ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1998, p. 2906–2913 Vol. 42, No. 11
0066-4804/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

# Mutations in *Bartonella bacilliformis gyrB* Confer Resistance to Coumermycin $A_1$

JAMES M. BATTISTI, LAURA S. SMITHERMAN, D. SCOTT SAMUELS, AND MICHAEL F. MINNICK*

*Division of Biological Sciences, The University of Montana, Missoula, Montana 59812-1002*

Received 17 April 1998/Returned for modification 1 June 1998/Accepted 13 August 1998

**This study describes the first isolation and characterization of spontaneous mutants conferring natural resistance to an antibiotic for any *Bartonella* species. The *Bartonella bacilliformis gyrB* gene, which encodes the B subunit of DNA gyrase, was cloned and sequenced. The *gyrB* open reading frame (ORF) is 2,079 bp and encodes a deduced amino acid sequence of 692 residues, corresponding to a predicted protein of ~77.5 kDa. Sequence alignment indicates that *B. bacilliformis* GyrB is most similar to the GyrB protein from *Bacillus subtilis* (40.1% amino acid sequence identity) and that it contains the longest N-terminal tail (52 residues) of any GyrB characterized to date. The cloned *B. bacilliformis gyrB* was expressed in an *Escherichia coli* S30 cell extract and was able to functionally complement a temperature-sensitive *E. coli* Cou$^r$ *gyrB* mutant (strain N4177). We isolated and characterized spontaneous mutants of *B. bacilliformis* resistant to coumermycin $A_1$, an antibiotic that targets GyrB. Sequence analysis of *gyrB* from 12 Cou$^r$ mutants of *B. bacilliformis* identified single nucleotide transitions at three separate loci in the ORF. The predicted amino acid substitutions resulting from these transitions are Gly to Ser at position 124 (Gly124→Ser), Arg184→Gln, and Thr214→Ala or Thr214→Ile, which are analogous to mutated residues found in previously characterized resistant *gyrB* genes from *Borrelia burgdorferi*, *E. coli*, *Staphylococcus aureus*, and *Haloferax* sp. The Cou$^r$ mutants are three to five times more resistant to coumermycin $A_1$ than the wild-type parental strain.**

Recent taxonomic reclassifications involving bacteria formerly constituting the *Rochalimaea* and *Grahamella* genera have rapidly expanded the number of species in the *Bartonella* genus (5, 8, 10, 23, 47). Of these 12 species, 5 are presently considered to be etiologic agents of emerging infectious disease in humans: *Bartonella bacilliformis*, *B. clarridgeiae*, *B. elizabethae*, *B. henselae*, and *B. quintana* (22, 23, 33). Hemotrophy and arthropod vector-mediated transmission are common parasitic strategies utilized by these small, gram-negative, facultatively intracellular pathogens.

Due to the lack of a system for site-specific genetic manipulation, few reports have been published concerning the molecular mechanisms involved in the pathogenesis, growth, and antibiotic resistance of *Bartonella* species (3, 15, 16, 24, 27, 29, 31, 34, 42, 46, 49). Therefore, we initially address this problem by molecularly characterizing the pathogens' *gyrB* gene. DNA gyrase is the bacterial type II topoisomerase responsible for introducing negative supercoiling into DNA (reviewed in references 20 and 37), and it is the target of several types of antimicrobial agents. The holoenzyme is an $A_2B_2$ complex encoded by the *gyrA* and *gyrB* genes; the A subunit is responsible for DNA breakage and reunion, whereas the B subunit harbors the ATP binding site. The coumarin antibiotics coumermycin $A_1$, novobiocin, and chlorobiocin impede DNA replication by inhibiting the ATP binding and hydrolysis catalyzed by GyrB (28). Several reports have demonstrated that single point mutations in the *gyrB* gene confer resistance to coumarin antibiotics (11, 13, 19, 36, 39, 44) providing a locus and selectable phenotype for allelic exchange experiments.

In this study, we describe the isolation and characterization of the first spontaneous mutants of any *Bartonella* species, as well as the first characterization of an antibiotic-resistant mutant. Analysis of coumermycin $A_1$-resistant mutants revealed single nucleotide lesions corresponding to specific amino acid substitutions in the N-terminal domain of GyrB. These mutations confer an approximately three- to fivefold increase in the MIC of coumermycin $A_1$ relative to the wild type. In addition, we show that the *B. bacilliformis gyrB* can functionally complement an *E. coli gyrB* mutant. Finally, we discuss the positions of the amino acid substitutions in *B. bacilliformis* GyrB as they relate to recently solved high-resolution crystal structures and enzyme function (26, 48).

* Corresponding author. Mailing address: Division of Biological Sciences, The University of Montana, Missoula, MT 59812-1002. Phone: (406) 243-5972. Fax: (406) 243-4184. E-mail: minnick@selway.umt.edu.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *E. coli* strains were grown overnight at 37°C in Luria-Bertani (LB) medium with standard antibiotic supplements when required (12). *B. bacilliformis* was grown and harvested as previously described (34).

To isolate coumermycin $A_1$-resistant mutants, suspensions of *B. bacilliformis* KC583 were plated on heart infusion agar supplemented with 5% erythrocytes and coumermycin $A_1$ (0.1 μg/ml; Sigma Chemical Co., St. Louis, Mo.). Coumermycin $A_1$-resistant mutants were usually observed after 5 days of growth and were harvested after 7 days. Resistant colonies were picked and resuspended in 150 μl of heart infusion broth. Resistant mutants were maintained in the presence of 0.04 μg of coumermycin $A_1$ per ml. Strains of *B. bacilliformis* and *Escherichia coli* used or generated in this study are summarized in Table 1.

**Preparation and manipulation of DNA.** Chromosomal DNA from *B. bacilliformis* for use in DNA hybridization or PCR analyses was prepared with CTAB (hexadecyltrimethyl ammonium bromide) by the methods of Ausubel et al. (2). Plasmid DNA extraction and isolation from *E. coli* for cloning were performed by the alkaline lysis procedure of Birnboim and Doly (4), and plasmid preparations for sequencing were made with either a Midi-Prep kit (Qiagen, Chatsworth, Calif.) or a Perfect Prep kit (5 PRIME-3 PRIME, Boulder, Colo.) as per the manufacturer's instructions. Cloning of individual DNA fragments was accomplished by two distinct methods. First, both λ-ZAP Express (Stratagene Cloning Systems, La Jolla, Calif.) and λ-GEM 11 (Promega, Madison, Wis.) genomic cloning systems were used as per the manufacturer's recommendations to obtain phagemid clones containing the *B. bacilliformis gyrB* gene for sequence analysis. Second, the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif.) was used as per the manufacturer's instructions to obtain a plasmid clone containing the entire wild-type *gyrB* open reading frame (ORF) for gene expression and functional complementation analyses. When required, DNA was purified from ethidium bromide-stained agarose gels or PCRs with either a GeneClean kit (Bio 101, Inc., La Jolla, Calif.) or by a QIAquick kit (Qiagen). Plasmids and recombinants used or constructed in this study are summarized in Table 1.

