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Conjugation between quinolone-susceptible bacteria can generate mutations in the quinolone resistance-determining region, inducing quinolone resistance

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ABSTRACT

Quinolones are an important group of antibacterial agents that can inhibit DNA gyrase and topoisomerase IV activity. DNA gyrase is responsible for maintaining bacteria in a negatively supercoiled state, being composed of subunits A and B. Topoisomerase IV is a homologue of DNA gyrase and consists of two subunits codified by the *parC* and *parE* genes. Mutations in *gyrA* and *gyrB* of DNA gyrase may confer resistance to quinolones, and the majority of resistant strains show mutations between positions 67 and 106 of *gyrA*, a region denoted the quinolone resistance-determining region (QRDR). The most frequent substitutions occur at positions 83 and 87, but little is known about the mechanisms promoting appearance of mutations in the QRDR. The present study proposes that some mutations in the QRDR could be generated as a result of the natural mechanism of conjugation between bacteria in their natural habitat. This event was observed following conjugation in vitro of two different isolates of quinolone-susceptible *Pseudomonas aeruginosa*, which transferred plasmids of different molecular weights to a recipient strain of *Escherichia coli* (HB101), also quinolone-susceptible, generating two different transconjugants that presented mutations in DNA gyrase and acquisition of resistance to all quinolones tested.

1. Introduction

Quinolones are a clinically important and useful group of antibacterial agents [1,2]. They inhibit DNA gyrase and topoisomerase IV activity and, as a consequence, inhibit DNA synthesis, resulting in bacterial death. The main quinolone resistance mechanisms are caused by mutations in DNA gyrase and topoisomerase IV, by an increase in AcrAB efflux pump activity, and by plasmid-mediated quinolone resistance (PMQR) mechanisms including *qnr*, *qepA* and *aac(6′)-Ib-cr* genes [3,4].

DNA gyrase is an ATP-dependent type II DNA topoisomerase responsible for maintaining bacteria in a negatively supercoiled state. Due to cleavage of the helix in both strands, it introduces changes in the topology of closed circular DNA, being related to DNA replication, recombination and gene expression [5]. This enzyme is composed of subunits A and B, containing two subunits of each and forming an A2B2 complex in the active enzyme [6].

Although *gyrA* and *gyrB* mutations may confer resistance to quinolones, the majority of resistant strains show mutations between positions 67 and 106 of *gyrA*. This region was denoted the quinolone resistance-determining region (QRDR) [7] and is in the N-terminal domain of *gyrA* [8]. The most frequent substitutions occur at positions 83 and 87, exhibiting replacement of serine (Ser83) to leucine (Leu) or tryptophan (Trp) and of aspartate (Asp87) to asparagine (Asn) or valine (Val) [9]. However, many other mutations have been described to date, many of which are silent mutations that do not appear to affect the functionality of the enzyme.

Topoisomerase IV is a homologue of DNA gyrase and consists of two subunits codified by the *parC* and *parE* genes, which have sequence identity with *gyrA* and *gyrB*. In *parC* and *parE*, most mutations occur mainly at positions Ser80 and Ser458, respectively [10], although other mutations have been described.

Substitution of amino acids in different positions of the QRDR has been described only as spontaneous mutations; however, little is known about the appearance of these mutations. The present study proposes that some mutations could be generated as a result of the natural mechanism of conjugation between bacteria in their

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Table 1
Primers used in this study.

Target gene	Primer sequence (5′→3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
aac(6′)-Ib-cr	F-TTGCGATGCTCTATGAGTGGCTA R-CTCGAATGCCTGGCGTGTTT	55	482	[13]
acrR	F-CTTGTTGGGCCTGTTTGTCGTCAC R-GCTTTTGTCGGCAGATCACCATTC	58	1147	[14]
gyrA	F-CATGAACGTATTGGGCAATG R-CCGTACCGTCATAGTTATCC	49	305	[15]
gyrB	F-CTCCTCCCAGACCAAAGACA R-TCACGACCGATACCACAGCC	58	447	[16]
parC	F-AAACCTGTTCAGCGCCGCATT R-GTGGTGCCGTTAAGCAAA	58	395	[16]
parE	F-GCCCAGCGCCGTATGCGTGC R-GTTCGGATCAAGCGTGGTTT	58	621	[17]
qnrA	F-AGAGGATTTCTCACGCCAGG R-TGCCAGGCACAGATCTTGAC	55	580	[18]
qnrB	F-GGMATHGAAATTCGCCACTG R-TTTGCYGYYCGCCAGTCGAA	55	264	[18]
qepA	F-TGGTCTACGCCATGGACCTCA R-TGAATTCGGACACCGTCTCCG	56	1137	[19]

natural habitat. This event was initially observed following conjugation in vitro of two different isolates of quinolone-susceptible *Pseudomonas aeruginosa*, which transferred plasmids of different molecular weights to a recipient strain of *Escherichia coli* (HB101), also quinolone-susceptible, generating two different transconjugants that presented mutations in DNA gyrase and acquisition of resistance to all quinolones tested.

