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gyrB Mutations in Coumermycin A_1-Resistant *Borrelia burgdorferi*

D. SCOTT SAMUELS,[1]* RICHARD T. MARCONI,[1] WAI MUN HUANG,[2] AND CLAUDE F. GARON[1]

Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840,[1] and Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, Utah 84132[2]

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We have isolated and characterized mutants of *Borrelia burgdorferi* that are resistant to the antibiotic coumermycin A_1, which targets the B subunit of DNA gyrase. Mutants had either 100- or 300-fold higher resistance to coumermycin A_1 than wild-type *B. burgdorferi*. In each case, a single point mutation in the *gyrB* gene converted Arg-133 to Gly or Ile. Mutations in the homologous Arg residue of *Escherichia coli* DNA gyrase are also associated with resistance to coumarin antimicrobial agents.

Lyme disease in North America is caused by the spirochete *Borrelia burgdorferi* (4, 38). *B. burgdorferi* has an unusual eubacterial genome composed of mostly linear DNA molecules (2, 3, 5, 10, 15, 18, 35, 36) with a few circular DNA molecules (2, 21, 33, 36). We have recently shown that *B. burgdorferi* is highly susceptible to growth inhibition by coumarin antimicrobial agents (33), which inhibit the enzyme DNA gyrase (13, 17, 24, 28, 42). Furthermore, the circular DNA molecules of *B. burgdorferi* are relaxed by coumermycin A_1 treatment (33).

DNA gyrase is a prokaryotic type II DNA topoisomerase, which introduces negative supercoiling into DNA by transiently nicking both strands of the helix and using ATP hydrolysis to pass another portion of the DNA molecule through the double-stranded break (9, 12, 16, 28). The enzyme, which is required for cell growth and replication, is a tetramer composed of two A subunits and two B subunits. The A subunit interacts with DNA and is responsible for the breaking-rejoining reaction, while the B subunit contains the ATPase activity. Coumarin drugs, such as coumermycin A_1, bind to the B subunit of DNA gyrase and inhibit its ATPase activity (17, 25, 37, 39), most likely by a noncompetitive mechanism that involves stabilizing a protein conformation with a low affinity for ATP (1, 24).

Resistance to coumarin drugs has been mapped to *gyrB*, the gene encoding DNA gyrase B (14, 17, 19, 27, 29). Molecular studies have demonstrated that mutations in the N-terminal domain of DNA gyrase B, which contains the ATP-binding site (1, 41), confer drug resistance (8, 11, 19, 40). The most common mutations in *Escherichia coli* are those that change Arg-136 (in the N-terminal domain) of DNA gyrase B to Leu, Cys, His, or Ser (8, 11). In addition, a mutation that changes Gly-164 to Val confers a temperature-sensitive resistance to the coumarin antibiotic chlorobiocin (8). A mutant of the halophilic archaebacterium *Haloferax* sp. resistant to the coumarin antibiotic novobiocin had mutations at three residues, which correspond to Gly-81 (which is not a conserved residue), Ser-121, and Arg-136 in *E. coli* (19). The mutation at the Arg residue is thought to be primarily responsible for the drug resistance (24).

There are very few mutants of *B. burgdorferi* derived from selection (6, 7, 30, 31) and none that are resistant to an antimicrobial agent. This dearth of mutants has hindered genetic experiments. We therefore isolated several coumermycin A_1-resistant variants, characterized their susceptibility to the selective agent, and found mutations in their *gyrB* genes at Arg-133, which corresponds to Arg-136 in *E. coli*.

