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Mutation in the *gyrA* Gene of Quinolone-Resistant Clinical Isolates of *Acinetobacter baumannii*

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The *gyrA* gene mutations associated with quinolone resistance were determined in 21 epidemiologically unrelated clinical isolates of *Acinetobacter baumannii*. Our studies highlight the conserved sequences in the quinolone resistance-determining region of the *gyrA* gene from *A. baumannii* and other bacteria. All 15 isolates for which the MIC of ciprofloxacin is ≥4 μg/ml showed a change at Ser-83 to Leu. Six strains for which the MIC of ciprofloxacin is 1 μg/ml did not show any change at Ser-83, although a strain for which the MIC of ciprofloxacin is 1 μg/ml exhibited a change at Gly-81 to Val. Although it is possible that mutations in other locations of the *gyrA* gene, the *gyrB* gene, or in other genes may also contribute to the modulation of the MIC level, our results suggest that a *gyrA* mutation at Ser-83 is associated with quinolone resistance in *A. baumannii*.

The incidence of nosocomial infections caused by *Acinetobacter baumannii* has been steadily rising in recent years (4, 29, 31). Several outbreaks of nosocomial infections caused by multiply resistant strains of *Acinetobacter* spp. have been documented (2, 7, 10, 13, 16, 22, 33). Many of these outbreaks have occurred in intensive care units in which extensive use of antibiotics can select for the emergence of multiply resistant strains (2, 7, 10, 13, 33). Until 1988, the new fluorinated quinolones presented very good activity against *Acinetobacter* strains (3, 11, 20, 28), even better than that of expanded-spectrum cephalosporins or aminoglycosides. However, resistance to these antibiotics rapidly emerged in clinical isolates (1, 32, 34). The emergence of fluoroquinolone resistance is of particular concern, given that relatively few antimicrobial agents are effective against *A. baumannii*.

In several bacteria, mutations conferring quinolone resistance have been found in both *gyrA* and *gyrB* genes of DNA gyrase, the intracellular target of quinolones. In *Escherichia coli*, several mutations have been identified in the *gyrA* gene (6, 14, 27, 35, 38, 40). Of these, mutations at Ser-83 and Asp-87 have been found with a higher frequency in quinolone-resistant *E. coli* clinical isolates than in susceptible isolates (35). Similar mutations have also been identified in quinolone-resistant isolates of *Staphylococcus aureus* (12, 19, 24, 30), *Campylobacter jejuni* (36), and *Pseudomonas aeruginosa* (21). Specific mutations in the *gyrB* gene also seem to be associated with quinolone resistance (35, 37, 39), although in quinolone-resistant *E. coli* clinical isolates, the frequency is very low (35). Alterations in drug permeation can also play a role in rendering bacteria quinolone resistant. In *E. coli*, this phenomenon has been associated with mutations leading to decreasing amounts of the outer membrane protein OmpF (9, 15, 17). However, alterations of outer membrane lipopolysaccharides and fluoroquinolone efflux at the inner membrane (8, 25) should also be considered as factors. It was observed that such an efflux system, in addition to DNA gyrase and outer membrane protein alterations, results in a high level of resistance (18). The molecular bases of quinolone resistance in *A. baumannii* remain to be investigated; therefore, the aim of this study was to determine whether there were *gyrA* mutations associated with quinolone resistance in epidemiologically unrelated clinical isolates of *A. baumannii*.

The clinical isolates of *A. baumannii* used in this study were collected from three Spanish hospitals. Identification of *A. baumannii* was based on standard biochemical reactions and the criteria of Bouvet and Grimont (5). All the clinical isolates studied were epidemiologically unrelated, as shown previously (23). Susceptibility testing was performed by an agar dilution method and in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (26). Approximately 10^4 CFU of each isolate was inoculated with a multipoint replicator onto freshly prepared medium containing serial dilutions of ciprofloxacin (Bayer, Leverkusen, Germany) or nalidixic acid (Prodesfarma, Barcelona, Spain). To identify *gyrA* mutations in resistant isolates, PCR and direct DNA sequencing were used. We utilized conserved amino acid sequence motifs found in diverse GyrA proteins to design oligonucleotide primers. Two primers, 5′AAATCTGCCCGT GTCGTTGGT3′ and 5′GCCATACCTACGGCGATACC3′ from Genosys Biotechnologies, Cambridge, United Kingdom, were used. The PCR was performed as follows. Half of a colony grown on MacConkey agar was resuspended in 25 μl of sterile distilled water and boiled for 10 min. After a short centrifugation step at 15,000 × *g*, 25 μl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, gelatin (0.1%, wt/vol), 400 μM deoxynucleoside triphosphates, and 1 μM each primer was added together with 2.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany). Each reaction mixture was overlaid with mineral oil and amplified at the following temperature profiles: 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification was performed in a DNA thermal cycler Model 480 (Perkin-Elmer Cetus). Amplified DNA products were resolved by electrophoresis in agarose (2%, wt/vol) gels containing 0.5 mg of ethidium bromide per liter. The PCR product was recovered from the agarose gel and purified with the Wizard DNA clean-up system according to the manufacturer's instructions (Promega Co., Madison, Wis.). The sample was then directly processed with the DNA sequencing kit and analyzed in an automatic DNA sequencer (Applied Biosystems 373A). When the PCR product was digested with *Hin*fI, 20 μl

