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Relationship between Mutations in the *gyrA* Gene and Quinolone Resistance in Clinical Isolates of *Corynebacterium striatum* and *Corynebacterium amycolatum*

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Quinolone susceptibility was analyzed in 17 clinical isolates of *Corynebacterium striatum* and 9 strains of *Corynebacterium amycolatum* by the E-test method in Mueller-Hinton agar plates. The *C. striatum* ATCC 6940 strain was used as a control strain. The amplified quinolone resistance determining regions of the *gyrA* genes of *C. amycolatum* and *C. striatum* were characterized. Four in vitro quinolone-resistant mutants of *C. amycolatum* were selected and analyzed. Both in vivo and in vitro quinolone-resistant strains of *C. amycolatum* showed high levels of fluoroquinolone resistance in strains with a double mutation leading to an amino acid change in positions 87 and 91 or positions 87 and 88 (unusual mutation) of GyrA, whereas the same concomitant mutations at amino acid positions 87 and 91 in GyrA of *C. striatum* produced high levels of resistance to ciprofloxacin and levofloxacin but only showed a moderate increase in the MIC of moxifloxacin, suggesting that other mechanism(s) of quinolone resistance could be involved in moxifloxacin resistance in *C. amycolatum*. Moreover, a PCR-RFLP-NcoI of the *gyrA* gene was developed to distinguish between *C. amycolatum* and *C. striatum* species.

Corynebacteria, other than *Corynebacterium diphtheriae*, are ubiquitous and are part of the normal flora of the skin and mucous membrane. The isolation of this type of microorganisms is not usually of clinical relevance and is attributable to the contamination of the sample. However, these microorganisms have increasingly been recognized as pathogens (5, 9). Several case reports have described *C. striatum* as a cause of infections such as pneumonia (30), vertebral osteomyelitis (6), septicemia (17), and endocarditis (4). Similarly, several authors have described *C. amycolatum* as a pathogen (3, 14). The rise in the clinical importance of these new pathogens makes it necessary to know their susceptibility to the antimicrobial agents currently available. Although the data are limited (9), *C. amycolatum* has shown a multidrug resistance phenotype, presenting high rates of resistance to antibiotics such as ampicillin, aminoglycosides, ofloxacin, and doxycycline and only showing susceptibility to glycopeptides (16), whereas the rates of resistance for *C. striatum* are lower, showing only high percentages of resistance to clindamycin and ampicillin (16). These figures on antimicrobial resistance are in accordance with those published by Funke et al. (8), who reported high rates of resistance to many classes of antimicrobial agents. Furthermore, high levels of resistance to ciprofloxacin have been described (17–21). A multidrug-resistant strain of *C. striatum* only susceptible to rifampin and vancomycin has recently been reported (30). In spite of these high levels of resistance to quinolones in species of the genus *Corynebacterium*, no reports have been published on the mechanisms of resistance. Moreover, the activity of the new fluoroquinolones in comparison with ciprofloxacin has been described (7, 25, 26). Rolston et al. (25, 26) compared the activity of several new fluoroquinolones such as levofloxacin, moxifloxacin, or trovafloxacin to the activity of ciprofloxacin in a small collection of *Corynebacterium* sp. strains (without species identification), with moxifloxacin being the quinolone with the best activity (i.e., an MIC at which 90% of the isolates tested are inhibited of 2 μg/ml). Moreover, on comparing the activity of gemifloxacin and levofloxacin with older quinolones among known species of *Corynebacterium*, Martinez-Martinez et al. (18, 20) showed that *C. amycolatum* had higher rates of resistance than *C. striatum*. Therefore, the main aim of the present study was to investigate the mechanisms of resistance to these antimicrobial agents in these microorganisms.

