Nonoptimal DNA topoisomerases allow maintenance of supercoiling levels and improve fitness of Streptococcus pneumoniae

Luz Balsalobre, María José Ferrándiz, Gabriela de Alba, Adela G de la Campa

Antimicrob Agents Chemother. 2011 Mar;55(3):1097-105.

Nonoptimal DNA topoisomerases allow maintenance of supercoiling levels and improve fitness of *Streptococcus pneumoniae*

Luz Balsalobre, María José Ferrándiz, Gabriela de Alba, and Adela G. de la Campa*

Unidad de Genética Bacteriana, Centro Nacional de Microbiología, Instituto de Salud Carlos III and CIBER Enfermedades Respiratorias, 28220 Majadahonda, Madrid, Spain

*To whom correspondence should be addressed. Tel: +34 91 509 7057: Fax: +34 91 509 7919; E-mail: agcampa@isciii.es.

Running title: Supercoiling levels influence viability

Key words: Fluoroquinolones, DNA topoisomerase IV, DNA gyrase, supercoiling, fitness

ABSTRACT

Fluoroquinolones, which target gyrase and topoisomerase IV, are used for treating *Streptococcus pneumoniae* infections. Fluoroquinolone-resistance in this bacterium can arise via point mutation or interspecific recombination with genetically-related streptococci. Our previous study on the fitness cost of resistance mutations and recombinant topoisomerases identified GyrAE85K as a high-cost change. However, this cost was compensated for by the presence of a recombinant topoisomerase IV (*parC* + *parE* recombinant genes) in strain T14. In this study we purified wild-type and mutant topoisomerases and compared their enzymatic activities. In strain T14, both gyrase carrying GyrAE85K and recombinant topoisomerase IV showed lower activities (between 2.0- to 3.7-fold) than the wild-type enzymes. These variations of *in vitro* activity corresponded to changes of *in vivo* supercoiling levels that were analyzed by two-dimensional electrophoresis of an internal plasmid. Strains carrying GyrAE85K and non-recombinant topoisomerasas had lower (11.1% to 14.3%) supercoiling density (σ) values than the wild-type. Those carrying GyrAE85K and recombinant topoisomerases showed either partial or total supercoiling level restoration, with σ values 7.9% (recombinant ParC) and 1.6% (recombinant ParC + recombinant ParE) lower than the wild-type. These data suggested that changes acquired by interspecific recombination might be selected because they reduce the fitness cost associated with fluoroquinolone-resistance mutations. An increase of fluoroquinolone resistance, even in the absence of further antibiotic exposure, is envisaged.

INTRODUCTION

In spite of the development of vaccines and chemotherapy, *Streptococcus pneumoniae* continues to be an main human pathogen, due in part to its high rate of resistance to antibiotics and in part to the low coverage and partial inefficiency of available vaccines. The World Health Organization estimates that about one million children aged <5 years die annually of pneumococcal pneumonia, meningitis, and/or sepsis worldwide (65). After the usage of the pneumococcal 7-valent conjugate vaccine in children, which includes most of the antibiotic-resistant serotypes, the incidence of invasive disease declined in both children and adults, reflecting herd immunity (34, 64). This was associated with a decline in penicillin resistance rates in many countries (17, 34, 52). However, emergence of serotypes not included in the vaccine, especially multiresistant serotype 19A, has been observed (9, 17, 44).

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

Pneumococcal resistance to β-lactams and macrolides has spread worldwide in the last three decades (27). Currently new respiratory fluoroquinolones, which target DNA gyrase (gyrase) and DNA topoisomerase IV (topo IV), the essential type II DNA topoisomerases, are recommended as therapeutic alternatives for treatment of adult patients with community-acquired pneumonia (38). DNA topoisomerases catalyze the interconversions of different topological DNA forms and thus solve the topological problems associated with DNA replication, transcription, and recombination (7). DNA supercoiling is maintained in bacteria homeostatically by the opposing activities of topoisomerases which relax DNA, and by gyrase, which introduces negative supercoils. In *Escherichia coli*, transcription of the DNA topoisomerase I gene increases when negative supercoiling increases (62), and that of the gyrase genes increases after DNA relaxation (41-43). Likewise, gyrase up-regulation in response to relaxation has also been

observed in *Streptomyces* and *Mycobacterium* (60, 63). We have recently described the transcriptional response to DNA relaxation that affects all *S. pneumoniae* topoisomerases, triggering the up-regulation of gyrase and the down-regulation of topoisomerases I and IV (19). We have showed that the pneumococcal genome is organized in topology-reacting gene clusters that share particular AT content and codon composition characteristics (19). *S. pneumoniae* is part of the commensal flora of the human nasopharinx. However, under specific circumstances, migrates to other niches (ear, lung, bloodstream, cerebrospinal fluid) causing diverse pathologies. Since the level of bacterial DNA supercoiling is affected by diverse environmental conditions (13, 56, 61), and global genome transcription is dependent on the degree of supercoiling (19, 25, 29, 51), changes in DNA topology would be crucial for cell viability and for the infective capacity of *S. pneumoniae* on their diverse niches (with diverse environmental conditions) in the human host.