2. Materials and methods

A total of seven conjugations were performed as described by Azevedo and da Costa [11] using two donor *P. aeruginosa* isolates (EW32 and EW33) and six different recipient strains [streptomycin-resistant *E. coli* HB101, nalidixic acid-resistant *E. coli* DH5α, two aztreonam-resistant environmental *P. aeruginosa* isolated from water (EW08 and EW10) and two aztreonam-resistant *P. aeruginosa* obtained from soil samples (S91 and S92)]. Donor and recipient strains were separately inoculated in Luria–Bertani (LB) broth (Oxoid Ltd., Basingstoke, UK) and were incubated at 37 °C for 18 h. Subsequently, 1 mL of each bacterial growth was centrifuged at 12,000 × *g* (Centrifuge 5415R; Eppendorf, Hamburg, Germany) for 30 s and the pellets were washed twice with 0.85% NaCl solution and re-suspended with 75 μL of LB broth. After this step, the donor isolate was mixed with the recipient strain and was then positioned on the surface of a membrane filter (nitrocellulose, 0.22 μm; Millipore, Billerica, MA) on a LB agar plate. Following 6 h at 30 °C, cell mixtures were re-suspended in 5 mL of LB broth, centrifuged at 12,000 × *g* for 120 s, washed twice with 1 mL of 0.85% NaCl solution, re-suspended in 100 μL of LB broth, and inoculated on the surface of a LB agar plate containing a suitable concentration of the resistance selectable markers of the donor and recipient isolates.

Antimicrobial susceptibility testing of the isolates and transconjugants was performed in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [12] by disc diffusion on Mueller–Hinton agar (Oxoid Ltd.) and by determination of the minimum inhibitory concentration (MIC) using a range from 0.2 μg/mL to 250 μg/mL of norfloxacin, levofloxacin and ciprofloxacin. The antimicrobial drug discs (Oxoid Ltd.) and their respective concentrations tested in this study were: amikacin, 30 μg; aztreonam, 30 μg; cefepime, 30 μg; ceftazidime, 30 μg; ciprofloxacin, 5 μg; gentamicin, 10 μg; imipenem, 10 μg; levofloxacin, 5 μg; lomefloxacin, 10 μg; meropenem, 10 μg; norfloxacin, 10 μg; ofloxacin, 5 μg; polymyxin B (300 U); piperacillin/tazobactam, 110 μg; streptomycin, 10 μg; tetracycline, 30 μg; ticarcillin/clavulanic acid, 85 μg; and tobramycin, 10 μg. *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PAO1 were used as quality control strains.

DNA was prepared with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of the DNA were determined using a NanoDrop® 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). PCR reactions were performed with a final volume of 50 μL containing 200 ng of DNA and 1.25 U of *Taq* DNA polymerase (Thermo Scientific) in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). PCR primers, annealing temperatures, amplicon sizes and references to investigated genes are shown in Table 1. PCR reactions were performed for all genes as follows: initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 1 min, annealing temperature at 60 °C for 1 min and extension at 72 °C for 1 min; and an additional extension at 72 °C for 5 min. Reactions without DNA were used as a negative control for all PCR assays. *E. coli* strain K12 was used as a positive control for the *acrR*, *gyrA*, *gyrB*, *parC* and *parE* genes. *E. coli* strain 64611 was used as positive control for *qepA*, *Klebsiella pneumoniae* Kp15 for *qnrB*, *E. coli* 16-00 for *qnrA* and *E. coli* 78-01 for *aac(6′)-Ib-cr*.

The amplified products of quinolone resistance genes of the transconjugants Tr32101 and Tr33101 and of the *E. coli* HB101 recipient strain were sequenced using the same amplification primers in an ABI 3130 Genetic Analyzer (Applied Biosystems, New York, NY). A comparative modelling study of the three-dimensional structures of the wild-type and mutated enzymes was performed using Modeller software [20].

3. Results

Transconjugants Tr32101 and Tr33101, obtained separately by conjugation between donor *P. aeruginosa* isolates (EW32 and EW33) and recipient *E. coli* HB101 strain, acquired plasmids of 22.5 MDa and 48 MDa, respectively, carrying genes for resistance to tetracycline [21]. Furthermore, a surprising result was that both transconjugants became resistant to all quinolones tested (ciprofloxacin, lomefloxacin, levofloxacin, norfloxacin and ofloxacin) by disc diffusion. Donor isolates were investigated by PCR for the presence of PMQR genes [*qnrA*, *qnrB*, *qepA* and *aac(6′)-Ib-cr*] and no amplification was observed. *P. aeruginosa* EW32 and EW33, the recipient strain *E. coli* HB101 and transconjugants Tr32101 and Tr33101 were also analysed by determination of the MIC of the antibiotics ciprofloxacin, levofloxacin and norfloxacin. The results demonstrated that only the transconjugants acquired resistance to these quinolones (Table 2). Donor and recipient