1-2003

gyrA Mutations in Ciprofloxacin-Resistant Bartonella Bacilliformis Strains Obtained in Vitro

Michael F. Minnick
University of Montana - Missoula, mike.minnick@mso.umt.edu

Zachary R. Wilson

Laura S. Smitherman

D. Scott Samuels
University of Montana - Missoula, scott.samuels@umontana.edu

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2003, p. 383–386 Vol. 47, No. 1
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.1.383–386.2003

gyrA Mutations in Ciprofloxacin-Resistant *Bartonella bacilliformis* Strains Obtained In Vitro

Michael F. Minnick,* Zachary R. Wilson, Laura S. Smitherman, and D. Scott Samuels

Division of Biological Sciences, The University of Montana, Missoula, Montana 59812

Received 2 April 2002/Returned for modification 5 August 2002/Accepted 1 October 2002

We isolated and characterized mutants of *Bartonella bacilliformis* that are resistant to the fluoroquinolone antibiotic ciprofloxacin, which targets the A subunit of DNA gyrase. Mutants had single point mutations in the *gyrA* gene that changed either Asp-90 to Gly or Asp-95 to Asn and had 3- or 16-fold higher resistance, respectively, to ciprofloxacin than did wild-type *B. bacilliformis*. Asp-95 is homologous to Asp-87 of *Escherichia coli* GyrA and is a common residue mutated in fluoroquinolone-resistant strains of other bacteria. This is the first report of a mutation at an Asp-90 homologue, which corresponds to Asp-82 in *E. coli* GyrA.

Bartonella bacilliformis is the bacterial agent of Carrión's disease in humans, an ailment endemic to the high-altitude regions of Ecuador, Colombia, and Peru (12). The pathogen is transmitted between humans through the bite of contaminated phlebotamine sand flies (22), and three outcomes of infection are possible. In the first syndrome, a patient develops chronic, asymptomatic bacteremia. Usually, these patients are indigenous to the area where the disease is endemic and may serve as reservoirs of infection (12). In the second syndrome, a patient develops chronic angiomatic lesions of the skin, referred to as verruga peruana, that are virtually indistinguishable from bacillary angiomatosis that can develop during infection by *Bartonella quintana* or *Bartonella henselae* (21). Although rarely fatal, verruga peruana lesions may scar the patient and can be accompanied by chronic bacteremia (22). The third syndrome, Oroya fever, produces a life-threatening course of acute hemolytic anemia characterized by disseminated erythrocyte infection and a severe reduction in hematocrit (16). Patients who present with Oroya fever are often not indigenous to areas where the disease is endemic (10, 17). Oroya fever is fatal in 40 to 88% of patients unless antibiotics are administered (10, 17, 22). Antimicrobial therapy varies with syndrome and includes the use of chloramphenicol for Oroya fever (4, 19) and of streptomycin or rifampin for verruga peruana (13). Reports of successful treatment of a limited number of infected patients with fluoroquinolones (ciprofloxacin) or macrolides (roxithromycin or erythromycin) hold promise for alternative therapeutic strategies (13).

The primary targets of fluoroquinolone antibiotics are the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (6, 9). Both enzymes catalyze the cleavage, passage, and reunion of double-stranded DNA in an ATP-dependent fashion (6, 15). However, DNA gyrase introduces negative supercoiling in order to relieve torsional stress imposed on DNA during transcription and replication, whereas topoisomerase IV decatenates interlinked daughter chromosomes following replication and is involved in relaxation (6, 15, 25). Both enzymes are A_2B_2 tetramers. The A subunit (GyrA or ParC) catalyzes DNA breakage and reunion, whereas the B subunit (GyrB or ParE) binds and hydrolyzes ATP to drive the process (15).