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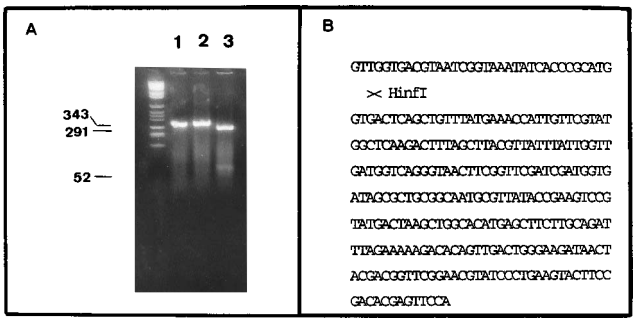

FIG. 1. Detection of the mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *A. baumannii* by *Hin*fI restriction fragment length polymorphism analysis of PCR products. (A) Agarose gel electrophoresis of *Hin*fI-digested PCR products of the *gyrA* gene. Lane 1, nondigested PCR product; lane 2, *Hin*fI-digested PCR product of quinolone-resistant clinical isolate; lane 3, *Hin*fI-digested PCR product of quinolone-susceptible clinical isolate; left lane, DNA molecular weight marker VI from Boehringer. Fragments of 343, 291, and 52 bp are shown (noted to the left of the gel). (B) Nucleotide sequence of a 285-bp fragment containing the putative quinolone resistance-determining region of the *gyrA* gene of *A. baumannii*. The location of the *Hin*fI site is noted.

of the PCR mixture was incubated for 2.5 h with 10 U of restriction enzymes under conditions recommended by the manufacturer, and the digestion products were separated by electrophoresis in 1.5% NuSieve plus 1% agarose gels. A fragment of the *gyrA* gene including the quinolone resistance-determining region was analyzed by PCR and automated direct DNA sequencing in 21 clinical isolates of *A. baumannii*. The PCR product had the expected size of 343 bp (Fig. 1A). After the fragment was eluted from the agarose gel, 285 bp of this amplicon was sequenced with the lower primer (Fig. 1B). The nucleotide sequence of a fragment of the *A. baumannii gyrA* gene including the quinolone resistance-determining region exhibits about 71% identity with the same region in the *gyrA* gene of *E. coli* and 69% identity with that of *P. aeruginosa*. When compared with other GyrA sequences (Table 1), the derived amino acid sequence of the *A. baumannii* GyrA protein showed 82% identity with that of *E. coli*, 74% with that of *S. aureus*, 75% with that of *C. jejuni*, and 83% with that of *P. aeruginosa*. Our studies highlight the sequence conservation of the quinolone resistance-determining region of the *gyrA* gene of *A. baumannii* and other bacteria. The *A. baumannii* GyrA protein carried conserved Asp-Ser residues at positions equivalent to 82 and 83 for *E. coli*; both codons form a *Hin*fI restriction site (GANTC). *Hin*fI digestion of the PCR product from a quinolone-susceptible *A. baumannii* strain generated two fragments of 291 bp and 52 bp. The *Hin*fI restriction site in isolates carrying a mutation at codon 83 was abolished, resulting in no digestion of the fragment containing the full-length PCR product (Fig. 1A). The presence or absence of *gyrA* mutations at codon 83 can be determined by the digestion of the PCR products with *Hin*fI, and this procedure may be used for the rapid screening of a large number of clinical isolates.