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| *B. bacilliformis* | | |
| KC583 | Wild-type strain | 7 |
| CR1, 2, 6, 8, 9 | KC583 GyrB$_{(Gly124\rightarrow Ser)}$ Cou$^r$ | This study |
| CR4, 7, 11, 12 | KC583 GyrB$_{(Arg184\rightarrow Gln)}$ Cou$^r$ | This study |
| CR3 | KC583 GyrB$_{(Thr214\rightarrow Ala)}$ Cou$^r$ | This study |
| CR5, 10 | KC583 GyrB$_{(Thr214\rightarrow Ile)}$ Cou$^r$ | This study |
| *E. coli* | | |
| HB101 | Host strain used for cloning | 6, Promega |
| TOP10F′ | TOPO TA Cloning Kit host strain | Invitrogen |
| N99 | Complementation analysis *strA galK* | 30 |
| N4177 | Isogenic to N99 except *gyrB*221 (Cou$^r$) and *gyrB*203 (TS) | 30 |
| Plasmids | | |
| pBK-CMV | Phagemid cloning vector | Stratagene |
| pCR2.1-TOPO | Cloning vector | Invitrogen |
| pGYRB1 | pBK-CMV recombinant containing 5′ portion of *B. bacilliformis gyrB* in an ~2,000-bp *Sau*3AI fragment; derived from λ-ZAP library | This study |
| pGYRB2 | pBK-CMV recombinant with an ~13-kb *Sac*I fragment containing the *B. bacilliformis gyrB*; derived from λ-GEM 11 library | This study |
| pGYRB3 | pCR2.1-TOPO recombinant containing entire *gyrB* gene in a 2,410-bp *Bam*HI fragment; derived from TA cloning strategy | This study |

**PCR and oligonucleotides.** PCR amplifications were achieved by using a Gene-Amp 2400 Thermocycler (Perkin-Elmer, Norwalk, Conn.) following procedures developed by Mullis et al. (35). Reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, a 200 μM concentration of each deoxynucleotide triphosphate, 4 mM $MgCl_2$, 2.5 U of Ampli*Taq* DNA polymerase (Roche Molecular Systems, Branchburg, N.J.), 1 to 100 ng of template DNA, and 0.1 μg of each primer. The reaction proceeded for 30 cycles of 1 min at 94°C, 1 min at 50 to 60°C (depending on calculated primer melting temperature), and 1 min at 72°C, with an initial 5-min denaturation at 94°C and a final 7-min extension at 72°C. Single-stranded degenerate oligonucleotide primers (based on regions of conserved homology [21]), GYRB5 (5′-AARMGNCCNGGNATGTAYATHGG-3′) and GYRB3 (5′-CCNACNCCRTGNARNCCNCC-3′), were synthesized by Gibco-BRL. Single-stranded oligonucleotide primers specific for the *B. bacilliformis gyrB* gene included the following: GYRB-F, nucleotide (nt) −219 to −187 (5′-CGCGGATCCCTGCGGAATAACAAATCATGGTG-3′); GYRB-R, nt 132 to 100 (5′-CGCGGATCCTATCGATAAAACGATCCATCTGGC-3′); LESION-F, nt 307 to 331 (5′-GCTGATTTGATTGATATAACATTGG-3′), and LESION-R, nt 711 to 688 (5′-TATAAATTTTTTCTGGGTCAAAAGC-3′).

**DNA hybridization analysis.** Total DNAs from *B. bacilliformis* KC583 and KC584 and *E. coli* HB101 were isolated, digested to completion with *Bam*HI, and then separated on an ethidium bromide-stained 1% (wt/vol) agarose gel. The gel was then blotted onto a nitrocellulose membrane (0.45-μm pore size; Schleicher & Schuell, Keene, N.H.) by the method of Southern (43) and baked for 1 h at 80°C. The 2,410-bp fragment used as the probe in this analysis was derived by PCR amplification using the amplimer set GYRB-F–GYRB-R and *B. bacilliformis* KC583 as template DNA. This 2,410-bp PCR fragment was subsequently labeled by random primer extension (14) with the Klenow fragment of *E. coli* polymerase I (Gibco-BRL) and [α-$^{32}$P]dCTP (New England Nuclear, Boston, Mass.). The blot was probed overnight at 50°C with the $^{32}$P-labeled 2,410-bp PCR fragment and then was washed and visualized as previously described (32).

DNA hybridization was also used for probing two separate λ genomic libraries to clone and sequence the *gyrB* gene. In these experiments, either the 300-bp PCR product derived from the degenerate amplimer set GYRB5-GYRB3 or the internal 1,101-bp *Hin*dIII fragment was labeled by random primer extension and used to screen the libraries.

**In vitro transcription-translation.** Expression of *gyrB* was done using an *E. coli* S30 cell in vitro transcription-translation system per the manufacturer's instructions (Promega). $^{35}$S-labelled proteins were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25) and autoradiography as previously described (34).

**In vivo complementation analysis.** A plasmid containing the cloned *gyrB* (pGYRB3) and the respective cloning vector (pCR2.1-TOPO) were separately introduced into strains N99 and N4177 by modifying the transformation procedure of Chung et al. (9) such that the culture temperature of N99 and N4177 was held below 30°C throughout the transformation procedure. Transformed clones of N99 or N4177 containing either pGYRB3 or pCR2.1-TOPO plasmids were selected by incubation at 30°C for 16 h in the presence of ampicillin (100 μg/ml). Immediately thereafter, clones of each of the four transformants (N99[pCR2.1-TOPO], N99[pGYRB3], N4177[pCR2.1-TOPO], and N4177[pGYRB3]) were simultaneously replica plated onto LB (supplemented with ampicillin [100 μg/ml]), and incubated at either 30°C (permissive temperature) or 42°C (restrictive temperature) for 20 h. Both *E. coli* host strains (N99 and N4177) were replica plated onto LB and LB-ampicillin (100 μg/ml) simultaneously for additional positive and negative controls, respectively. Replica-plated clones were scored after 20 h of growth by estimating relative colony size.

**Antibiotic susceptibility testing.** MICs were determined by two methods. Initial determination of the MIC of coumermycin $A_1$ for the wild type was achieved by plating 100 μl of *Bartonella* suspensions containing $10^5$ CFU/ml on heart infusion agar supplemented with 5% erythrocytes and coumermycin $A_1$ concentrations ranging from 0.01 to 1.0 μg/ml. Second, determination of the MICs for Cou$^r$ mutants was accomplished by an agar dilution technique previously described for *Bartonella* (27). Briefly, resistant strains were harvested after 5 days of incubation, washed, and resuspended in phosphate-buffered saline (pH 7.5). The suspensions were then equilibrated to a McFarland 0.5 standard at an optical density at 600 nm. Aliquots (10 μl) were applied to heart infusion agar supplemented with 5% erythrocytes and coumermycin $A_1$ concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 μg/ml. The MIC is defined as the concentration of coumermycin $A_1$ at which no growth is detected following 7 days of routine incubation. MICs were obtained by three independent determinations.