Fluoroquinolones effectively inhibit type II DNA topoisomerases by disrupting DNA breakage-reunion reactions (6, 15). The result is an accumulation of lethal, double-stranded breaks (11, 24). Resistance to these drugs is typically conferred by point mutations in the quinolone resistance-determining region (QRDR) located near the N terminus of the A subunits of both gyrase (GyrA) and topoisomerase IV (ParC) (6, 23). The specific target of fluoroquinolones, either DNA gyrase or topoisomerase IV, varies among different bacterial species as well as with different fluoroquinolones (1, 6–8, 14). Given the growing potential of ciprofloxacin for treatment of bartonellosis (13), this study was undertaken to genetically determine the specific target and frequency of mutations that confer resistance to this drug. We hypothesized that mutations in *gyrA* of *B. bacilliformis* would cause resistance to ciprofloxacin.

GyrA, a target of fluoroquinolone antibiotics, has not previously been described in any species of *Bartonella*. The entire *gyrA* gene of strain KC583 (ATCC 35685) was analyzed to obtain a wild-type sequence for comparison with fluoroquinolone-resistant mutants. The *gyrA* gene was cloned from a λ Zap Express (Stratagene) genomic library of *B. bacilliformis* and sequenced as previously described (3). The gene contains 2,784 bp and encodes a protein of approximately 103 kDa. The open reading frame is characterized by a GTG initiation codon and is preceded by a putative strong promoter region spanning nucleotides −16 to −65 (promoter neural network score = 0.93). The *gyrA* gene is flanked upstream by a 560-bp gene

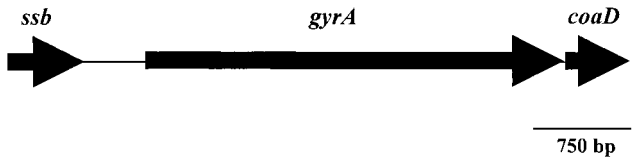

FIG. 1. Linkage map of the *B. bacilliformis gyrA* locus, including two putative flanking genes encoding a single-stranded DNA binding protein (*ssb*) and phosphopantetheine adenylyltransferase (*coaD*).

* Corresponding author. Mailing address: Division of Biological Sciences, The University of Montana, Missoula, MT 59812-4824. Phone: (406) 243-5972. Fax: (406) 243-4184. E-mail: mike.minnick@mso.umt.edu.