MATERIALS AND METHODS

Bacterial strains. *C. amycolatum* strains were isolated in the Hospital Universitario Virgen Macarena, Seville, Spain. *C. striatum* strains were isolated in the Departments of Microbiology of Hospital Clínic, Barcelona, Spain, Hospital Universitario Virgen Macarena, Seville, Spain, and Hospital Monte Naranco, Oviedo, Spain. All of the strains were identified by the API Coryne system (bioMérieux, Marcy l'Étoile, France), and two additional tests were performed to differentiate *C. amycolatum* and *C. striatum*, as described elsewhere (21). The *C. striatum* ATCC 6940 strain was used as a control strain.

Susceptibility testing. The MICs of ciprofloxacin, levofloxacin, and moxifloxacin were determined by the E-test method (AB Biodisk, Solna, Sweden) in Mueller-Hinton agar plates at 37°C in an aerobic atmosphere. There are no specific guidelines by the National Committee for Clinical Laboratory Standards for the susceptibility testing of corynebacteria.

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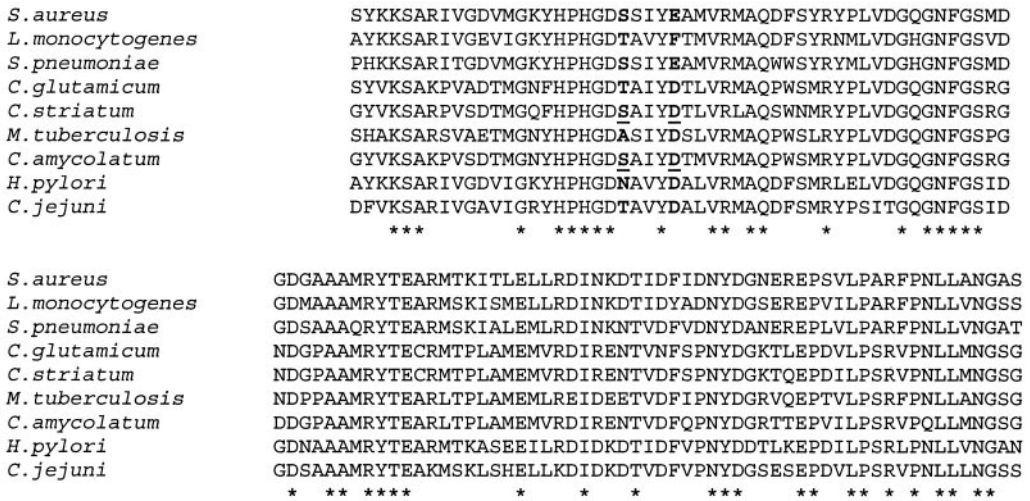

FIG. 1. Comparison of the amino acid sequence of the QRDR of the *gyrA* gene. The protein sequences were obtained based on the indicated GenBank accession numbers: *C. amycolatum* (AAS75324), *C. striatum* (AAS75323), *C. glutamicum* (NP599264), *S. aureus* (BAB41222), *S. pneumoniae* (NP358692), *L. monocytogenes* (AAM48483.1), *C. jejuni* (NP282177), *H. pylori* (NP207495), and *M. tuberculosis* (NP214520).

Amplification and sequence of QRDR of the *gyrA* gene. To amplify the quinolone resistance determining region (QRDR) of the *gyrA* gene of *C. amycolatum* and *C. striatum*, a couple of degenerate primers for *gyrA* were first used. Based on this sequence a novel pair of specific primers for corynebacteria was designed: CorynA1 (GCG GCT ACG TAA AGT CC) and CorynA2 (CCG CCG GAG CCG TTC AT). The PCRs were performed as follows. One colony was resuspended in 25 μl of water and boiled for 10 min. Afterward, 25 μl of the 2× PCR mixture was added. The final concentrations were 0.5 μM for each primer, 200 mM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase. The PCR program was 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles, with a final extension of 72°C for 5 min. The PCR products were visualized in an agarose gel containing 0.5 μg of ethidium bromide/ml. The PCR products were then cleaned up with a commercial kit (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, Wis.), and 4 μl was used to sequence the recovered PCR product with a BigDye sequence kit (v3.1; Applied Biosystems, Foster City, Calif.).