TABLE 1. Comparison of the amino acid sequences of the region containing Ser-83 and four GyrA subunits[a]

E.coli	VGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAMR
S.aureus	VGDVMGKYHPHGDSSIYEAMVRMAQDFSYRYPLVDGQGNFGSMDGDGAAAMR
C.jejuni	VGAVIGRYHPHGDTAVYDALVRMAQDFSMRYPSITGQGNFGSIDGDSAAAMR
P.aeruginosa	VGDVIGKYHPHGDTAVYDTIVRMAQPFSLRYMLVDGQGNFGSVDGDNAAAMR
A.baumannii	VGDVIGKYHPHGDSAVYETTVRMAQDFSLRYLLVDGQGNFGSIDGDSAAAMR
	** * * ****** * ***** ** ** ******* *** *****
	YTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIP
	YTEARMTKITLELLRDINKDTIDFIDNYDGNEREPSVLPARFP
	YTEAKMSKLSHELLKDIDKDIVDFVPNYDGSESEPDVLPSRVP
	YTEVRMAKLAHELLADLEKETVDWVPNYDGTEQIPAVMPTQIP
	YTEVRMTKLAHELLADLEKDIVDWEDNYDGSERIPEVLPTRVP
	*** * * * * * * **** * * * * *

[a] Asterisks indicate positions in the alignment that are perfectly conserved in all five GyrA proteins. Bold letters indicate an amino acid equivalent to Ser-83 in *E. coli*.

TABLE 2. Quinolone resistance mutations in the *gyrA* gene of clinical isolates of *A. baumannii*

Strain(s)	MIC (μg/ml)[a]		Amino acid change[b]
	CIP	NAL	
167	0.125	2	
58 and 201	0.125	8	
88	0.5	8	
77	1	8	
93	1	8	Gly-81 to Val
33 and 661	4	256	Ser-83 to Leu
34	8	512	Ser-83 to Leu
175	8	64	Ser-83 to Leu
29	16	256	Ser-83 to Leu
547	32	>1,024	Ser-83 to Leu
31, 2545, 24, 13, and 30	64	>1,024	Ser-83 to Leu
65	64	>1,024	Ser-83 to Leu and Ala-84 to Pro
14, 522, and 1543	128	>1,024	Ser-83 to Leu

[a] CIP, ciprofloxacin; NAL, nalidixic acid.
[b] Amino acid position equivalent to Ser-83 of *E. coli.*

The mutations in the *gyrA* gene leading to amino acid changes are shown in Table 2. All clinical isolates for which the MIC of ciprofloxacin is ≤1 μg/ml had identical *gyrA* sequences, except for strain 58, which had a mutation at amino acid codon Ala-119 that changed it to Ser. Two other resistant strains (strains 661 and 34) also presented this mutation (data not shown). Although it is very close to the active-site tyrosine 122, a novel mutation at amino acid codon Ala-119 found in these three clinical isolates of *A. baumannii* does not seem to play a particularly important role in the acquisition of resistance since it is found in either susceptible or resistant strains. Those clinical isolates for which the MIC of ciprofloxacin is ≤1 μg/ml did not show any change at Ser-83, although a strain for which the MIC of ciprofloxacin is 1 μg/ml exhibited a change at Gly-81 to Val. All 15 isolates for which the MIC of ciprofloxacin is ≥4 μg/ml presented a change at Ser-83 to Leu. A strain for which the MIC of ciprofloxacin is 64 μg/ml showed a double change of Ser-83 to Leu and Ala-84 to Pro. It has been found that the amino acid residues most frequently mutated in spontaneous *gyrA* mutations and in clinical isolates of *E. coli* and *S. aureus* are Ser-83 and Ser-84 (6, 14, 27, 35, 38, 40). Similar mutations for *A. baumannii* have been found in our study. In these mutations, a change at Ser-83 leads to a high level of resistance to ciprofloxacin and nalidixic acid. Gly-81, Ala-84, and Gln-106 residues, for which substitutions are associated with quinolone resistance in *E. coli*, are all conserved in *A. baumannii* gyrase A. However, only changes at Gly-81 and Ala-84 have been found in 2 isolates of the 21 clinical isolates analyzed, suggesting that their contribution to the resistance mechanism, if any, is of little importance. The residue Asp-87, another important amino acid in the acquisition of a

high level of resistance to ciprofloxacin in *E. coli* (35), is changed in *A. baumannii* by Glu, the same as in *S. aureus* (36). However, no mutations affecting this amino acid have been found in any of clinical isolates analyzed.

Although we have only examined a small region of the *gyrA* gene sequence and it is possible that mutations at other locations of the *gyrA* gene, the *gyrB* gene, or in other genes may also contribute to the modulation of the MIC level, our results strongly suggest that a *gyrA* mutation at a codon equivalent to Ser-83 in *E. coli* is responsible for, or at least contributes to, ciprofloxacin and nalidixic acid resistance in *A. baumannii*.

Nucleotide sequence accession number. The EMBL accession number for the partial sequence of the quinolone-susceptible (HCP-77) and quinolone-resistant (VH-525) *A. baumannii gyrA* gene is X82165.

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