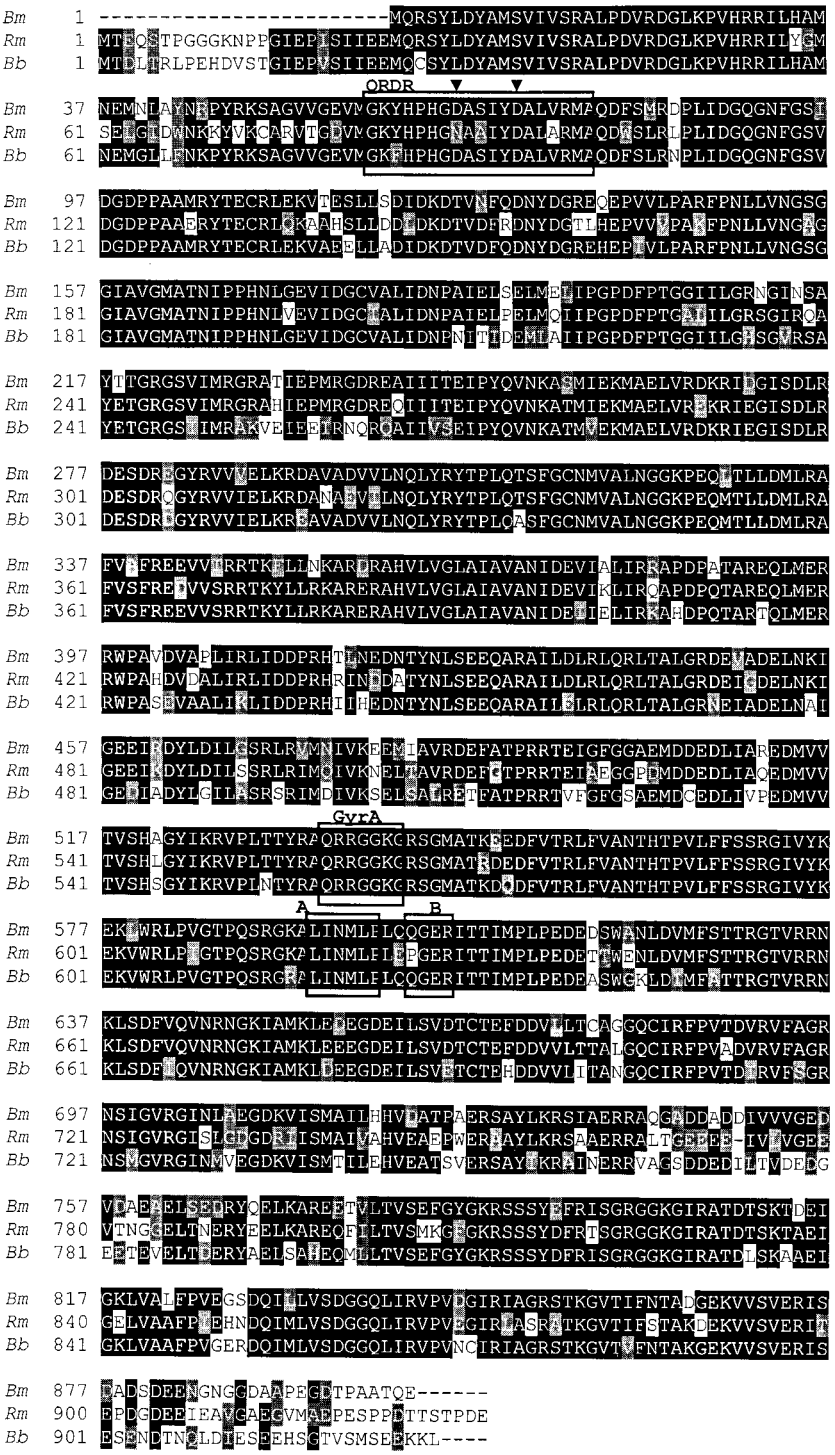

FIG. 2. Multiple-sequence alignment of *B. bacilliformis* GyrA (*Bb*) with GyrA proteins from *Rhizobium meliloti* (*Rm*) and *Brucella melitensis* (*Bm*). Identical amino acid residues are shown in black, conserved residues are shown in gray, and introduced gaps are indicated with hyphens. The GenBank accession numbers for the *Rm* and *Bm* homologues are NP385666 and NP539801, respectively. The QRDR is boxed and the nested ciprofloxacin resistance mutations observed at Asp-90 and Asp-95 are indicated by arrowheads. The highly conserved GyrA, A, and B boxes of the GyrA subunit are also shown.

encoding a putative single-stranded DNA binding protein (*ssb*) and is followed immediately by a 492-bp gene encoding the phosphopantetheine adenylyltransferase enzyme (*coaD*) (Fig. 1). The encoded GyrA protein contains the GyrA-specific consensus sequences, including the A and B boxes plus the GyrA box (Fig. 2), all of which are elements that are absent in ParC (20). BLASTp searches suggest that GyrA homologues from other α-*Proteobacteria*, including *Brucella melitensis* and *Agrobac-*

terium tumefaciens (81 and 77% amino acid identities, respectively), are the closest relatives, while *E. coli* GyrA shares 55% identity with this protein (Fig. 2). Unlike GyrB of *B. bacilliformis* (2), GyrA does not have an unusually long N terminus compared with those of closely related homologues (Fig. 2).

Wild-type *B. bacilliformis* strain KC583 was routinely grown for 4 days at 30°C and 100% relative humidity on heart infusion agar (Difco, Detroit, Mich.) supplemented with 4% sheep erythrocytes and 2% sheep serum (Quad Five, Ryegate, Mont.). Antibiotic-resistant mutants were obtained by plating cells on medium supplemented with 0.4 μg of ciprofloxacin (Pentex; Miles Inc., Kankakee, Ill.)/ml. Twenty of the resulting mutants were isolated. The putative QRDR sequence of *B. bacilliformis gyrA* (Fig. 2) was amplified by PCR using primers QRDR-F (CATGCGATGAATGAAATGGGACTTTTG) and QRDR-R (AAACGACATTCCGTGTAACGCATCGC) and sequenced on both strands as previously described (2).