Selection of in vitro fluoroquinolone-resistant mutants of *C. amycolatum*. An inoculum of ca. 10^9 CFU of the quinolone-susceptible *C. amycolatum* strain 1008 was spread on blood agar plates containing 1 mg of ciprofloxacin/ml (15 times the MIC), followed by incubation overnight to obtain first-step mutants. Second-step mutants were obtained by culturing the previously obtained mutant strain on blood agar plates containing 32 μg of ciprofloxacin/ml. The sequence of the *gyrA* gene, as well as the MICs of the fluoroquinolones of the first- and second-step mutants, was determined as described above.

PCR-RFLP of the QRDR of the *gyrA* gene. The PCR product of the *gyrA* gene described above was subjected to digestion with the NcoI restriction enzyme. A total of 25 μl of the recovered PCR product was taken, and 2.5- and 1-μl portions of the restriction buffer and NcoI restriction enzyme were added, followed by incubation for 3 h at 37°C. The restriction patterns were then visualized in a 2% agarose gel containing 0.5 μg of ethidium bromide/ml.

RESULTS

Sequences of the QRDR of the *gyrA* gene. The amplified fragment of the *gyrA* gene sequences obtained from the quinolone-susceptible *C. amycolatum* clinical strain and the *C. striatum* ATCC 6940 strain were added to GenBank under accession numbers AY559039 and AY559038. These sequences were used as wild-type sequences to find mutations in resistant strains.

The amino acid sequence of the fragment corresponding to the QRDR was compared to those of the GyrA from other gram-positive bacteria, such as *Corynebacterium glutamicum*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Listeria monocytogenes*, and to other microorganisms that do not have the *parC* gene, such as *Helicobacter pylori*, *Campylobacter jejuni*, and *Mycobacterium tuberculosis* (Fig. 1). Similarities in nucleotides and amino acid sequences in these sequences are presented in Table 1.

The nucleotide sequence of the *gyrA* gene of *C. striatum* and *C. amycolatum* showed a homology of 80.7%. Comparison of these sequences with those of other microorganisms demonstrated a high homology with the *gyrA* genes of *C. glutamicum* (85.7 and 81.3%, respectively) and *M. tuberculosis* (72.1 and 72.7%, respectively). It is interesting that there was a high degree of homology with *M. tuberculosis*, which also lacks the *parC* gene. The remaining compared sequences presented homologies of between 64 and 53%. Furthermore, when the amino acid sequences of these microorganisms were compared, similar results were found.

MICs of fluoroquinolones and mutations in the *gyrA* gene. Two of nine strains of *C. amycolatum* isolates presented the

TABLE 1. Comparison of nucleotide and amino acid sequences of the QRDRs of the *gyrA* genes of *C. striatum* and *C. amycolatum* with other microorganisms

Strain	% Homology[a]			
	C. striatum		*C. amycolatum*	
	nt	aa	nt	aa
C. striatum				
C. amycolatum	80.7	86		
C. glutamicum	85.7	90	81.3	85.5
S. aureus	59	62.1	59.6	63.9
S. pneumoniae	59.6	58.5	59.6	60
M. tuberculosis	72.1	74.7	72.7	77.7
H. pylori	55	63	60.2	61.2
C. jejuni	54	62.1	53.4	60
L. monocytogenes	57.2	56.7	59.6	58.5

[a] nt, nucleotides; aa, amino acids.