**Nucleotide sequencing and computer analysis.** The inserts of overlapping clones derived from λ-ZAP Express (pGYRB1) and λ-GEM 11 (pGYRB2) genomic libraries were sequenced separately to obtain the nucleotide sequence for the entire *gyrB* gene. Templates were primed with M13 universal primers or with synthetic oligonucleotides prepared with a DNA synthesizer (model 394; Applied Biosystems, Foster City, Calif.). The nucleotide sequences for both DNA strands of the *gyrB* gene were then determined by the dideoxy chain-termination method of Sanger et al. (41) using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit as per the manufacturer's instructions (Applied Biosystems). Sequencing was done on an Applied Biosystems Automated DNA Sequencer (model 373A). Sequence data were compiled and analyzed by using PC/GENE 6.8 software (Intelligenetics, Mountain View, Calif.) for restriction site determination and ORF identification, BLAST (1) for database searches, CLUSTAL W 1.6 (45) for multiple sequence alignments, and BOXSHADE 3.21 (18) for sequence alignment formatting.

**Nucleotide sequence accession number.** The GenBank accession number for the *Bartonella bacilliformis gyrB* nucleotide sequence is U82225.

## RESULTS

**Cloning the *gyrB* gene.** Two clones were required for sequence analysis of this gene. A positive plaque with the cloned *B. bacilliformis gyrB* gene was isolated from a λ-ZAP Express library (Stratagene) by probing with a [α-$^{32}$P]dCTP-labeled 300-bp PCR product generated from *B. bacilliformis* KC583 template DNA by using the degenerate oligonucleotide primers GYRB5 and GYRB3. A pBK-CMV phagemid clone was excised from the λ-ZAP Express clone and termed pGYRB1. Nucleotide sequence analysis revealed that only the 5′ portion (1094 bp) of the *gyrB* gene was present in the ~2,000-bp *Sau*3AI insert of pGYRB1.

To obtain the remainder of the sequence for *gyrB*, the 1,101-bp *Hin*dIII fragment of pGYRB1 containing the 5′ portion of the *gyrB* gene was labeled by random primer extension and used to probe a λ-GEM 11 genomic library (Promega) in hopes of obtaining a λ clone with a larger insert containing the entire *gyrB* gene. A second λ clone was identified and found to contain the entire *gyrB* gene in an ~13-kbp *Sac*I fragment by DNA hybridization. The *Sac*I fragment was excised and cloned into pBK-CMV to generate pGYRB2. The insert in pGYRB2 was used to complete the nucleotide sequencing of the wild-type *B. bacilliformis gyrB* gene.

The complete *gyrB* gene (2,410 bp) was amplified from *B. bacilliformis* KC583 DNA by using the amplimer set GYRB-

F–GYRB-R and cloned into pCR2.1-TOPO. This *gyrB* recombinant was designated pGYRB3.

**Nucleotide sequence of the *gyrB* gene.** The nucleotide sequence of the wild-type (coumermycin $A_1$-sensitive) *B. bacilliformis gyrB* gene was determined from both DNA strands and is presented in Fig. 1. Computer-assisted analysis of the *gyrB* gene showed a 2,079-bp ORF. This ORF is characterized by a common initiation codon, ATG, that is preceded by putative −35 (TTCAAA) and −10 (GATAAT) consensus regulatory elements and a potential ribosomal binding site (AGTA) (Fig. 1).

Further analysis of the ORF indicated that the encoded protein had a deduced length of 692 amino acid residues and a predicted molecular mass of approximately 77.5 kDa. BLAST (1) homology searches indicate that *B. bacilliformis* GyrB is most similar to *Bacillus subtilis* GyrB, with an amino acid sequence identity of 40.1%, whereas *B. bacilliformis* GyrB has only 18.4% identity with *B. subtilis* ParC, a GyrB homolog. The *B. bacilliformis* subunit has 34.1% identity with *E. coli* GyrB. Alignment of the deduced amino acid sequence from *B. bacilliformis gyrB* with the known amino acid sequences of GyrBs from *E. coli*, *B. subtilis*, and *Mycobacterium tuberculosis* (using CLUSTAL W 1.6) indicates multiple areas of strong homology (Fig. 2) and reveals that the *B. bacilliformis* GyrB has an unusually long N terminus. Sequence analysis of ~600 bp of flanking sequence indicate a possible gene upstream of *gyrB* with homology to lipoate-protein ligase B, whereas 3′ flanking sequence produces no areas of strong homology to database sequences (data not shown).

**DNA hybridization analysis.** In order to verify that the *gyrB*-containing fragment was of *Bartonella* origin, DNA hybridization analysis was done with *Bam*HI-digested DNA from *B. bacilliformis* strains KC583 and KC584 and from *E. coli* HB101. As shown in Fig. 3B, Southern blots probed at high stringency (7% mismatch) with a $^{32}$P-labeled 2,410-bp PCR fragment derived from *B. bacilliformis* KC583 template (by using amplimers GYRB-F and GYRB-R) clearly demonstrated single hybridization bands from both strains of *B. bacilliformis* (Fig. 3B, lanes 3 and 4). No signal was observed in *Bam*HI-digested *E. coli* HB101 DNA (Fig. 3, lane 2). In addition, the G+C content of the ORF (38.4 mol%) is in good agreement with the overall G+C content (39 mol%) of *B. bacilliformis* (7).

**In vitro expression of *gyrB*.** To determine if *E. coli* transcription-translation machinery would express the cloned *B. bacilliformis gyrB*, an *E. coli* S30 cell DNA expression kit (Promega) was used to produce polypeptides in vitro. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins expressed from pGYRB3 revealed a protein product consistent with the predicted molecular mass for GyrB of 77.5 kDa that was not expressed from the pCR2.1-TOPO control by this system (data not shown). The 77.5-kDa protein was the largest protein encoded, although additional insert-specific protein bands of approximately 68, 65, 52, and 38 kDa were observed and may have been produced by the *E. coli* S30 extract from anomalous ORFs on the noncoding strand of pGYRB3 or may be degradation products.

**Functional complementation analysis.** Since the S30 extract expressed the cloned *gyrB*, an isogenic pair of *E. coli* strains first described by Menzel and Gellert (30) was used to evaluate the in vivo function of the cloned *B. bacilliformis gyrB*. *E. coli* N99 carries a wild-type *gyrB*, and strain N4177 has two *gyrB* mutations, which together confer a coumermycin $A_1$-resistant (Cou$^r$) and temperature-sensitive (TS) phenotype. Growth of strain N4177 is permissive at 30°C but is restricted at 42°C unless a functional *gyrB* is supplied in *trans* to complement the Cou$^r$ TS mutation. Therefore, we wanted to determine whether *B. bacilliformis gyrB* could functionally complement strain N4177. To address this question, the *B. bacilliformis gyrB* recombinant pGYRB3 was introduced into strains N99 and N4177, selected at 30°C, and subsequently replica plated and separately incubated at both permissive (30°C) and restrictive (42°C) temperatures. The *B. bacilliformis gyrB* recombinant, pGYRB3, was shown to increase the growth rate of strain N4177 at 42°C by approximately threefold relative to negative controls (Table 2). The presence of plasmids or varied incubation temperature did not affect the relative growth rates of host strain N99. The pattern of growth for this analysis was consistent and reproducible and shows that *B. bacilliformis gyrB* can functionally complement the Cou$^r$ TS mutation of *E. coli* N4177.

