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Amino Acid Variation in the GyrA Subunit of Bacteria Potentially Associated with Natural Resistance to Fluoroquinolone Antibiotics

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In studies of genetic diversity in natural microbial populations, we have analyzed nucleotide sequences in the quinolone resistance-determining region of the bacterial *gyrA* gene in ciprofloxacin-resistant and nonselected soil bacteria obtained from the environment. It is apparent that this sequence is highly variable, and resistance to fluoroquinolone antibiotics occurring in environmental populations of bacteria is due at least in part to natural sequence variation in this domain. We suggest that the development of new antimicrobial agents, including completely synthetic antimicrobials such as the fluoroquinolones, should incorporate the analysis of resistance mechanisms among microbes in natural environments; these studies could predict potential mechanisms of resistance to be encountered in subsequent clinical use of the agents and would guide chemical modification designed to evade resistance development.

The fluoroquinolones are potent broad-spectrum antimicrobial agents that are increasingly used in the treatment of human and veterinary infections (4, 6). The primary target of fluoroquinolone action is DNA gyrase, a type II bacterial topoisomerase (3, 16) composed of two A subunits and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. While most wild-type strains encountered in clinical situations are highly sensitive to the fluoroquinolones, the extensive use of these agents has led to the development of resistant strains, the majority of which are due to mutational alteration of the GyrA subunit (14). There have been many reports of the isolation of such mutants of gram-negative (23) and gram-positive (8, 15) pathogens, and these studies have identified a 41-amino-acid quinolone resistance-determining region (QRDR) encoded by the *gyrA* gene (22). In *Escherichia coli*, this region of the *gyrA* gene, corresponding to nucleotides 199 to 318, coding for amino acids 67 to 106 of the GyrA protein, is situated within a highly conserved domain of the N-terminal portion of the A subunit which contains the catalytic site. The level of resistance to quinolones has been correlated with the type and number of alterations at key amino acid positions within the QRDR (2, 5, 17, 19).

In connection with studies of the genetic diversity of natural microbial populations, we have analyzed nucleotide sequences in the QRDR motif in random bacterial isolates cultured from soil and in ciprofloxacin-resistant isolates and QRDR sequences determined by amplification and cloning of extracted soil community DNA. Suspensions of soils from the Vancouver, British Columbia, area of Canada were plated onto tryptic soy agar (BBL) containing ciprofloxacin (Bayer Leverkusen). At concentrations up to 5 μg/ml, the selection plates were often nearly confluent, but when the drug concentration was increased to 10 μg/ml, resistant strains were readily isolated. Fatty acid methyl ester (10) and 16S ribosomal DNA sequence (9, 12) analyses were carried out with template DNA prepared from single colonies by being heated with InstaGene matrix (Bio-Rad) according to the supplier's protocol. Partial sequences of PCR products were obtained with an automated sequencer using ABI Prism dye terminator cycle sequencing reaction mix (Perkin-Elmer). Sequences were compared to the nucleotide sequence databases by BLAST analysis (1). The most frequent fluoroquinolone-resistant isolates obtained were *Arthrobacter* spp., aureobacteria, or flavobacteria; one gram-negative isolate remains unidentified. It is likely that other genera would have been obtained by using different selective media.

A 420-bp segment of the N-terminal region of the *gyrA* genes of the resistant isolates was amplified for sequencing from the same template DNA preparations with a pair of degenerate PCR primers based on consensus amino acid motifs of bacterial type II topoisomerases (7). Determinations of MICs were made for the resistant isolates by a macrodilution broth method using tryptic soy broth containing 0.5, 1.0, and 2.5 μg of ciprofloxacin per ml and further doubling dilutions up to 160 μg/ml. The MIC was defined as the lowest concentration of ciprofloxacin which inhibited development of visible growth in 20 h at 30°C.

The deduced amino acid sequences of the amplified QRDR from each of the resistant isolates are shown in Fig. 1. As indicated, the QRDR for most of the isolates varied from the *E. coli* fluoroquinolone-sensitive prototype in having amino acid substitutions at codon 83 and also at one or more of the positions (codons 84 and 87) previously identified to be associated with fluoroquinolone resistance. While the degenerate primers used could potentially amplify the QRDR of the *parC* gene also (7), the amino acid substitutions in these sequences resemble those reported for GyrA more closely than the alterations in ParC found in quinolone-resistant clinical isolates (20). The soil isolates that were most resistant to ciprofloxacin had substitutions at both positions 83 and 84. However, for none of the isolates described has it been demonstrated that the fluoroquinolone-resistant phenotype is due to the QRDR alterations; other targets of the fluoroquinolone antibiotics have been identified, notably topoisomerase IV in the streptococci (11, 13). Mutations in *gyrB* have been found in a number of cases (8), and enhanced drug efflux is involved in the multifactorial process of high-level resistance to the fluoroquino-