TABLE 2. Relationship between mutations in the QRDRs of the *gyrA* genes and the MICs for *C. amycolatum*

Strain[a]	GyrA (aa) sequence[b]					MIC (μg/ml)[c]		
	87-S	88-A	89-I	90-Y	91-D	CIP	LEV	MOX
1008	S	A	I	Y	D	0.064	0.094	0.016
1186	S	A	I	Y	D	0.047	0.094	0.016
1049	R	A	I	Y	D	>32	8	2
1011	R	A	V	Y	D	>32	24	6
1009	R	P	I	Y	D	>32	>32	>32
1028	R	P	I	Y	D	>32	>32	>32
1044	R	P	I	Y	D	>32	>32	>32
1067	R	P	I	Y	D	>32	>32	>32
1189	R	P	I	Y	D	>32	>32	>32
CA1*	R	A	I	Y	D	>32	8	2
CA32A*	R	P	I	Y	D	>32	>32	>32
CA32B*	R	A	I	Y	Y	>32	>32	>32
CA32C*	R	P	I	Y	D	>32	>32	>32

[a] *, In vitro fluoroquinolone-resistant mutants.
[b] aa, amino acid.
[c] CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin.

TABLE 3. Relationship between mutations in the QRDRs of the *gyrA* gene and the MICs for *C. striatum*

Strain	GyrA (aa)[a]		MIC (μg/ml)[b]		
	Position 87	Position 91	CIP	LEV	MOX
ATCC	S	D	0.094	0.125	0.038
441	S	D	0.125	0.125	0.047
488	S	D	0.125	0.125	0.047
420	S	G	6	1.5	0.38
197-H	S	Y	3	1.5	0.5
536	F	D	1	1.5	0.19
462	F	D	1.5	2	0.25
473	F	D	1.5	1.5	0.38
449	F	D	3	2	0.25
379	V	D	>32	8	0.75
424	V	D	>32	8	0.75
474	V	D	>32	8	0.75
628-H	F	A	>32	>32	8
629-H	F	A	>32	>32	6
635-H	F	A	>32	>32	6
845-H	F	A	>32	>32	8
849-H	F	A	>32	>32	6
39488	F	A	>32	>32	8

[a] See Table 1, footnote *b*.
[b] See Table 1, footnote *c*.

lowest MICs of quinolones. The MICs of ciprofloxacin for these two strains were of 0.064 and 0.047 μg/ml; the MIC of levofloxacin was 0.094 μg/ml, and finally the MIC of moxifloxacin was 0.016 μg/ml. One strain showed a mutation in the amino acid codon Ser-87, generating a change to Arg and drastically increasing the MIC of ciprofloxacin to >32 μg/ml. The MICs of levofloxacin and moxifloxacin were also increased to 8 and 2 μg/ml, respectively. (The number of the amino acid position was taken from the amino acid sequence of GyrA from *C. glutamicum*.)

Two double mutations in the *gyrA* gene were observed in six *C. amycolatum* clinical isolates. The first double mutant observed in one strain showed a mutation at amino acid codon Ser-87 to Arg plus a novel mutation in an unusual position: amino acid codon Ile-89 to Val. In this strain the MIC of ciprofloxacin was of 32 μg/ml, and the MICs of levofloxacin and moxifloxacin increased to 24 and 6 μg/ml, respectively.

For the remaining five strains, the MICs of the three fluoroquinolones were determined to be >32 μg/ml. These strains carried a double mutation in the amino acid codon Ser-87 and Ala-88 of the *gyrA* gene, producing a change from Ser-87 to Arg and another unusual change from Ala-88 to Pro (Table 2).

Fluoroquinolone-resistant mutants of *C. amycolatum* were obtained in vitro to ensure that the mutations observed in the clinical strains are sufficient to produce these high levels of MIC of fluoroquinolones. Three first-step, in vitro-selected ciprofloxacin resistant mutants, named CA1, CA2, and CA3, which were grown in 1 μg of ciprofloxacin/ml, were chosen for further analysis. All had one mutation in the amino acid codon Ser-87 changing to Arg, as observed in the clinical strains, and the MICs of the three fluoroquinolones—ciprofloxacin (>32 μg/ml), levofloxacin (8 μg/ml), and moxifloxacin (2 μg/ml)—were also the same. Second-step mutants were selected after culture of the first-step mutant (CA1) in 32 μg of ciprofloxacin/ml. In this case, the MICs of the fluoroquinolones tested for the three selected mutants (CA32A, CA32B, and CA32C) chosen for further analysis were >32 μg/ml, but the mutations were different. Mutants CA32A and CA32C presented the same double mutations as the clinical isolates (Ser87 to Arg and Ala-88 to Pro), whereas the other mutant (CA32B) showed a mutation in the amino acid codon Ser-87 and the second mutation in the amino acid codon Asp-91, changing to Tyr (Table 2).

