

Mutation in the DNA Gyrase A Gene of *Escherichia coli* That Expands the Quinolone Resistance-Determining Region

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In three *Escherichia coli* mutants, a change (Ala-51 to Val) in the gyrase A protein outside the standard quinolone resistance-determining region (QRDR) lowered the level of quinolone susceptibility more than changes at amino acids 67, 82, 84, and 106 did. Revision of the QRDR to include amino acid 51 is indicated.

The quinolones are antibacterial agents that act by forming ternary complexes with DNA gyrase and DNA topoisomerase IV on chromosomal DNA. Resistance to the compounds is generally associated with amino acid substitutions in portions of the GyrA (gyrase) and ParC (topoisomerase IV) proteins called the quinolone resistance-determining regions (QRDRs) (20). In *Escherichia coli*, the GyrA QRDR spans amino acids 67 to 106, with alteration at positions 83 and 87 often associated with clinical resistance (19). A similar association with resistance has been observed for a variety of pathogens (1, 6, 12, 16, 17), suggesting that resistance is due to altered drug targets. The use of fluoroquinolones that mitigate the protective effects of alterations at positions 83 and 87 (7, 21) may cause other alleles to assume a more important role in reducing the level of susceptibility (4). To help define additional sites that may contribute to resistance, we examined three nalidixic acid-resistant mutants of *E. coli* that are also thermotolerant (2, 3).

Independent, nalidixic acid-resistant (Nal^r) strains of *E. coli* CGSC 6353 (3) were obtained by selecting for growth on Luria-Bertani (LB) agar (9) containing 20 µg of nalidixic acid per ml, and members of the thermotolerant (T/r⁺) subset were identified by growth on LB agar plates at 48°C. These strains were designated MF1, MF3, and MF4-1; a Nal^r strain that lacked thermotolerance was designated MF13. Nal^r was mapped by P1-mediated transduction (18), DNA was isolated by phenol extraction, PCR was used to amplify regions of the *gyrA* gene, and nucleotide sequences in amplified regions were determined by automated sequencing. The protective effect of the *gyrA* mutations was compared by determining the fluoroquinolone concentration required to inhibit colony formation by 99% (MIC₉₉) rather than by standard MIC determinations to focus more sharply on bacteriostatic activity. For this measurement cells grown to the stationary phase in LB medium were diluted and applied to quinolone-containing agar plates; the colonies were counted after incubation at 37°C for 1 day. Preliminary determinations with twofold dilutions of the fluoroquinolone provided an approximate value for the MIC₉₉; a second measurement, plus a replicate, then used linear drug

concentration increments that were 10 to 20% of the MIC₉₉. The numbers of colonies recovered were plotted against the drug concentration to determine the MIC₉₉ by interpolation.

P1-mediated transduction showed that a *gyrA* mutation was associated with nalidixic acid resistance. In this experiment a *zfa-3145::Tn10 Kan^r* marker, which is 70% cotransducible with *gyrA*, was first transferred by transduction from strain CAG 12183 (Nal^s Kan^r) (14) into strain MF4-1 (Nal^r T/r⁺). About 30% of the Kan^r transductants retained the Nal^r phenotype, indicating that a mutation at or near *gyrA* can confer nalidixic acid resistance. Then, the Nal^r marker was transferred into wild-type cells. Two Kan^r Nal^r transductants (KD1719 and KD1720) from the initial transduction were used to prepare phage lysates that were used to infect the parental Nal^s strain (CGSC 6353) and two other wild-type strains, DM4100 (15) and C600 (5). Cotransduction frequencies between Kan^r and Nal^r were 70, 80, and 70% for the three recipient strains, respectively. Thus, a mutation at or near *gyrA* was sufficient to confer nalidixic acid resistance.

The change associated with Nal^r in the *gyrA* QRDR was determined by nucleotide sequence analysis following PCR with primers 1037 and 1038 (Table 1). As shown in Table 2, the parental strain (CGSC 6353) had a predicted amino acid sequence identical to a sequence found in GenBank (accession no. X06744). In contrast, Nal^r T/r⁺ mutants (strains MF4-1, MF1, and MF3) contained a change that altered amino acid 51

TABLE 1. Oligonucleotides used for PCR and sequence determination

Oligonucleotide no.	Nucleotide sequence	Position in <i>gyrA</i>
1037	5'-AGTGGATCCGTAATTGGCAAG ACAAACGAG-3'	-110 to -134
1038	5'-CGGCCATCAGTTCATGGGCA-3'	390 to 409
1042	5'-TTGAATTCAAACAAGGGAGAT AGCTCC-3'	2651 to 2674
1043	5'-TACGGAATCCGTCTGGCGA-3'	366 to 385
1044	5'-TCATTAACGTCGCGGT-3'	665 to 684
1045	5'-TCCCAGACCCAGTTGCAGGT-3'	964 to 984
1046	5'-TGCTCGAACGTGCTGGCGAC-3'	1268 to 1288
1047	5'-CGCCAACAGCGCAGACATCA-3'	1565 to 1585
1048	5'-GTCAACCTGCTGCCGCTGGA-3'	1858 to 1878
1049	5'-TCGCGGTATTTCGCTTAGGTG-3'	2163 to 2183
1050	5'-CAGGGCGTGATCCTCATCCG-3'	2458 to 2478