**Isolation of coumermycin $A_1$-resistant mutants.** Spontaneous coumermycin $A_1$-resistant mutants were observed 7 days after inoculation and occurred at a frequency of $\sim 6 \times 10^{-9}$ when selected in the presence of 0.1 μg of coumermycin $A_1$ per ml. After initial selection, mutant strains were cultured on heart infusion agar supplemented with 0.04 μg of coumermycin $A_1$ per ml. A total of 12 *B. bacilliformis* KC583 coumermycin-resistant mutants were selected in this manner and designated CR1 through CR12 (Table 1). In the absence of coumermycin $A_1$, the growth rate and gross morphology of the Cou$^r$ colonies were indistinguishable from those of wild-type strains.

**Coumermycin $A_1$ resistance is correlated with mutations in the *gyrB* gene.** Genomic DNA was isolated from wild-type *B. bacilliformis* KC583 and the 12 coumermycin $A_1$-resistant mutants. The region of the *gyrB* gene encoding the N-terminal domain was amplified by PCR with LESION-F and LESION-R primers and subsequently sequenced with the LESION-F primer. Further analysis of these sequences revealed single nucleotide transitions at three separate loci that resulted in four distinct amino acid substitutions. First, in 5 of the 12 coumermycin $A_1$-resistant strains (CR1, CR2, CR6, CR8, and CR9), identical G-to-A transitions at base 370 of the 2,079-bp ORF resulted in a deduced Gly124-to-Ser (Gly124→Ser) substitution. Second, 4 of the 12 resistant strains (CR4, CR7, CR11, and CR12) carried a G-to-A transition at base 550 that resulted in a deduced Arg184→Gln substitution. The third loci at which lesions were detected occurred in the Thr214 codon, in which two different transitions were observed with two distinct deduced substitutions; the ACA-to-GCA transition resulted in a Thr214→Ala substitution (CR3), whereas the ACA-to-ATA transition resulted in a Thr214→Ile substitution (CR5, CR10). These data demonstrate that spontaneous coumermycin $A_1$-resistant mutants are correlated with specific and localized lesions in the *gyrB* gene. Table 3 summarizes several genotypic and phenotypic attributes of the coumermycin $A_1$-resistant strains.

**In vitro coumermycin $A_1$ susceptibilities.** We assessed the antibiotic susceptibility of wild-type *B. bacilliformis* KC583 to coumermycin $A_1$ by using agar dilution techniques. At coumermycin $A_1$ concentrations above 0.03 μg/ml, growth rates were noticeably decreased, and at those above 0.06 μg/ml, growth appeared to be completely inhibited. Thus, the MIC for KC583 was determined to be 0.06 μg/ml. One representative of each of the four different *gyrB* mutant types was assayed for coumermycin $A_1$ susceptibility. MICs for mutant strains CR3, CR4, and CR9 were 0.2 μg/ml, whereas CR5 demonstrated a slightly higher level of resistance, with a MIC of 0.3 μg/ml (Table 3).