Two of seventeen strains of *C. striatum* and the control strain ATCC 6940 were associated with the lowest quinolone MICs. The MIC of ciprofloxacin was between 0.094 to 0.125 μg/ml, and the MICs of levofloxacin and moxifloxacin were of 0.125 and 0.047 μg/ml, respectively. These three strains did not show any mutation in the QRDR of the *gyrA* gene. Four strains had a mutation at amino acid codon Ser-87 that changed the amino acid to Phe. The ciprofloxacin and levofloxacin MICs for these strains showed moderate increases from 1 to 3 and from 1 to 2 μg/ml, respectively. However, the MICs of moxifloxacin were between 0.19 and 0.38 μg/ml. The MICs for two strains were higher: ciprofloxacin at 3 and 6 μg/ml, levofloxacin at 1.5 μg/ml, and moxifloxacin at 0.5 and 0.38 μg/ml. These strains presented a mutation at position Asp-91, changing the amino acid to Tyr and Gly, respectively. Three strains presented a mutation at the amino acid codon 87, changing Ser to Val; the MIC of ciprofloxacin was >32 μg/ml, the MIC of levofloxacin was 8 μg/ml, and the MIC of moxifloxacin was 0.75 μg/ml. Finally, the ciprofloxacin and levofloxacin MICs were highest (>32 μg/ml) for the remaining six strains, and the MIC of moxifloxacin remained between 6 and 8 μg/ml. These strains presented a double mutation at positions 87 and 91, with a change from Ser to Phe in the amino acid codon 87 and from Asp to Ala at position 91. The relationships between the MICs and the mutations in target genes are summarized in Tables 2 and 3.

PCR-RFLP of the QRDR of the *gyrA* gene. The differences in the QRDR sequence of the *gyrA* gene between *C. amycolatum* and *C. striatum* allow differentiation of these two species by PCR-restriction fragment length polymorphism (RFLP) analysis using the NcoI restriction enzyme. This enzyme has different restriction sites in the sequence of the *gyrA* gene de-

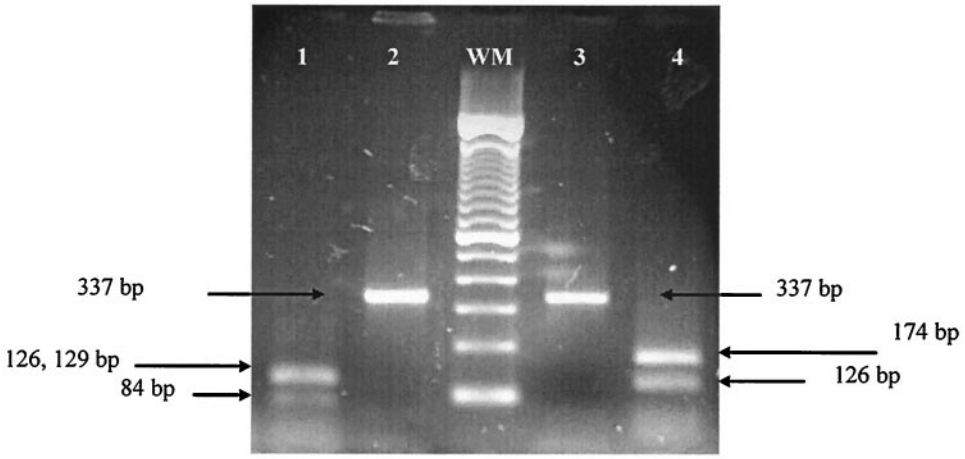

FIG. 2. PCR-RFLP-NcoI analysis of the *gyrA* gene of *C. amycolatum* and *C. striatum*. Lanes 2 and 3, undigested fragments of the QRDR of the *gyrA* gene of *C. amycolatum* and *C. striatum*, respectively; lanes 1 and 4, NcoI-digested fragments of the QRDR of the *gyrA* gene of *C. amycolatum* and *C. striatum*, respectively. WM, molecular weight markers (100 bp; Gibco-BRL, Gaithersburg, Md.).