Gyrase ($GyrA_2GyrB_2$) introduces negative supercoils into DNA (23) and topo IV ($ParC_2ParE_2$) acts mainly in chromosome partitioning (31). Fluoroquinolones inhibit these enzymes by forming a ternary complex of drug, enzyme, and DNA. Cellular processes acting on this complex would yield to the formation of irreparable double-stranded DNA breaks that cause bacterial death (14). Genetic and biochemical studies have shown that ciprofloxacin (CIP) and levofloxacin, target primarily topo IV and secondarily gyrase in *S. pneumoniae* (18, 28, 45, 48, 59). However, for moxifloxacin, gyrase is the primary target (26).

Fluoroquinolone-resistant clinical isolates carry mutations in the quinolone resistance-determining regions (QRDRs), which are located in the N-terminus of ParC or GyrA and in the C-terminus of ParE. Although CIP is not an effective antipneumococcal drug, we used it to detect fluoroquinolone resistance, and considered a CIP-resistance breakpoint MIC $\geq$4 μg/ml to

improve detection of first-step mutant strains (8, 10, 11). Low-level (MIC of 4-8 μg/ml) CIP-resistant strains had mutations altering the QRDRs of one of the two subunits of topo IV: S79, S80 or D83 of ParC (10, 28, 45), D435 of ParE (50). High-level (MICs ≥ 16 μg/ml) CIP-resistant strains had additional GyrA changes (S81 or E85) (28, 45).

A study (years 2004-2005) including 15 European countries (54) showed a low level (< 3%) of fluoroquinolone resistance in *S. pneumoniae*, with the exception of Poland (4.4%), Finland (6.6%), and Italy (7.2%). Higher rates have been detected in some Asian countries (21), as well as in Canada (1). In Canada, an increase of CIP resistance between 1998 (0.6%) and 2006 (7.3%) occurred in conjunction with increased consumption (1). In Spain, two epidemiological studies performed in 2002 (11) and 2006 (10) showed a stable low rate of CIP resistance (≤ 2.3%), maybe influenced by the stabilization of CIP consumption in this period. However, an increase in resistance in some European countries in which fluoroquinolone use has increased (data from European surveillance of antimicrobial consumption, http//www.esac.ua.ac.be) is not unexpected.

Resistance changes can be acquired either by spontaneous mutation or by intra-specific (58) or inter-specific recombination with related streptococci of the *mitis* group (SGM) (5, 11, 20, 58). The fluoroquinolone-resistant pneumococcal recombinant isolates studied by our group have acquired portions of either *parE* (unpublished results), *parC* (11), or *parE* plus *parC* (5, 11, 20) from SMG. In the latter case, given the presence of the *ant* gene in the intergenic *parE-parC* region of SMG, recombinants acquired an extra gene in the recombination process and consequently, had larger intergenic *parE-parC* regions (1.1 to 7.2 Kb) than non-recombinant pneumococci (0.4 Kb).

Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)
Con formato: Inglés (Estados Unidos)

The evolution of antibiotic resistance depends on the balance between antibiotic use and the fitness cost (typically observed as a reduced growth rate) imposed by resistance mutations (2). A direct relation between fluoroquinolone consumption and increase in resistance in *S. pneumoniae* has been described (8, 36). In addition, fitness depends on the specific drug/ mutation combination (15, 22, 33, 37), and compensatory mutations have been described that ameliorate the fitness loss (6). There are a few reports on fitness cost of fluoroquinolone-resistance mutations in *S. pneumoniae* (4, 24, 30, 55) and only one in which recombinant strains were considered (4). In our previous study we analyzed a set of 24 CIP-resistant isogenic strains and categorized the mutations as conferring no-, low-, or high-biological cost to the strains (4). The mutation causing the GyrAE85K change was identified as a high-cost mutation. However, the fitness cost imposed by this change was compensated by the presence of a recombinant topo IV in strain T14. A single study, in which enzymatic activity determinations were not made, has related fitness cost of quinolone resistance mutations in *E. coli* gyrase and supercoiling level measured in mono-dimensional agarose gel electrophoresis (3). In this study we combined high-resolution two-dimensional agarose gel electrophoresis with enzymatic assays to get insight into the compensation of the fitness cost imposed by GyrAE85K by the presence of a recombinant topo IV enzyme.