## DISCUSSION

We have described the first isolation and molecular characterization of spontaneous mutant strains conferring natural

```
                                                                                      -35                -10      RBS
  -90 atgtttctta agtgcattga tttttaaata aaaaataggc ataaagcttt attttaagaa aattcaaatt taggtagata attaagtaat

    1 ATGAGCAATG ACAATAAAGA TCTTTTTAGT GTTCTAAATC ATGCTCAGTC CCGTATAGAC AGGAAAGAAA ACACTCAATA TACTTCAGCG
       M  S  N   D  N  K  D   L  F  S   V  L  N   H  A  Q  S   R  I  D   R  K  E   N  T  Q  Y   T  S  A

   91 CACTCAGAAA TAGTCGTTCC AGCCGTTCCG TTATCTTCAC CCCATCATCA TAAAGAAGAC AGCACCTATA ATGCCTCATC TATTCGAATA
       H  S  E   I  V  V  P   A  V  P   L  S  S   P  H  H  H   K  E  D   S  T  Y   N  A  S  S   I  R  I

  181 CTTGAAGGTT TAGAACCTGT ACGTTTACGA CCTGGAATGT ACATCGGTGG CACAGATAGC AAAGCACTCC ACCATTTATT CTCTGAAATT
       L  E  G   L  E  P  V   R  L  R   P  G  M   Y  I  G  G   T  D  S   K  A  L   H  H  L  F   S  E  I

  271 ATTGACAATG CGATGGACGA AGCCGTTGCA GGTTATGCTG ATTTGATTGA TATAACATTG GACAGCAACA ATTATCTGAC TGTCACAGAT
       I  D  N   A  M  D  E   A  V  A   G  Y  A   D  L  I  D   I  T  L   D  S  N   N  Y  L  T   V  T  D

  361 AATGGACGTG GCATTCCTAT TGAAAATCAT CCCCAAATAC CGGATAAATC TACCCTTGAA GTCATTATGA CACATCTTCA TTCAGGTGGA
       N  G  R   G  I  P  I   E  N  H   P  Q  I   P  D  K  S   T  L  E   V  I  M   T  H  L  H   S  G  G

  451 AAATTTGATG GAAAAGCCTA TCAAACTTCT GGTGGATTAC ATGGAGTGGG CATTTCTGTC GTTAACGCCC TCTCTGATGA TATGGAAGTA
       K  F  D   G  K  A  Y   Q  T  S   G  G  L   H  G  V  G   I  S  V   V  N  A   L  S  D  D   M  E  V

  541 GAAGTCGCAC GAGAGCGCAA ACTTTATCGC CAACGTTTCT CACGCGGAAT TCCTCAATCT GGGCTAGAAG AATTAGGCGA TGTTTATAAT
       E  V  A   R  E  R  K   L  Y  R   Q  R  F   S  R  G  I   P  Q  S   G  L  E   E  L  G  D   V  Y  N

  631 CGTCGTGGTA CACGAGTTTG TTTTCATCCT GATAGTCAAA TTTTTGGCGA AAACACAGCT TTTGACCCAG AAAAAATTTA TAAAATAGCG
       R  R  G   T  R  V  C   F  H  P   D  S  Q   I  F  G  E   N  T  A   F  D  P   E  K  I  Y   K  I  A

  721 CGCTCTAAAG CCTATCTCTT CAATGGAGTG AAAATTCGTT GGAATTGTGA TCCTGCGGCA CTTAAAGATG CAAAAAACAT CCCTGAAAAA
       R  S  K   A  Y  L  F   N  G  V   K  I  R   W  N  C  D   P  A  A   L  K  D   A  K  N  I   P  E  K

  811 GATGTTTTTT ACTTTCCAGA TGGACTGAAA GATTATTTAT CATTATCACT GAAAAATAAA CATCTTGTAA CAGCTGAAAT TTTTTCTGGT
       D  V  F   Y  F  P  D   G  L  K   D  Y  L   S  L  S  L   K  N  K   H  L  V   T  A  E  I   F  S  G

  901 AAAACACAAC AGCTTAGTGG CCATGGTTCA GTTGAATGGG CGATAGCTTG GCACAATGGT GATGCCTATA TACAATCTTA CTGTAATACC
       K  T  Q   Q  L  S  G   H  G  S   V  E  W   A  I  A  W   H  N  G   D  A  Y   I  Q  S  Y   C  N  T

  991 ATTCCTACTG AAGAAGGTGG AACACATGAA ACAGGACTAA GACAAACTCT TCTCCGTGGA TTGAAAGCTT ATGCTGAATT AATAGGAAAT
       I  P  T   E  E  G  G   T  H  E   T  G  L   R  Q  T  L   L  R  G   L  K  A   Y  A  E  L   I  G  N

 1081 AAGCGTGCCT CGATCATTAC TTCTGATGAT GTTATGGCTT CAACAGTTGT AATGCTCTCA GTCTTTATTA AAGATCCTCA GTTTGTTGGA
       K  R  A   S  I  I  T   S  D  D   V  M  A   S  T  V  V   M  L  S   V  F  I   K  D  P  Q   F  V  G

 1171 CAAACAAAAG ATCGATTAGC CACAACTGAA GCACAACGTA TCGTTGAAAA TGCAATACGT GATCCTTTCG ATCATTGGCT AGCTAATTCT
       Q  T  K   D  R  L  A   T  T  E   A  Q  R   I  V  E  N   A  I  R   D  P  F   D  H  W  L   A  N  S

 1261 CCCCATGAAG CAACAAAACT ACTAAATTGG GTTATTGAAC GAGCTGAAGA ACGTCTCAAA CGACGTCAAG ATAGAGAAAT AAATCGAAAA
       P  H  E   A  T  K  L   L  N  W   V  I  E   R  A  E  E   R  L  K   R  R  Q   D  R  E  I   N  R  K

 1351 ACTGCCGTAC GTAAATTACG CTTACCTGGA AAATTAGCAG ATTGTAGCCA AAATTCTGCC GCTGGTGCTG AATTATTTAT TGTTGAAGGT
       T  A  V   R  K  L  R   L  P  G   K  L  A   D  C  S  Q   N  S  A   A  G  A   E  L  F  I   V  E  G

 1441 GACTCTGCTG GTGGTTCTGC TAAACAAGCG CGTAATAGAA CAAATCAAGC AATTTTACCT CTGCGTGGAA AAATCTTAAA TGTAGCAAGT
       D  S  A   G  G  S  A   K  Q  A   R  N  R   T  N  Q  A   I  L  P   L  R  G   K  I  L  N   V  A  S

 1531 GCTGCACGTG AAAAAATGAG TTCAAGCCAA ACGATCGCCG ACCTAATACT CGCACTTGGA TGTGGAACGC GTTCTAAATA TCGTGAAGAA
       A  A  R   E  K  M  S   S  S  Q   T  I  A   D  L  I  L   A  L  G   C  G  T   R  S  K  Y   R  E  E

 1621 GATCTCAGGT ACGAACGTAT TATCATTATG ACCGATGCAG ATGTTGACGG TGCGCATATT GCTTCACTCT TAATTACTTT CTTCTTTCAA
       D  L  R   Y  E  R  I   I  I  M   T  D  A   D  V  D  G   A  H  I   A  S  L   L  I  T  F   F  F  Q

 1711 GAAATACCTG ATCTTATTCG TGCAGGACAT CTGTATCTCG CTGTGCCTCC CCTTTACAGA ATATCACAAG GAGGAAAGGT TGCTTACGCA
       E  I  P   D  L  I  R   A  G  H   L  Y  L   A  V  P  P   L  Y  R   I  S  Q   G  G  K  V   A  Y  A

 1801 CGCGACGATT CTCATAAAGA CGAGTTGCTA AAAACTGAAT TTACTGGAAA AGGTAAAATT GAAATTGGAC GTTTTAAAGG CCTTGGAGAA
       R  D  D   S  H  K  D   E  L  L   K  T  E   F  T  G  K   G  K  I   E  I  G   R  F  K  G   L  G  E

 1891 ATGCGTGCCG AGCAACTTAA AGAAACAACG ATGAATCCTA AAAAACGTAC ACTTTTACGT GTTTCTATTG ATACTTTTGA AATGCAAGAA
       M  R  A   E  Q  L  K   E  T  T   M  N  P   K  K  R  T   L  L  R   V  S  I   D  T  F  E   M  Q  E

 1981 ACTAAAGAAA CAGTGCAAAA TCTTATGGGA ACTAAACCGG AAGAACGGTT CCGCTTTATA CAAGAAAGCT CTACTTTCGC AAATAATTTA
       T  K  E   T  V  Q  N   L  M  G   T  K  P   E  E  R  F   R  F  I   Q  E  S   S  T  F  A   N  N  L

 2071 GATATCtgat tttcaaaagt tagtttttaa tctactgctg ctgcatcaat aacccgcagt tgcggagtaa tcgttccatt ccagtaattt
       D  I  *
```

FIG. 1. Nucleotide and predicted amino acid sequence of *B. bacilliformis gyrB*. The nucleotide sequence of a 2,250-bp fragment containing the wild-type coumermycin $A_1$-sensitive *B. bacilliformis gyrB* is shown. Nucleotides within the 2,079-bp ORF are given in uppercase letters, and the deduced 692-residue amino acid sequence is shown below each corresponding codon. Putative consensus regulatory elements are indicated (−35, −10, ribosomal binding site [RBS]). The stop codon is marked with an asterisk. The three codons (and their corresponding amino acids) in which single nucleotide substitutions resulting in coumermycin $A_1$ resistance were found are boxed. The unusually long 52-residue N terminus is shown in boldface type. The predicted molecular mass of the mature protein is 77.5 kDa. The GenBank accession number for the *gyrB* gene is U82225.