pending on whether the *gyrA* sequence belongs to *C. striatum* or *C. amycolatum* (Fig. 2).

In both cases three bands were obtained after restriction. In the case of *C. striatum* the bands had sizes of 37, 126, and 174 bp. The minor band was difficult to see. Meanwhile, the sizes of the bands obtained from the QRDR of the *gyrA* gene of *C. amycolatum* were 82, 126, and 129 bp. In this case only two bands were visualized in the agarose gel because the 126- and 129-bp bands were observed as only one.

DISCUSSION

Resistance to quinolones is mainly related to the acquisition of point mutations in the sequence of the QRDR of the *gyrA* and *parC* genes, as for most microorganisms, such as, for example, *Enterobacteriaceae* and gram-positive cocci (13). However, a special group of microorganism, such as *Mycobacterium* spp., *H. pylori*, or *Campylobacter* spp. do not have the *parC* gene; therefore, resistance is related to the mutations in the *gyrA* gene (1, 10, 29). In our study, the *parC* gene of *C. striatum* and *C. amycolatum* could not be amplified with degenerate primers. Moreover, using a GenBank search (www.ncbi.nih.nlm.org), the QRDR sequences of the *grlA* gene of *S. aureus* and the *parC* genes of *S. pneumoniae* and *Escherichia coli* were compared over the complete genome sequence of *C. glutamicum*, with no matches being found, and the only sequence matched was the *gyrA* gene. Therefore, *Corynebacterium* spp. are other microorganisms belonging to this group of microorganisms, which do not have topoisomerase IV.

The role of mutation(s) in the *gyrA* gene in the development of quinolone resistance is similar to that of other microorganisms lacking the *parC* gene in their genome, as in *Campylobacter* spp. (1, 24) or *H. pylori* (22, 29). In these microorganisms the mutations in the gene encoding the subunit A of the DNA gyrase can confer quinolone resistance and overexpression of efflux pump(s) may play a complementary role in quinolone resistance acquisition. Overall, the level of quinolone resistance seems to depend on the type of amino acid substitution and on the amino acid substituted. In the case of *C. amycolatum* only one change in position 87 of GyrA was able to confer resistance to all of the quinolones tested, although with a higher level of resistance to ciprofloxacin than to levofloxacin or moxifloxacin. This is supported by the results obtained with the first-step mutant of *C. amycolatum*. However, a mutation in the amino acid codon Ser-87 or in the amino acid codon Asp-91 *C. striatum* only increased the MICs of ciprofloxacin and levofloxacin, whereas the strain remained susceptible to moxifloxacin.

In *C. jejuni*, one mutation at amino acid codon Asp90 generated a lower MIC than a mutation in the amino acid Thr86 (1). Other microorganisms, lacking the *parC* gene, such as *H. pylori* and *M. tuberculosis* showed the same behavior (15, 29).