Selection of coumermycin A_1-resistant variants. *B. burgdorferi* B31 (ATCC 35210) has been extensively passaged in culture and was grown at 32 to 34°C in Barbour-Stoenner-Kelly (BSK) II medium lacking gelatin (33). Coumermycin A_1 (Sigma) in dimethyl sulfoxide was added directly to *B. burgdorferi* cultures in BSK II medium (yielding a final dimethyl sulfoxide concentration of less than 0.02%). *B. burgdorferi* was grown in solid medium as previously described (22, 23). Briefly, 240 ml of P-BSK (75 g of bovine serum albumin [fraction V; Pentex, Miles] per liter, 7.5 g of Neopeptone [Difco] per liter, 9 g of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] per liter, 1.1 g of sodium citrate per liter, 7.5 g of glucose per liter, 1.2 g of sodium pyruvate per liter, 0.6 g of *N*-acetyl-D-glucosamine per liter, 3.3 g of sodium bicarbonate per liter, 3.8 g of TC Yeastolate [Difco] per liter, NaOH to pH 7.5), 38 ml of 10× CMRL-1066 (without L-glutamine and sodium bicarbonate; Life Technologies), 12 ml of rabbit serum (trace hemolyzed; Pel-Freez), 20 ml of fresh 5% sodium bicarbonate, and 200 ml of 1.7% agarose (high-strength analytical grade; Bio-Rad) were mixed at 55°C; 35 ml was poured into 150-mm-diameter dishes and allowed to solidify. The medium was equilibrated to 42°C, and 45 ml was mixed with 2.5 ml of *B. burgdorferi* in liquid BSK II and poured on top of the solid bottom layer. Plates were incubated at 32 to 34°C in a humidified 5% CO_2 atmosphere. Coumermycin A_1-resistant variants were selected by plating 2.5 ml of a log-phase culture of *B. burgdorferi* B31 (~10^8 bacteria per ml) in 0.1 μg of coumermycin A_1 per ml (top and bottom agarose), which inhibits growth of the parent cells by ~80% (33). Ten isolated colonies were picked (with a Pasteur pipet) from five dishes after 2 to 4 weeks and grown in liquid BSK II with 0.1 μg of coumermycin A_1 per ml. Cultures of coumermycin A_1-resistant *B. burgdorferi* were replated, and single colonies were picked a second time from top agarose containing 0.1 μg of coumermycin A_1 per ml.

The gross morphology and plasmid content of the spontaneous coumermycin A_1-resistant variants were indistinguishable from those of the parental B31 strain (data not shown). However, the generation time of all 10 variants was 11 h,

* Corresponding author. Phone: (406) 363-9224. Fax: (406) 363-9204. Electronic mail address: scott@rml.niaid.nih.gov.

TABLE 1. Rate of growth, susceptibility to coumermycin A_1, and amino acid residue at position 133 in the B subunit of DNA gyrase for wild-type and coumermycin A_1-resistant mutants of *B. burgdorferi*[a]

Strain	Doubling time (h [SE])	IC_{90} (μg/ml)	DNA gyrase B residue 133
B31	10 (0.1)	0.2	Arg
CR8A	11 (0.2)[b]	20	Gly
CR10E	11 (0.3)[b]	60	Ile

[a] Growth was assayed in 10-ml cultures by centrifuging the cells, resuspending them in 1 ml of Dulbecco's phosphate-buffered saline, and determining the A_{600} as described previously (33). Inhibitory concentrations of coumermycin A_1 were determined by inoculating 10 ml of BSK II medium with 10^6 bacteria per ml in the presence or absence of coumermycin A_1, incubating the cells at 34°C for 72 to 75 h (at which time the cultures in the absence of antibiotics reached 1×10^8 to 1.5×10^8 bacteria per ml), and assaying growth. The IC_{90} (previously termed MIC) was defined as the concentration of antibiotic that inhibited growth by 90% relative to growth in the absence of antibiotic (33).

[b] $P \leq 0.05$ compared with B31 as determined by *t* tests on the means of four independent experiments.

compared with 10 h for B31 (Table 1). The variants could be divided into two groups, based on the level of resistance to coumermycin A_1. Variant CR8A had an IC_{90} (the concentration that inhibits the growth of 90% of the cells) of 20 μg/ml, 100-fold higher than that of parental B31 (Table 1). The second group, consisting of the other nine variants (represented by CR10E), had an IC_{90} of 60 μg of coumermycin A_1 per ml, 300-fold higher than that of the parental strain (Table 1). All of the variants maintained coumermycin A_1 resistance after at least 30 generations in the absence of selection. Growth was inhibited by 50% in the presence of 0.02, 3, and 10 μg of coumermycin A_1 per ml for B31, CR8A, and CR10E, respectively.