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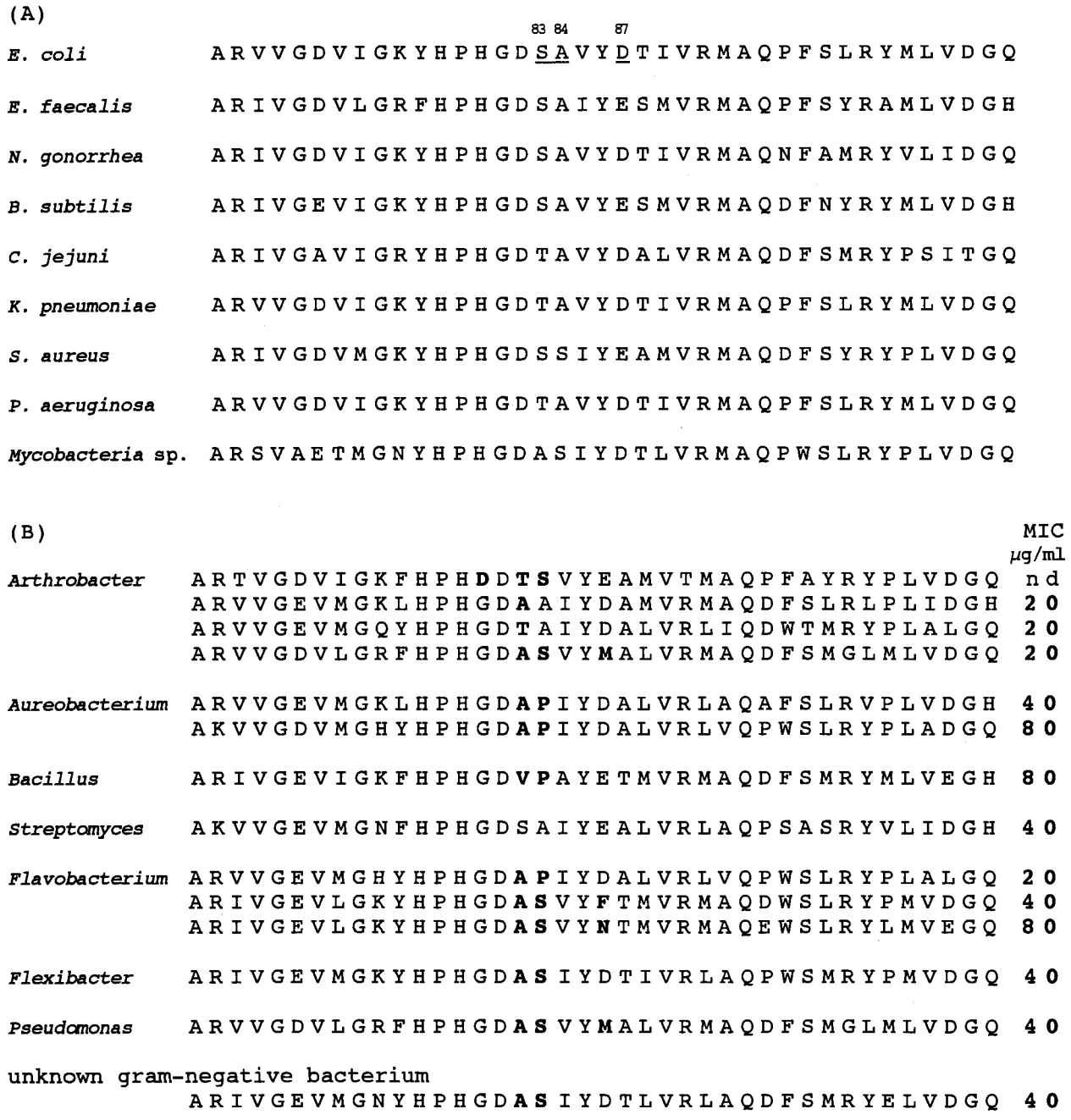

FIG. 1. Alignment of the deduced amino acid sequences of the QRDRs of several species of fluoroquinolone-sensitive bacteria (A) with those of ciprofloxacin-resistant soil isolates (B). Significant amino acid variations from the *E. coli*-sensitive prototype (underlined) are indicated (boldface), and the corresponding MICs are given on the right.

lone antibiotics in clinical isolates (18, 24). The one fluoroquinolone-resistant streptomycete (MIC > 40 μg/ml) has none of the alterations in the *gyrA* gene normally associated with fluoroquinolone-resistant mutants. This isolate will be investigated further. The fluoroquinolones are potent synthetic antimicrobials that have no known analogs among microbial biosynthetic products. The isolation of fluoroquinolone-resistant strains from the environment might be employed to identify bacteria that produce gyrase inhibitors related to the fluoroquinolones in mode of action.

For comparison, random soil bacterial isolates representative of different species were examined, and most of them were highly sensitive to ciprofloxacin (susceptible to <0.5 μg/ml). The QRDR sequences were determined in each case (Fig. 2), and for 9 of the 15 isolates the deduced amino acid sequences had substitutions in the positions associated with fluoroquinolone resistance. In seven of the strains, serine 83 was replaced with another amino acid, but most of the strains remained sensitive. Two of the isolates with amino acid substitutions at both positions 83 and 106 showed reduced susceptibility.