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TABLE 2. Properties of nalidixic acid-resistant mutants

Strain	Relevant phenotype	Nucleotide sequence (amino acid) in GyrA	
		Codon 51	Codon 87
CGSC 6353	Nal ^s T/r ⁻	GCC (Ala)	GAC (Asp)
MF4-1	Nal ^r T/r ⁺	GTC (Val)	GAC (Asp)
MF1	Nal ^r T/r ⁺	GTC (Val)	GAC (Asp)
MF3	Nal ^r T/r ⁺	GTC (Val)	GAC (Asp)
MF13	Nal ^r T/r ⁻	GCC (Ala)	TAC (Tyr)

from alanine to valine. The Nal^r mutant that was not thermotolerant, strain MF-13, had a 5' base change (G to T) in codon 87 expected to reduce the level of quinolone susceptibility by substituting tyrosine for aspartic acid (11).

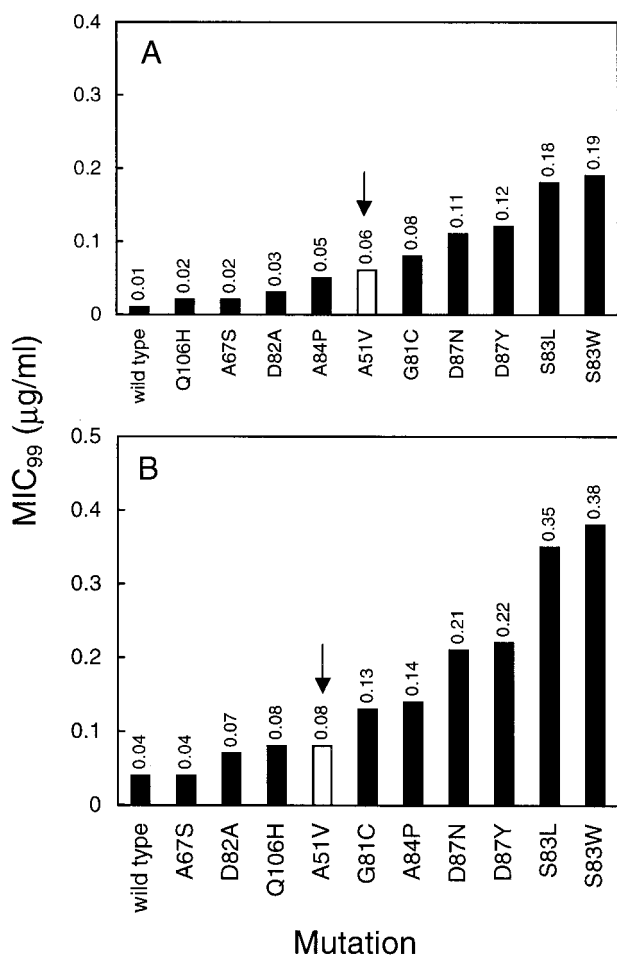


FIG. 1. Relative susceptibilities of gyrase mutants to fluoroquinolones. The MIC₉₉s (see text) of ciprofloxacin (A) and gatifloxacin (B) were determined for a series of GyrA variants and are indicated above each bar (each determination was made twice with similar results). For illustrative purposes, the results for the mutant with the Ala-51-to-Val substitution are shown in white and are indicated by the arrows. All strains are *gyrA* Nal^r transductants of wild-type strain DM4100, as described in the text or elsewhere (8). Amino acid changes in the GyrA QRDR and strain numbers (in parentheses) are as follows: S83L (KD66), A51V (KD1721), A67S (KD1911), G81C (KD1915), S83W (KD1909), D87N (KD1913), Q106H (KD1917), D82A (KD1973), A84P (KD1975), and D87Y (KD1977).