Mutations in the *gyrB* gene correlated with coumermycin A_1 resistance. DNA was isolated from wild-type and coumermycin A_1-resistant *B. burgdorferi* as described previously (33). The gyrase genes have been mapped to near the center of the linear chromosome (5, 26). The region of the *gyrB* gene encoding a portion of the N-terminal domain was amplified by PCR, using a GeneAmp kit (Perkin-Elmer Cetus) with 292F (5′-GGTG GTAAGTTTAATAAAGGCACG) and 582R (5′-GTTTA AAAAAGCAAGCTCTTTAAG) as primers (20, 26). We determined the sequence of the *gyrB* gene from nucleotides 316 to 558 (Fig. 1A), using a dsDNA Cycle Sequencing System (Life Technologies/BRL). This N-terminal 81-amino-acid region shares 50% amino acid identity with the *E. coli* protein. This conservation provides an unequivocal alignment of the *gyrB* gene products in which the *B. burgdorferi* Arg-133 corresponds to Arg-136 of *E. coli* (Fig. 1B). This Arg residue was conserved in *B. burgdorferi* 212 (26), B31 (Fig. 1B), JD-1, Sh-2-82, and CA-11.2A (data not shown). The B31 sequence was identical to the 212 sequence (GenBank accession number L14948) between nucleotides 316 and 558. CR8A, which is 100-fold more resistant than the wild type, was found to have an A-to-G transition (Fig. 1A) that resulted in an Arg-133-to-Gly change. All nine members of the CR10E group, which are 300-fold more resistant than the wild type, were found to have a G-to-T transversion (Fig. 1A) that converted Arg-133 to Ile. No other mutations were found in the region of *gyrB* that encodes amino acid residues 106 to 186 (which includes Ser-118 and Gly-161, corresponding to Ser-121 and Gly-164, respectively, in *E. coli*) in any of the coumermycin A_1-resistant variants (Fig. 1A). In addition, Southern blotting indicates that there is only one copy of the *gyrB* gene per chromosome in CR10E (34).

A

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B31  TATAAAGTTT CTGGGGGACT TCATGGCGTT GGAATTTCGG TTGTAAATGC  365
8A   .......... .......... .......... .......... ..........
10E  .......... .......... .......... .......... ..........

B31  TCTATCTTCG TTTTTAGAGG TTTATGTTAA TAGAGATGGA AAAATTTTTA  415
8A   .......... .......... .......... .G........ ..........
10E  .......... .......... .......... ..T....... ..........

B31  GGCAAACTTT TTCAAAAGGT ATTCCGACTT CTAAAGTAGA AGTTGTGGGG  465
8A   .......... .......... .......... .......... ..........
10E  .......... .......... .......... .......... ..........

B31  GAATCTTCTG TTACGGGGAC TAAGGTTACT TTTTTGGCGG ATTCTGAAAT  515
8A   .......... .......... .......... .......... ..........
10E  .......... .......... .......... .......... ..........

B31  TTTTGAAACT TTAGATTATA ATTTCGATGT TCTTGAAAAA AGG          558
8A   .......... .......... .......... .......... ...
10E  .......... .......... .......... .......... ...
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B

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ECOGYRB   YKVSGGLHGVGVSVVNALSQKLELVIQREGKIHRQIYEHGVPQ----AP  153
BBUGYRB   ..........I.......SF..VYVN.D...F..TFSK.I.TSK----  150
HALGYRB   .Q................SE..VEVK.D.AVWTHRF.V.E..VEEFER  158
SAUGYRB   ..........S........D..VYVH.NET.YH.A.KK....FD----  161
BSUGYRB   ..........A.......TE.DVTVH.D......T.KR...VTD----  155
PPUGYRB   ..................E..V.TVR.S...WE.T.V.....-----..  155
NGOGYRB   ..I..............DWVT.T.Y.D..E.FVRFVR.ETE----E.  157
MPNGYRB   ..........A.......SSFKVWVA..HQQYFLAFHN.GEVIGDL--  159
CCRGYRB   ............D.....KW...L.H.N..V.QMRF.R.DAVTS----  174
PMIGYRB   ..................E....T.H.D....Q...RS...D----DR  153