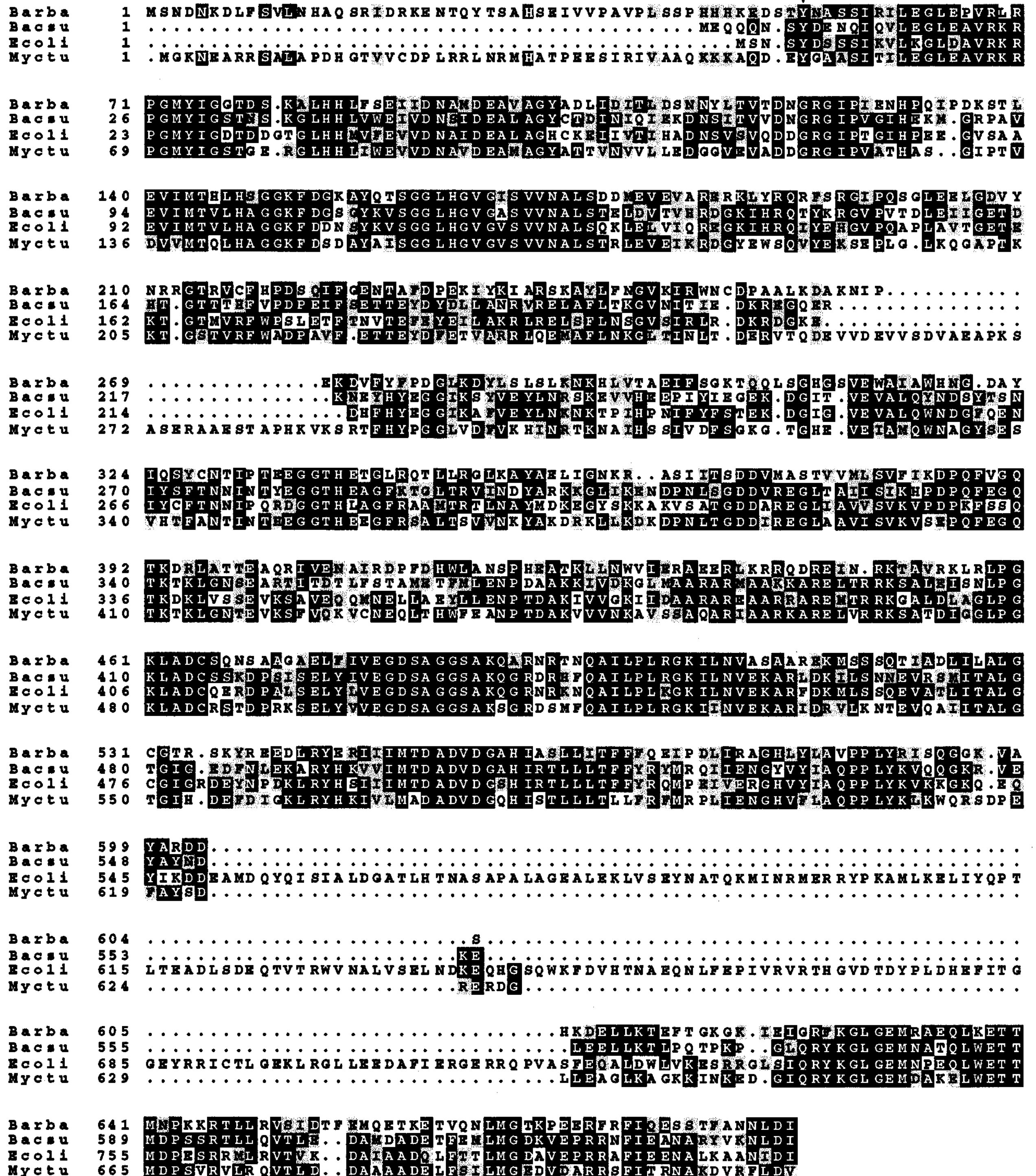

FIG. 2. Multiple alignment of *B. bacilliformis* GyrB with *B. subtilis*, *E. coli*, and *M. tuberculosis* GyrB. Multiple alignment of *B. bacilliformis* GyrB (Barba) with *B. subtilis* GyrB (Bacsu), *E. coli* GyrB (Ecoli), and *M. tuberculosis* (Myctu) generated with CLUSTAL W 1.6 (45) and formatted with BOXSHADE 3.21 (18). Identical amino acid residues are shown as white on black, conserved residues are shown as black on grey, and introduced gaps are shown as dots. Note the unusual 53-residue N-terminal extension that is similar in length to the N terminus of *M. tuberculosis* GyrB. The first universally conserved residue (*E. coli* of Tyr5) is indicated by the arrowhead. GenBank accession numbers for these GyrB sequences are U82225 (*B. bacilliformis*) D26185 (*B. subtilis*), AE000447 (*E. coli*), and X78888 (*M. tuberculosis*).

resistance to an antibiotic for any *Bartonella* species. Generation of the mutant strains was accomplished by exposure to inhibitory (0.1-μg/ml) levels of the DNA gyrase inhibitor coumermycin $A_1$ and occurred at a frequency of $\sim 6 \times 10^{-9}$.

Based upon amino acid sequence alignments, *B. bacilliformis* GyrB belongs to the shorter, 650-amino-acid size class represented by homologs of enzymes from *B. subtilis*, *Mycoplasma pneumoniae*, *Staphylococcus aureus*, *Borrelia burgdorferi*, and

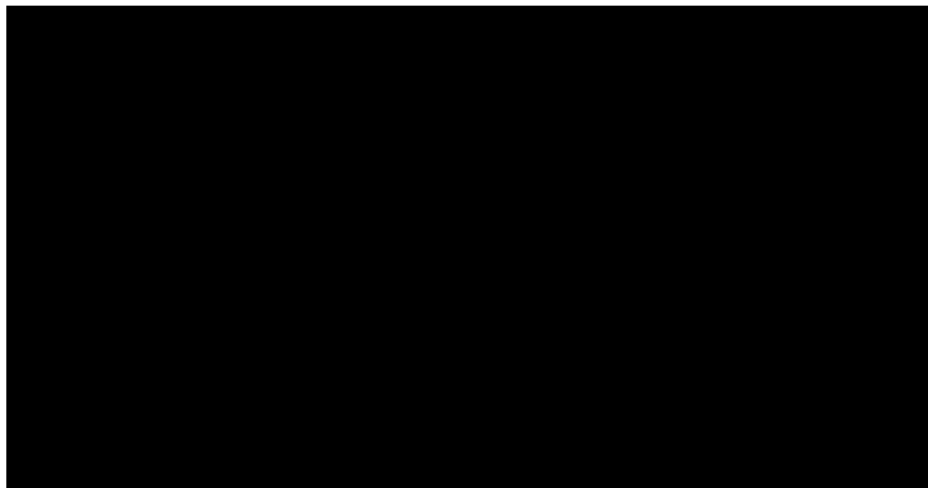

FIG. 3. Detection of the *gyrB* gene in the *B. bacilliformis* chromosome by DNA hybridization. (A) Ethidium bromide-stained agarose gel (1%, wt/vol) containing λ *Hin*dIII size standards (lane 1), *Bam*HI-digested chromosomal DNA of *E. coli* HB101 (lane 2), *Bam*HI-digested chromosomal DNA of *B. bacilliformis* KC583 (lane 3), *Bam*HI-digested chromosomal DNA of *B. bacilliformis* KC584 (lane 4), no DNA (lane 5), and 2,410-bp PCR fragment containing the entire *B. bacilliformis gyrB* ORF. (B) The corresponding autoradiograph following DNA hybridization with the described 2,410-bp PCR fragment labeled with [$^{32}$P]dCTP. The lanes are the same as those panel A. Note the hybridization signal in both *B. bacilliformis* strains (lanes 3 and 4).

*Haloferax* sp. (20). In the larger, 800-amino-acid size class, represented by *E. coli*, an extra 150-amino-acid block is found in the C-terminal domain of the protein (20) (Fig. 2). The commonly recognized ATP binding motif GXXGXG is found at positions 162 to 167 of *B. bacilliformis* GyrB, corresponding to positions 114 to 119 of *E. coli* GyrB.