In addition, total (community) DNA was isolated from a soil sample (21), amplified by PCR with the QRDR primers, and

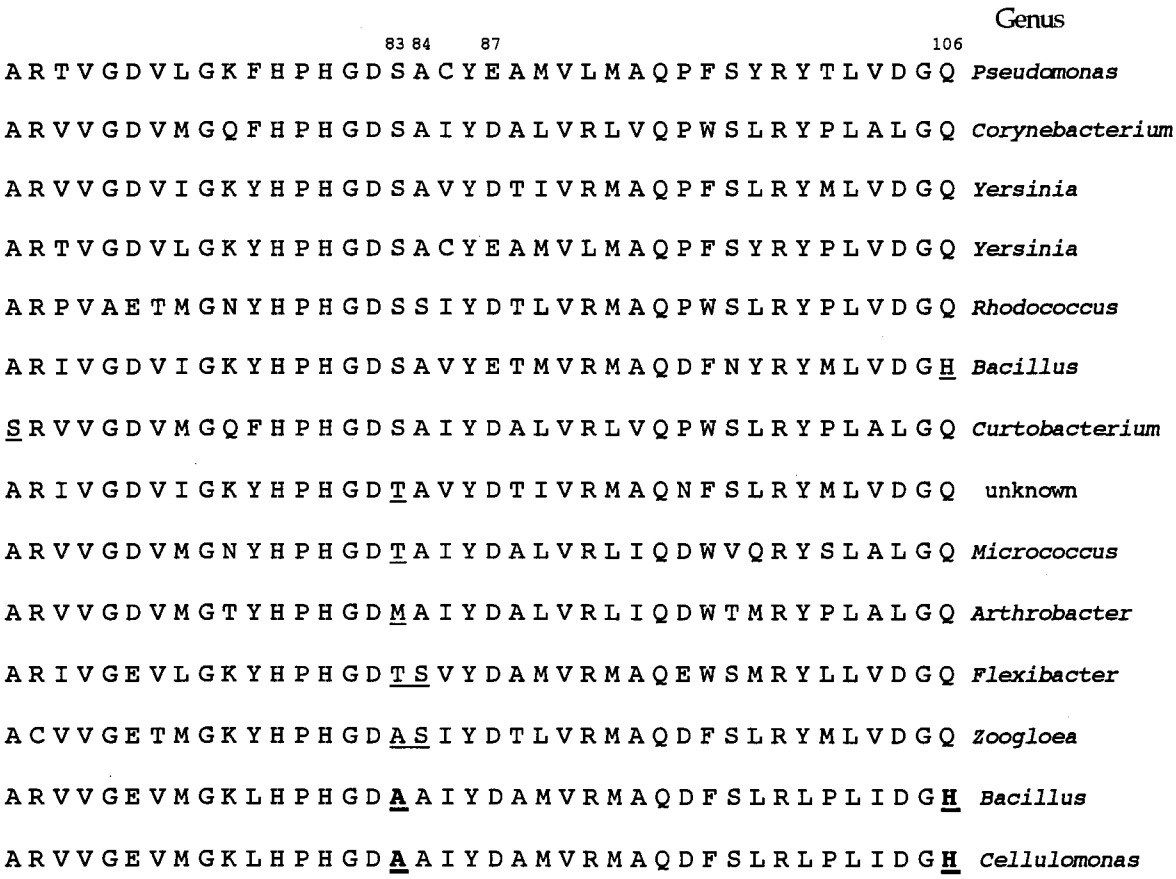

FIG. 2. Alignment of the deduced amino acid sequences of the QRDRs of several species of soil bacteria isolated in the absence of antibiotic selection. Substitutions in the fluoroquinolone-resistant positions were noted for several isolates (underlined). The two isolates with substitutions at both codons 83 and 106 (boldface) showed reduced ciprofloxacin susceptibility.

cloned; 20 random *E. coli* transformants were chosen, and the QRDRs were sequenced. The results (not shown) demonstrated considerable diversity in sequence, and none of the QRDRs obtained were identical matches for any published sequence. Position 83 was serine in only 5 of 20 cases; we predict that such strains would likely exhibit reduced sensitivity to ciprofloxacin.

This study is a report of our initial investigations of natural fluoroquinolone resistance. Surprisingly, a number of species were refractory to this synthetic antimicrobial, and the majority possessed one or more alterations in the QRDR, particularly in the key amino acid residues at positions 83, 84, and 87. It is apparent that the QRDR of GyrA is a highly variable sequence and that amino acid substitutions occurring as a result of allellic variations within the *gyrA* gene might lead to reduced susceptibility to fluoroquinolones without apparent selection.

Sequence analysis of microbes that are naturally resistant to potential antimicrobials would be valuable in predicting mechanisms of resistance likely to be encountered in subsequent clinical use. Such early warning would permit rational design of agents active against resistant strains, and biochemical analysis of the resistant isolates might be expected to identify the target of the drug at an early stage in drug development. Expansion of knowledge of microbial genome sequences and a heightened awareness of the extent of genetic diversity in the microbial world will enhance such applications.

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