In a study by Bachoual et al. (1), *C. jejuni* mutants resistant in vitro were selected with ciprofloxacin and moxifloxacin. The increase in resistance was related to alterations in the QRDR in the *gyrA* gene, i.e., a mutation at amino acid position 87 selected in the first-step mutant with ciprofloxacin, changing the amino acid from Ala to Pro. The MICs of both quinolones showed a slight increase, whereas in the second-step mutant a concomitant mutation was found at amino acid position 86 changing Thr to Ile. This new mutation in combination with the previous one resulted in increased resistance, with MICs of 64 μg/ml for ciprofloxacin and 32 μg/ml for moxifloxacin. This is probably what occurred with *C. amycolatum*, which showed a second mutation in the amino acid codon 88, changing Ala to Pro. This substitution is adjacent to Ser-87, which is the target for the acquisition of resistance. This is a very unusual mutation associated with quinolone resistance, and these concomitant mutations at amino acid codons Ser-87 and Ala-88 could explain the high MICs (>32 μg/ml) observed with all of the quinolones. It is well known that among the 20 coded amino acids proline has a very distinct conformational behavior (2). More precisely, it has a strong tendency to disrupt α-helices. If we take into account that the DNA gyrase quinolone-binding motif, which spans the sequence from Ser-87 to Asp-91, has an α-helical structure (23), our results strongly suggest that a change in Ser-87 to Arg leads Asp-91 to bind the quinolone to

the DNA gyrase, whereas the conformational distortion caused by Pro adjacent to Ser-87 leads to no amino acid being involved in DNA gyrase quinolone binding, thereby increasing the MICs of the three quinolones to >32 μg/ml. Second-step quinolone-resistant mutants also showed this second mutation in Ala-88, which was changed to Pro, and is probably the most frequently found in clinical isolates. However, other mutations, such as that affecting Asp-91, can also be obtained in vitro.

The increment in the MIC of ciprofloxacin, which is attributable to the mutation of amino acid codon Ser-87 to Phe in *C. striatum*, is comparable to the mutations found in *Salmonella* spp. (11, 12) and *Neisseria gonorrhoeae* (27, 28), which also consisted of a substitution of Ser to Phe in the amino acid equivalent to Ser-87 of *C. striatum*. In *Salmonella* spp., the MIC of ciprofloxacin obtained with Ser to Phe mutation varied from 0.25 to 8 μg/ml (11, 12). Otherwise, for *N. gonorrhoeae*, the MIC of ciprofloxacin ranged from 0.12 to 1 μg/ml (27, 28). The ranges in MICs may be explained by a concomitant action of one or more efflux pumps.

The activity of new fluoroquinolones against *C. striatum* and *C. amycolatum* has rapidly decreased during the last few years. Comparing data from previous studies performed in 1999 and 2001, respectively (18, 20), increases in the MICs at which 50% of the isolates are inhibited of ciprofloxacin from 2 μg/ml for *C. amycolatum* and from 4 μg/ml for *C. striatum* to ≥16 μg/ml for both species were observed. This fact may be related to the increase in prescribed fluoroquinolones in Spain during the last few years and to the ability to acquire quinolone resistance.

The difference in the nucleotide sequences of the QRDR of the *gyrA* gene of *C. striatum* and *C. amycolatum* allowed the designing of a PCR-RFLP of the *gyrA* gene with the restriction enzyme NcoI to differentiate these species. This method is more specific and rapid than the additional tests usually done to differentiate these two species These tests are (i) the morphology test, which shows that the colony is dry with a sugary texture in the case of *C. amycolatum* and creamy and pale yellow in the case of *C. striatum*, and (ii) the biochemical test, which consists of the hydrolysis of tyrosine, being positive for *C. striatum* and negative for *C. amycolatum* (21). The amplified rDNA restriction analysis (ARDRA) of the 16S gene has also been developed to differentiate *Corynebacterium* spp. (31) and *Acinetobacter* spp. (32).

In summary, whereas a mutation in the *gyrA* gene of *C. amycolatum* was sufficient to result in high MICs of ciprofloxacin, levofloxacin, and moxifloxacin, two mutations are necessary to result in high MICs of ciprofloxacin and levofloxacin, whereas the moxifloxacin MIC for *C. striatum* remains lower. Moreover, a rapid PCR-RFLP NcoI of the QRDR of the *gyrA* gene has been developed to differentiate both species of *Corynebacterium*. This molecular technique could supplement the phenotypic and biochemical tests currently used to identify these two species correctly.

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