCDDDFFT_FFN3T	617
	E.coli	581 · FKLUSFVNATOKMINEMFERVDKAMLKELIVODTUTEADI.SDEOTVTDWNAIUSEINDK	640
	S. aureus	587: -N-ADOLWET-TM-NP-EHRALLOVKLEDATEADOTFEMI.MGDVVENBROFI_EDNAV	638
	Stations	* * * * * * * * * * * * * * * * * * *	0.50
	P fragilig	648.VANIDA	657
	E.coli	641 · FORGSOWKEDUHTNA FONLEED I UDUDTUCUDTUCUDTUCUTUTUTUTUTUTUTUTUTUTUTU	700
	S. aureus	639: VANLDE	644
	2+441645	0.0	044
	B.fragilis	654:	653
	E.coli	701: LEEDAFIERGERRQPVASFEQALDWLVKESRRGLSIQRYKGLGEMNPEQLWETTMDPESR	760
	S.aureus	645:	644
	B.fragilis	654:	653
	E.coli	761:RMLRVTVKDAIAADQLFTTLMGDAVEPRRAFIEENALKAANIDI	804
	S.aureus	645:	644

FIG. 3-Continued.

from L1-1 and L1-2 were identical to that of ATCC 25285. However, PCR products from second-step mutants carried a single-nucleotide change compared to the wild type. A TCTto-TTT alteration was found at codon 82, which would result in a Ser-to-Phe substitution in GyrA. Sequence analysis of PCR products from third- and fourth-step mutants (in each case, two mutants were examined) did not reveal further mutations in this region of the *gyrA* gene. The QRDR of *gyrB* (residues 436 to 467) of levofloxacin-resistant mutants was also amplified and analyzed. The nucleotide sequence of this region of all mutants was identical to that of ATCC 25285.

## DISCUSSION

We have cloned and characterized the gyrA and gyrB genes of B. fragilis. Assignment was based on close sequence homology to *E. coli* DNA gyrase subunits and the demonstration that when expressed in *E. coli*, the reconstituted GyrA and GyrB proteins showed ATP-dependent supercoiling activity, which is characteristic of DNA gyrase. The QRDR is highly conserved among *B. fragilis*, *E. coli*, and *S. aureus*. Ser-82 and Tyr-121, which are reported to be sites important in quinolone resistance and DNA breakage reunion (12, 21), respectively, were conserved among the three strains.

We isolated a series of *B. fragilis* ATCC 25285 mutants resistant to levofloxacin by stepwise selection on plates containing increasing drug concentrations (Table 1). These strains also exhibited cross-resistance to other quinolones. By examining *gyrA* genes in the quinolone-resistant ATCC 25285 mutants, we found mutation of Ser-82 to Phe in GyrA. As this residue is equivalent to the resistance hot spot Ser-83 of GyrA in *E. coli* (5, 10, 23, 31), the mechanisms of quinolone resistance resistance for the plane of the spot series of the s

St. i a	MIC (µg/ml) <sup>b</sup>			Mutation		
Strain	LVFX	STFX	CPFX	SPFX	gyrA	gyrB
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) $\rightarrow$ 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

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

<sup>a</sup> Two clones were analyzed for each strain.

<sup>b</sup> 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



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.

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.

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