ECOGYRB   LAVTGETEKTGTMVRFWPSLETFTNVTEFEYEILAKR  190
BBUGYRB   VE.V..SSV...K.T.LADS.I.-ETLDYNFDV.E..  186
HALGYRB   VRDLEPG.D...TI....DDGI.-ET...DFKT.EN.  194
SAUGYRB   .KEV.T.D....VI..KADG.I..ET.VYN..T.QQ.  198
BSUGYRB   .EII...DH...TTH.V.DP.I.SET..YD.DL..N.  192
PPUGYRB   M..V..S.T...HIH.K..A...K.I-H.SWD.....  191
NGOGYRB   .KIV.DSD.K..T...LAGT...G.I-.YSFD.....  193
MPNGYRB   VNEGKCDKEH..K.E.V.DFTVM-EKSDYKQTVI.S.  195
CCRGYRB   .K...DSPVR-.EGPK-AG-..L.G-..VTFF       202
PMIGYRB   .K.I...D.S..F.......D..KGE...          182
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FIG. 1. (A) Nucleic acid sequence of a 243-bp region of the *gyrB* gene from parental B31 and coumermycin A_1-resistant *B. burgdorferi*. Nucleotides 316 to 558 from *gyrB* of strains B31, CR8A, and CR10E, which encode a region of the N-terminal domain of DNA gyrase B, are shown. The sequences of the other eight variants of the CR10E group are identical to the sequence of CR10E. Dots indicate nucleotide identity. (B) Comparison between the protein sequences of an 81-amino-acid region of DNA gyrase B from *E. coli* (ECO), *B. burgdorferi* (BBU), *Haloferax* sp. (HAL), *Staphylococcus aureus* (SAU), *Bacillus subtilis* (BSU), *Pseudomonas putida* (PPU), *Neisseria gonorrhoeae* (NGO), *Mycoplasma pneumoniae* (MPN), *Caulobacter crescentus* (CCR), and *Proteus mirabilis* (PMI). The GenBank accession numbers are X04341, L14948, M38373, X71437, X02369, X54631, M59981, X53555, U00592, and M58352, respectively. The CLUSTAL program from PC/Gene 6.26 was used to compare predicted sequences. The *P. mirabilis* and *C. crescentus* sequences are not complete. The conserved Arg residue is in boldface; dots indicate amino acid identity, and dashes indicate introduced gaps.

Conclusions and discussion. Coumermycin A_1 is an antibiotic that interacts with the B subunit of DNA gyrase. We have isolated coumermycin A_1-resistant variants of *B. burgdorferi* and have mapped single point mutations correlating with drug resistance to Arg-133 of DNA gyrase B. This is the first report of a mutation in a Lyme disease agent that confers resistance to an antibiotic. The site of mutation is consistent with mutations at the conserved Arg residue previously observed in *E. coli* (Arg-136) and *Haloferax* sp. (Arg-137), which confer resistance to coumarin antibiotics (24). A corresponding Arg residue is found in all DNA gyrase B proteins whose sequence is known (Fig. 1B) except that from *Streptomyces sphaeroides*, which is the producer of the coumarin antibiotic novobiocin (40). In *E. coli*, mutations of Arg-136 to His, Arg-136 to Ser or Cys, and Arg-136 to Leu confer 5-, ~20-, and 64-fold resistance, respectively, to coumarin drugs (8, 11), while the three

mutations, including Arg-137 to His, confer ~1,000-fold resistance to coumarin drugs in *Haloferax* sp. (19). The mutation in *B. burgdorferi* that correlates with coumermycin A_1 resistance is Arg-133 to Ile or Gly, neither of which has been previously described in any other bacteria. The level of resistance in these variants is 100- to 300-fold relative to the wild-type level. We did not identify any mutations of Arg-133 to Ser, which is possible with a single base change from the *B. burgdorferi* Arg codon (AGA). This may be because the Arg-133-to-Ser change does not confer enough resistance to allow for selection under our conditions (despite the low concentration of coumermycin A_1 used). On the other hand, an Arg-136-to-Gly mutation has not been detected in *E. coli* in spite of extensive searches (24). This may be due to differences between the DNA gyrase B proteins of the two bacteria: although they share 54% overall identity, they are significantly different in size. The crystal structure of the N-terminal domain of the B subunit in *E. coli* indicates that Arg-136 interacts with Tyr-5 (41) and may, therefore, have an indirect role in forming the ATP binding pocket (24).

The coumermycin A_1-resistant mutants grew slightly slower than the wild-type strain. This slower growth rate may be due to the decreased activity of the drug-resistant DNA gyrase, although some coumarin-resistant strains of *E. coli* grow at the same rate as wild-type strains (8). The slower growth phenotype may either be unique to *B. burgdorferi* or not detected in *E. coli* because of its rapid generation time relative to *B. burgdorferi*. Preliminary results suggest that the level of supercoiling in the coumermycin A_1-resistant *B. burgdorferi* mutants CR8A and CR10E are lower than in wild-type B31 (32), indicative of a mutant DNA gyrase (8). We are currently using coumarin-resistant *gyrB* as a selectable marker for genetic studies in *B. burgdorferi* (34).

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