The mutation rate was calculated to be 6×10^{-9} ciprofloxacin-resistant mutants per generation, a value that is about 10-fold higher than the estimated point mutation rate of *E. coli* (5) and that is the same as the rate we observed for coumermycin A_1 resistance in *B. bacilliformis gyrB* (2). Twenty ciprofloxacin-resistant mutants were obtained in vitro, and their QRDR regions were characterized by sequence analysis. Of these, four contained transitions of GAT to AAT (G_{283} to A), encoding predicted substitutions of Asn for Asp-95. A single mutant contained a transition of GAT to GGT (A_{269} to G), encoding a predicted substitution of Gly for Asp-90. Asp-90 and Asp-95 are homologous to Asp-82 and Asp-87 of *E. coli* GyrA, respectively. The level of ciprofloxacin resistance of the mutant with the A_{269}-to-G mutation was threefold higher (MIC of ciprofloxacin, 0.9 μg/ml) than that of the wild-type parental strain, for which the MIC was 0.3 μg/ml (a previous study [18] obtained a similar MIC [0.25 μg/ml] for the same wild-type strain), and the level for the mutant with the G_{283}-to-A mutation was approximately 16-fold higher (MIC, 4.7 μg/ml) (these MICs were determined as described previously [2]). The remaining 15 ciprofloxacin-resistant mutants apparently possess mutations that map outside the *gyrA* QRDR, possibly being located in *gyrB*, *parC*, or other regions of *gyrA* (6). The replacement of Asp-95 by Asn that was observed in the majority of ciprofloxacin QRDR mutants of *B. bacilliformis* is similar to the mutation of Asp-87 to Asn commonly observed in *E. coli* and the homologous Asp-94 and Asp-95 substitutions reported for several other bacteria (6). Mutations at Asp-90 or its homologues, including Asp-82 of *E. coli* GyrA, that are associated with fluoroquinolone resistance have not, to our knowledge, been reported previously in the literature.

This study includes the first characterization of a *gyrA* gene for the *Bartonella* genus, a group responsible for several emerging infectious diseases of humans. The sequence data will be useful for future studies examining mechanisms of fluoroquinolone resistance in these pathogens. Our results also show that *B. bacilliformis* mutations occur in the *gyrA* QRDR of ciprofloxacin-resistant mutants, particularly at residue Asp-95 and infrequently at Asp-90. Our data also suggest that fluoroquinolone resistance in *B. bacilliformis* arises more frequently from mutations that map outside the *gyrA* QRDR. Although genetic tools are limited in *B. bacilliformis*, the association of these two mutations within the well-established QRDR of GyrA is strong evidence for their involvement in fluoroquinolone resistance. This is the first description of mutations in a *Bartonella* species that may cause resistance to a clinically useful antimicrobial agent. These data may have clinical relevance as ciprofloxacin and other fluoroquinolones gain popularity as treatment regimens for bartonellosis.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper is listed in GenBank under accession no. AF469609.

We thank Patty McIntire of the Murdock Molecular Biology Facility at The University of Montana for her assistance in nucleotide sequence analysis and Peter Heisig for helpful discussions.

Research support was provided by Public Health Service grant AI45534 and American Heart Association grant 9940002N (to M.F.M.) and National Science Foundation grant MCB-9722408 (to D.S.S.). Z.R.W. was a recipient of an NSF-EPSCoR undergraduate research assistantship.