The structure of the *B. bacilliformis* GyrB is unusual in two ways. First, in GyrBs sequenced to date, the first N-terminal amino acid that demonstrates universal conservation throughout bacteria is a Tyr residue represented by *E. coli* Tyr5, corresponding to Tyr53 of *B. bacilliformis* (Fig. 2). The side chain of Tyr5 hydrogen bonds to the bound ATP analog (48). The number of amino acids preceding this conserved Tyr is less than 13 for nearly all bacteria examined to date. *B. bacilliformis* GyrB is unusual in this respect in that 52 amino acid residues precede the *E. coli* Tyr5 homolog, making it the longest N-terminal extension reported to date. Only *M. tuberculosis* has an N-terminal extension of this magnitude, with 50 amino acids (Fig. 2); however, the two extensions are not homologous. The crystal structure of the *E. coli* GyrB N-terminal domain complexed with a nonhydrolyzable ATP analog shows that the N-terminal 13 residues form a protrusion that interacts with the other GyrB protomer (48). This interaction stabilizes the dimer interface and forms part of the ATP binding site (48). However, the N terminus is apparently not ordered in the cocrystal structure with the coumarin inhibitor novobiocin (26). The function of the unusually long N-terminal extensions of *M. tuberculosis* and *B. bacilliformis* GyrBs is intriguing and remains to be determined. A second primary structural feature of *B. bacilliformis* GyrB that we have noted is Glu128 (*E. coli* equivalent, Gly81). In all wild-type GyrBs reported thus far, this residue is either glycine or aspartate, with the exception of those found in the *Mycobacteria*, which have alanine or glutamate at this position. In this respect, *B. bacilliformis* GyrB is also more similar to the mycobacterial GyrB. This position is one of three loci that is mutated in a novobiocin-resistant *Haloferax* (Asp82→Gly) (19), although it is distant from the coumarin binding site (26). Although both *B. bacilliformis* and *M. tuberculosis* are slow-growing bacteria and have several similar GyrB structural features, the effect of these properties on interactions with ATP or coumarins is unknown.

TABLE 2. Complementation with *B. bacilliformis gyrB*

| *E. coli* strain | Growth at 42°C with the following plasmid[a]: | | |
|---|---|---|---|
| | None | pCR2.1-TOPO | pGYRB3 |
| N99 | +++ | +++ | +++ |
| N4177 | +/− | +/− | +++ |

[a] Growth is based upon colony size and logarithmic growth kinetics as measured by optical density at 600 nm. The temperature chosen (42°C) is the restrictive temperature for N4177 (30). Symbols: +++, robust growth; +/−, slight growth.

The mechanism of coumermycin $A_1$ resistance in *B. bacilliformis* mutants was identified by sequencing PCR fragments generated with primers amplifying the portion of the *gyrB* gene that encodes the N-terminal domain. We have isolated 12 coumermycin $A_1$-resistant mutants and have identified single nucleotide transitions at three separate loci resulting in single amino acid substitutions in the N-terminal domain of the GyrB protein. Lesions detected in the resistant *B. bacilliformis gyrB* genes are analogous in location and residue substitution to previously characterized resistant *gyrB* genes (11, 13, 19, 39, 40, 44). The crystal structure has revealed important interactions for each of the lesion sites. First, the side group of the *E. coli* Arg136 residue (*B. bacilliformis* Arg184) makes critical hydrogen bonds with the coumarins and with *E. coli* Tyr5 (*B. bacilliformis* Tyr53) on the other protomer (which is involved in ATP binding) (26). The second and third residues associated with coumarin resistance, *E. coli* Gly77 (*B. bacilliformis* Gly124) and *E. coli* Thr165 (*B. bacilliformis* Thr214), specifically interact with each other as well as stabilize interactions with ATP and coumarins (26).

These data demonstrate that the *B. bacilliformis* DNA gyrase B protein is a target for coumarin antibiotics. Wild-type *B. bacilliformis* (MIC, 0.06 μg/ml) was shown to be more susceptible to growth inhibition by coumermycin $A_1$ than almost all other bacteria tested (50) and is 250 times more susceptible than *E. coli* (17). These data are consistent with the finding that *Bartonella* is extremely susceptible to a variety of antibac-

TABLE 3. Genotypic and phenotypic analysis of *B. bacilliformis gyrB* mutants

| Residue | Substitution | Frequency[a] | MIC (μg/ml) of coumermycin $A_1$[b] | Homologous GyrB lesions (reference[s]) |
|---|---|---|---|---|
| Gly124 | Ser | 41.6 | 0.2 | *B. burgdorferi* Gly74→Ser (40)<br>*S. aureus* Gly85→Ser (44) |
| Arg184 | Glu | 33.3 | 0.2 | *E. coli* Arg136→Leu, Cys, His, Ser (11, 13)<br>*B. burgdorferi* Arg133→Gly, Ile (39)<br>*S. aureus* Arg144→Ile (44)<br>*Haloferax* sp. Arg137→His (19) |
| Thr214 | Ala | 8.3 | 0.2 | *B. burgdorferi* Thr162→Ile (40)<br>*S. aureus* Thr173→Asn (44) |
| Thr214 | Iso | 16.6 | 0.3 | |

[a] The frequency is expressed as a percentage of the 12 total isolates.
[b] The MIC for wild-type KC 583 was 0.06 μg/ml. The MIC was determined for CR3, CR4, CR5, and CR9, which are representative of each of the mutant types.

terial agents in vitro (27). The mutant strains demonstrated an approximately fivefold increase in resistance levels. The MICs for GyrB mutants represented by strains CR3, CR4, and CR9 were determined to be 0.2 μg/ml, whereas the MIC for CR5 was 0.3 μg/ml. This suggests that a Thr214→Ile substitution confers a higher level of resistance than Thr214→Ala, Gly124→Ser, or Arg184→Gln, consistent with findings in *B. burgdorferi* (40).

The transition between the diverse thermal environments of the arthropod vector and the human host, as well as the presentation of the verruga peruana on the extremities (<37°C), suggests that there is a close relationship between temperature and gene expression in *B. bacilliformis. Yersinia enterocolitica* DNA gyrase mutants simulate thermoinduced alterations of DNA supercoiling with coincident phenotypic changes (38). Likewise, DNA topology regulated by DNA gyrase may play an important role in the survival or virulence of *B. bacilliformis* in both the vector and host, and DNA gyrase mutants may provide a method for analysis of thermoregulation.

## ACKNOWLEDGMENTS

We thank Joan Strange (The University of Montana Murdock Molecular Biology Facility) for her excellent technical assistance with nucleic acid sequencing and Marty Gellert and Mary O'Dea for their contribution of strains N99 and N4177. D.S.S. thanks Tony Maxwell for useful discussions.

This work was supported by Public Health Service grants AI34050 and RR10169 (to M.F.M.) and AI39695 (to D.S.S.) from the National Institutes of Health (NIAID) and National Science Foundation grant MCB-9722408 (to D.S.S.).

## REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Short protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Benson, L. A., S. Kar, G. McLaughlin, and G. M. Ihler.** 1986. Entry of *Bartonella bacilliformis* into erythrocytes. Infect. Immun. **54:**347–353.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Birtles, R. J., T. G. Harrison, N. A. Saunders, and D. H. Molyneux.** 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int. J. Syst. Bacteriol. **45:**1–8.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
7. **Brenner, D. J., S. P. O'Connor, D. G. Hollis, R. E. Weaver, and A. G. Steigerwalt.** 1991. Molecular characterization and proposal of a neotype strain for *Bartonella bacilliformis*. J. Clin. Microbiol. **29:**1299–1302.
8. **Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt.** 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int. J. Syst. Bacteriol. **43:**777–786.
9. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
10. **Clarridge, J. E., T. J. Raich, D. Pirwani, B. Simon, L. Tsai, M. C. Rodriguez-Barradas, R. Regnery, A. Zollo, D. C. Jones, and C. Rambo.** 1995. Strategy to detect and identify *Bartonella* species in routine clinical laboratory yields *Bartonella henselae* from human immunodeficiency virus-positive patient and unique *Bartonella* strain from his cat. J. Clin. Microbiol. **33:**2107–2113.
11. **Contreras, A., and A. Maxwell.** 1992. *gyrB* mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. Mol. Microbiol. **6:**1617–1624.
12. **Davis, R. W., D. Bostein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **del Castillo, I., J. L. Vizan, M. C. Rodriguez-Sainz, and F. Moreno.** 1991. An unusual mechanism for resistance to the antibiotic coumermycin $A_1$. Proc. Natl. Acad. Sci. USA **88:**8860–8864.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
15. **Garcia, F. U., J. Wojta, K. N. Broadly, J. M. Davidson, and R. L. Hoover.** 1990. *Bartonella bacilliformis* stimulates endothelial cells *in vitro* and is angiogenic *in vivo*. Am. J. Pathol. **136:**1125–1135.
16. **Garcia, F. U., J. Wojta, and R. L. Hoover.** 1992. Interactions between live *Bartonella bacilliformis* and endothelial cells. J. Infect. Dis. **165:**1138–1141.
17. **Gellert, M., M. H. O'Dea, T. Itoh, and J.-I. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73:**4474–4478.
18. **Hofmann, K., and M. D. Baron.** 1996. BOXSHADE 3.21. http://www.isrec.isb-sib.ch/software/BOX_form.html.
19. **Holmes, M. L., and M. L. Dyall-Smith.** 1991. Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria. J. Bacteriol. **173:**642–648.
20. **Huang, W. M.** 1994. Type II DNA topoisomerase genes, p. 201–222. *In* L. F. Liu (ed.), DNA topoisomerases, biochemistry and molecular biology. Academic Press, San Diego, Calif.
21. **Huang, W. M.** 1992. Multiple DNA gyrase-like genes in eubacteria, p. 39–48. *In* T. Andoh, H. Ideda, and M. Oguro (ed.), Molecular biology of DNA topoisomerases and its application to chemotherapy: proceedings of the International Symposium on DNA Topoisomerases in Chemotherapy, Nagoya, Japan. CRC Press, Boca Raton, Fla.
22. **Koehler, J. E.** 1996. *Bartonella* infections. Adv. Pediatr. Infect. Dis. **11:**1–27.
23. **Kordick, D. L., E. J. Hilyard, T. L. Hadfield, K. H. Wilson, A. G. Steigerwalt, D. J. Brenner, and E. B. Breitschwerdt.** 1997. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch fever). J. Clin. Microbiol. **35:**1813–1818.
24. **Kreier, J. P., and M. Ristic.** 1981. The biology of hemotrophic bacteria. Annu. Rev. Microbiol. **35:**325–338.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
26. **Lewis, R. J., O. M. P. Singh, C. V. Smith, T. Skarzynski, A. Maxwell, A. J. Wonacott, and D. B. Wigley.** 1996. The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. EMBO J. **15:**1412–1420.
27. **Maurin, M., S. Gasquet, C. Ducco, and D. Raoult.** 1995. MICs of 28 antibiotic compounds for 14 *Bartonella* (formerly *Rochalimea*) isolates. Antimicrob. Agents Chemother. **39:**2387–2391.

28. **Maxwell, A.** 1993. The interaction between coumarin drugs and DNA gyrase. Mol. Microbiol. **9:**681–686.
29. **McGinnis Hill, E., A. Raji, M. S. Valenzuela, F. Garcia, and R. Hoover.** 1992. Adhesion to and invasion of cultured human cells by *Bartonella bacilliformis*. Infect. Immun. **60:**4051–4058.
30. **Menzel, R., and M. Gellert.** 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell **34:**105–113.
31. **Mernaugh, G., and G. M. Ihler.** 1992. Deformation factor: an extracellular protein synthesized by *Bartonella bacilliformis* that deforms erythrocyte membranes. Infect. Immun. **60:**937–943.
32. **Minnick, M. F., R. A. Heinzen, M. E. Frazier, and L. P. Mallavia.** 1990. Characterization of the *cbbE′* gene of *Coxiella burnetii*. J. Gen. Microbiol. **136:**1099–1107.
33. **Minnick, M. F.** *Bartonella* species. *In* M. Sussman (ed.), Molecular medical microbiology, in press. Academic Press, London, United Kingdom.
34. **Mitchell, S. J., and M. F. Minnick.** 1995. Characterization of a two-gene locus from *Bartonella bacilliformis* associated with the ability to invade human erythrocytes. Infect. Immun. **63:**1552–1562.
35. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. **155:**335–350.
36. **Muñoz, R., M. Bustamante, and A. G. de la Campa.** 1995. Ser-127-to-Leu substitution in the DNA gyrase B subunit of *Streptococcus pneumoniae* is implicated in novobiocin resistance. J. Bacteriol. **177:**4166–4170.
37. **Reece, R. J., and A. Maxwell.** 1991. DNA gyrase: structure and function. Crit. Rev. Biochem. Mol. Biol. **26:**335–375.
38. **Rohde, J. R., J. M. Fox, and S. A. Minnich.** 1994. Thermoregulation in *Yersinia enterocolitica* is coincident with changes in DNA supercoiling. Mol. Microbiol. **12:**187–199.
39. **Samuels, D. S., R. T. Marconi, W. M. Huang, and C. F. Garon.** 1994. *gyrB* mutations in coumermycin $A_1$-resistant *Borrelia burgdorferi*. J. Bacteriol. **176:**3072–3075.
40. **Samuels, D. S., J. Alverson, S. W. Knight, C. H. Eggers, C. F. Garon, W. M. Huang, and B. J. Kimmel.** Unpublished data.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Scherer, D. C., I. DeBuron-Connors, and M. F. Minnick.** 1993. Characterization of *Bartonella bacilliformis* flagella and effect of antiflagellin antibodies on invasion of human erythrocytes. Infect. Immun. **61:**4962–4971.
43. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
44. **Stieger, M., P. Angehrn, B. Wohlgensinger, and H. Gmünder.** 1996. GyrB mutations in *Staphylococcus aureus* strains resistant to cyclothialidine, coumermycin, and novobiocin. Antimicrob. Agents Chemother. **40:**1060–1062.
45. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
46. **Walker, T. S., and H. H. Winkler.** 1981. *Bartonella bacilliformis*: colonial types and erythrocyte adherence. Infect. Immun. **31:**480–486.
47. **Weiss, E., and G. A. Dasch.** 1982. Differential characteristics of strains of *Rochalima*: *Rochalima vinsonii* sp. nov., the Canadian vole agent. Int. J. Syst. Bacteriol. **32:**305–314.
48. **Wigley, D. B., G. J. Davies, E. J. Dodson, A. Maxwell, and G. Dodson.** 1991. Crystal structure of the N-terminal domain of the DNA gyrase B protein. Nature **351:**624–629.
49. **Xu, Y.-H., Z.-Y. Lu, and G. M. Ihler.** 1995. Purification of deformin, an extracellular protein synthesized by *Bartonella bacilliformis* which causes deformation of erythrocyte membranes. Biochim. Biophys. Acta **1234:**173–183.
50. **Zimmer, C., K. Storl, and J. Storl.** 1990. Microbial DNA topoisomerases and their inhibition by antibiotics. J. Basic Microbiol. **30:**209–224.