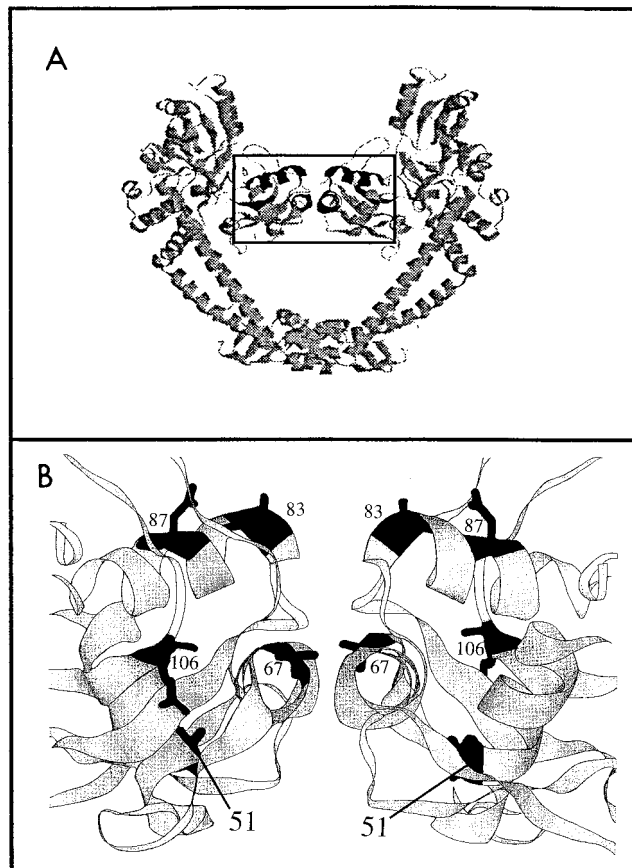


FIG. 2. Structure of the GyrA59 dimer. The figure shows a ribbon representation (generated in RasMol) of the GyrA59 fragment (10), courtesy of J. G. Heddle (John Innes Centre, Norwich, United Kingdom). (A) The entire GyrA59 dimer. (B) An enlargement of the boxed region in panel A. Amino acids that change to confer quinolone resistance are indicated in black and by the amino acid numbers. Amino acid 51 is in helix 2, amino acid 67 is in helix 3, and amino acids 83 and 87 are in helix 4.

To determine whether a Nal^r T/r⁺ isolate contains additional changes in *gyrA*, we determined the sequence of the entire gene for parental (CGSC 6353) and mutant (MF4-1) strains. The *gyrA* gene was amplified with primers 1037 and 1042 (Table 1) and sequenced with primers 1043 through 1050 (Table 1). No further differences were observed between these two strains. Thus, an Ala-51-to-Val change in GyrA is sufficient to confer nalidixic acid resistance.

The Ala-51-to-Val substitution was neither necessary nor sufficient to confer thermotolerance: a Nal^s transductant of MF4-1 (strain KD1567) retained thermotolerance. Moreover, the Nal^r transductants of CGSC 6353, DM4100, and C600 were not thermotolerant: Nal^r transductants and their parental strains were indistinguishable with respect to growth on salt-free LB agar plates at both 43.5 and 46°C. In previous work (3), we reported that a plasmid-borne, wild-type *gyrA* gene suppressed both Nal^r and T/r⁺, suggesting that a GyrA alteration was necessary for thermotolerance. Suppression of thermotolerance by expression of GyrA from a plasmid probably arises from the presence of multiple copies of the *gyrA* gene. The genetic basis for thermotolerance remains unclear.

We compared the loss of quinolone susceptibility associated with the Ala-51-to-Val change to that seen with other GyrA variants. As shown in Fig. 1, Val-51 was associated with intermediate susceptibility to ciprofloxacin and gatifloxacin that was greater than that seen for four other amino acid changes generally considered to be within the QRDR (Ala-67 to Ser, Asp-82 to Ala, and Gln-106 to His). In the case of ciprofloxacin, this was also true for the Ala-84-to-Pro change. We conclude that the QRDR should be expanded to include position 51.

Examination of the crystal structure of the GyrA59 dimer (10) reveals that Ala-51 lies in helix 2, which is below the DNA recognition helix (helix 4; Fig. 2). Changes at positions 83 and 87 that cause the greatest loss in quinolone susceptibility are located on the surface of the recognition helix (Fig. 2), where quinolone binding may occur (8, 10, 13, 22). We speculate that the Ala-51-to-Val substitution distorts the region, altering its ability to interact with fluoroquinolones.