REFERENCES

1. **Bagel, S., V. Hullen, B. Wiedemann, and P. Heisig.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob. Agents Chemother. **43:**868–875.
2. **Battisti, J. M., L. S. Smitherman, D. S. Samuels, and M. F. Minnick.** 1998. Mutations in *Bartonella bacilliformis gyrB* confer resistance to coumermycin A_1. Antimicrob. Agents Chemother. **42:**2906–2913.
3. **Carroll, J. A., S. A. Coleman, L. S. Smitherman, and M. F. Minnick.** 2000. Hemin-binding surface protein from *Bartonella quintana*. Infect. Immun. **68:**6750–6757.
4. **Cuadra, M. S.** 1956. Salmonellosis complication in human bartonellosis. Tex. Rep. Biol. Med. **14:**97–113.
5. **Drake, J. W.** 1991. A constant rate of spontaneous mutation in DNA-based microbes. Proc. Natl. Acad. Sci. USA **88:**7160–7164.
6. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
7. **Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13:**641–653.
8. **Fournier, B., and D. C. Hooper.** 1998. Mutations in topoisomerase IV and DNA gyrase of *Staphylococcus aureus*: novel pleiotropic effects on quinolone and coumarin activity. Antimicrob. Agents Chemother. **42:**121–128.
9. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. I. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
10. **Gray, G. C., A. A. Johnson, S. A. Thornton, W. A. Smith, J. Knobloch, P. W. Kelley, L. Obregon Escudero, M. Arones Huayda, and F. S. Wignall.** 1990. An epidemic of Oroya fever in the Peruvian Andes. Am. J. Trop. Med. Hyg. **42:**215–221.
11. **Khodursky, A. B., and N. R. Cozzarelli.** 1998. The mechanism of inhibition of topoisomerase IV by quinolone antibacterials. J. Biol. Chem. **273:**27668–27677.
12. **Kreier, J. P., and M. Ristic.** 1981. The biology of hemotrophic bacteria. Annu. Rev. Microbiol. **35:**325–338.
13. **Maguina Vargas, C.** 1988. Bartonellosis or enfermedad de Carrión. Nuevos aspectos de una vieja enfermedad. A.F.A. Editores Importadores S.A., Lima, Peru.
14. **Pan, X. S., and L. M. Fisher.** 1998. DNA gyrase and topoisomerase IV are dual targets of clinafloxacin action in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **42:**2810–2816.
15. **Reece, R. J., and A. Maxwell.** 1991. DNA gyrase: structure and function. CRC Crit. Rev. Biochem. Mol. Biol. **26:**335–375.
16. **Reynafarje, C., and J. Ramos.** 1961. The hemolytic anemia of human bartonellosis. Blood **17:**562–578.
17. **Schultz, M. G.** 1968. A history of bartonellosis (Carrion's disease). Am. J. Trop. Med. Hyg. **17:**503–515.
18. **Sobraquès, M., M. Maurin, R. J. Birtles, and D. Raoult.** 1999. In vitro susceptibilities of four *Bartonella bacilliformis* strains to 30 antibiotic compounds. Antimicrob. Agents Chemother. **43:**2090–2092.
19. **Urteaga, B. O., and E. H. Payne.** 1955. Treatment of the acute febrile phase of Carrion's disease with chloramphenicol. Am. J. Trop. Med. **4:**507–511.
20. **Ward, D., and A. Newton.** 1997. Requirement of topoisomerase IV *parC* and *parE* genes for cell cycle progression and developmental regulation in *Caulobacter crescentus*. Mol. Microbiol. **26:**897–910.
21. **Webster, G. F., C. J. Cockerell, and A. E. Friedman-Kien.** 1992. The clinical spectrum of bacillary angiomatosis. Br. J. Dermatol. **126:**535–541.
22. **Weinman, D.** 1965. The *bartonella* group, p. 775–785. *In* R. J. Dubos and

J. G. Hirsch (ed.), Bacterial and mycotic infections of man. Lippincott, Philadelphia, Pa.
23. **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.
24. **Yoshida, H., M. Nakamura, M. Bogaki, H. Ito, T. Kojima, H. Hattori, and S. Nakamura.** 1993. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **37:**839–845.
25. **Zechiedrich, E. L., A. B. Khodursky, S. Bachellier, R. Schneider, D. Chen, D. M. Lilley, and N. R. Cozzarelli.** 2000. Roles of topoisomerases in maintaining steady-state DNA supercoiling in *Escherichia coli*. J. Biol. Chem. **275:**8103–8113.