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REFERENCES

- Bachoual, R., L. Dubreuil, C.-J. Soussy, and J. Tankovic. 2000. Roles of *gyrA* mutations in resistance of clinical isolates and in vitro mutants of *Bacteroides fragilis* to the new fluoroquinolone trovafloxacin. *Antimicrob. Agents Chemother.* **44**:1842–1845.
- Friedman, S. M., M. L. Droffner, and N. Yamamoto. 1991. Thermotolerant nalidixic acid-resistant mutants of *Escherichia coli*. *Curr. Microbiol.* **22**:311–316.
- Friedman, S. M., M. Malik, and K. Drlica. 1995. DNA supercoiling in a thermotolerant mutant of *Escherichia coli*. *Mol. Gen. Genet.* **248**:417–422.
- Ince, D., and D. Hooper. 2000. Mechanisms and frequency of resistance to premarloxacin in *Staphylococcus aureus*: novel mutations suggest novel drug-target interactions. *Antimicrob. Agents Chemother.* **44**:3344–3350.
- Jendrisak, J., R. Young, and J. Engel. 1978. In S. Berger and A. Kimmel (ed.) *Guide to molecular cloning techniques*, p. 359–371. Academic Press, Inc., New York, N.Y.
- Jones, M. E., D. F. Sahn, N. Martin, S. Scheuring, P. Heisig, C. Thornsberry, K. Köhrer, and F.-J. Schmitz. 2000. Prevalence of *gyrA*, *gyrB*, *parC*, and *parE* mutations in clinical isolates of *Streptococcus pneumoniae* with decreased susceptibilities to different fluoroquinolones and originating from worldwide surveillance studies during the 1997–1998 respiratory season. *Antimicrob. Agents Chemother.* **44**:462–466.
- Kitamura, A., K. Hoshino, Y. Kimura, I. Hayakawa, and K. Sato. 1995. Contribution of the C-8 substituent of DU-6859a, a new potent fluoroquinolone, to its activity against DNA gyrase mutants of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **39**:1467–1471.
- Lu, T., X. Zhao, and K. Drlica. 1999. Gatifloxacin activity against quinolone-resistant gyrase: allele-specific enhancement of bacteriostatic and bactericidal activity by the C-8-methoxy group. *Antimicrob. Agents Chemother.* **43**:2969–2974.
- Miller, J. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Morais-Cabral, J. H., A. P. Jackson, C. V. Smith, N. Shikotra, A. Maxwell, and R. C. Liddington. 1997. Crystal structure of the breakage-reunion domain of DNA gyrase. *Nature* **388**:903–906.
- Nakamura, S. 1997. Mechanisms of quinolone resistance. *J. Infect. Chemother.* **3**:128–138.
- Schmitz, F.-J., A. Fluit, S. Brisse, J. Verhoef, K. Koher, and D. Milatovic. 1999. Molecular epidemiology of quinolone resistance and comparative in vitro activities of new quinolones against European *Staphylococcus aureus* isolates. *FEMS Immunol. Med. Microbiol.* **26**:281–287.
- Sindelar, G., X. Zhao, A. Liew, Y. Dong, J. Zhou, J. Domagala, and K. Drlica. 2000. Mutant prevention concentration as a measure of fluoroquinolone potency against mycobacteria. *Antimicrob. Agents Chemother.* **44**:3337–3343.
- Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross. 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**:1–24.
- Sternglanz, R., S. DiNardo, K. A. Voelkel, Y. Nishimura, Y. Hirota, A. K. Becherer, L. Zumstein, and J. C. Wang. 1981. Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affecting transcription and transposition. *Proc. Natl. Acad. Sci. USA* **78**:2747–2751.
- Sullivan, E. A., B. N. Kreiswirth, L. Palumbo, V. Kapur, J. M. Musser, A. Ebrahimzadeh, and T. R. Frieden. 1995. Emergence of fluoroquinolone-resistant tuberculosis in New York City. *Lancet* **345**:1148–1150.
- Tanaka, M., H. Nakayama, M. Haraoka, T. Saika, I. Kobayashi, and S. Naito. 2000. Susceptibilities of *Neisseria gonorrhoeae* isolates containing amino acid substitutions in *gyrA*, with or without substitutions in *parC*, to newer fluoroquinolones and other antibiotics. *Antimicrob. Agents Chemother.* **44**:192–195.
- Wall, J. D., and P. D. Harriman. 1974. Phage P1 mutants with altered transducing abilities for *Escherichia coli*. *Virology* **59**:532–544.
- Yoo, J.-H., D.-H. Huh, J.-H. Choi, W.-S. Shin, J.-W. Kang, C.-C. Kim, and D. J. Kim. 1997. Molecular epidemiological analysis of quinolone-resistant *Escherichia coli* causing bacteremia in neutropenic patients with leukemia in Korea. *Clin. Infect. Dis.* **25**:1385–1391.
- 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.
- Zhao, X., C. Xu, J. Domagala, and K. Drlica. 1997. DNA topoisomerase targets of the fluoroquinolones: a strategy for avoiding bacterial resistance. *Proc. Natl. Acad. Sci. USA* **94**:13991–13996.
- Zhou, J.-F., Y. Dong, X. Zhao, S. Lee, A. Amin, S. Ramaswamy, J. Domagala, J. M. Musser, and K. Drlica. 2000. Selection of antibiotic resistance: allelic diversity among fluoroquinolone-resistant mutations. *J. Infect. Dis.* **182**:517–525.