MATERIALS AND METHODS

Bacterial strains, growth and transformation of bacteria. *S. pneumoniae* strains used were wild-type R6 and its isogenic derivatives Tr7, T1, T4, T9 and T14 (4). They were grown in a casein hydrolysate-based medium with 0.3% sucrose (AGCH) as energy source and transformed as described previously (35). *E. coli* was grown in Luria-Bertani (LB) broth and

transformed as described (57). The pQE1 vector/M15 *E. coli* host system (Qiagen) was used to over-express *S. pneumoniae* GyrB, GyrA, ParC, and ParE proteins in *E. coli*.

Cloning of topoisomerase genes, protein over-expression and purification. Genes were amplified from chromosomal DNA of strains R6, T1, T9 or T14. Forward oligonucleotides were previously phosphorylated and reverse primers contained *Sph*I restriction sites in their 5´-ends (Table 1). Amplifications (50 µl) were performed with 2.5 U of *Pfu* DNA polymerase (Fermentas), 0.1 µg of template DNA, 1 µM (each) of oligonucleotide primers, and 0.2 mM (each) deoxynucleoside triphosphates. PCR reactions included 1 cycle of 5 min denaturation at 94ºC; 30 cycles of 1 min at 94ºC, 1 min at 55 or 45ºC, 3 min polymerase extension step at 72ºC; a final 5 min extension step at 72ºC and slow cooling at 4ºC. Oligonucleotides were removed (QIAquick PCR Purification Kit, Qiagen), PCR products were cut with *Sph*I, cloned into plasmid pQE1 digested with *Sph*I + *Pvu*II, and established into *E. coli* M15 (pREP4). Transformants were selected in LB plates containing ampicillin (100µg/ml, for pQE1 selection) and kanamycin (25 µg/ml, for pREP4 selection). The pQE1 vector/ M15 (pREP4) system permits the controlled hyperproduction of proteins with an N-terminal Met-Lys-$(His)_6$-Gln- fusion encoded by genes placed under the control of a phage T5 promoter and two *lac* operator sequences. Plasmid pREP4 constitutively expresses the LacI repressor. Expression of recombinant proteins cloned into pQE vectors is induced by isopropyl-β-D-thiogalactoside (IPTG) which binds to LacI and inactivates it. This inactivation allows the host cell's RNA polymerase to transcribe the sequences downstream from the T5 promoter. The various *E. coli* M15 (pREP4) strains harbored pQE1 recombinant plasmids carrying either *parC* (from R6, T1, T9 and T14), *parE* (from R6 and T14), *gyrA* (from R6 and T14) or *gyrB* (from R6) genes. Strains were grown overnight at 37ºC in LB

medium, diluted 20-fold in 200-ml medium and grown at 37ºC (for GyrB or ParE overproduction) or at 30ºC (for GyrA or ParC) until $OD_{600\ nm}$ = 0.6. At this moment, 1 mM IPTG was added, and growth was continued for another 30 min. Bacteria were collected by centrifugation and suspended in 4 ml of 50 mM NaH_2PO_4, 300 mM NaCl (column buffer) containing 10 mM imidazole prior to flash freezing in dry ice. The suspension was thawed at 0ºC and incubated for 30 min with lysozyme (1 mg/ml) and Triton X-100 (0.2%). Cell debris was removed by centrifugation (10,000 x g for 20 min at 4ºC), and the supernatant mixed with 2 ml of 50% Ni-nitriloacetic acid resin slurry (Qiagen) by slow agitation on a rotary shaker at 4ºC for 1 h. The mixture was packed in a column and washed with 8 ml of column buffer containing 20 mM imidazole. His-tagged proteins were eluted with a linear gradient 50-250 mM imidazole in column buffer. Protein fractions were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and those containing proteins of the expected size were pooled and dialyzed overnight at 4ºC against 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 50% glycerol.

Topoisomerase catalytic assays. Gyrase and topo IV were reconstituted by incubation of their corresponding subunits with an excess of GyrB or ParE subunits (1:3.8 ratio) for 1 h at 4ºC. Gyrase-mediated supercoiling reactions (100 µl) were performed during 1 h at 37ºC as described (18) using 0.4 µg of relaxed pBR322 DNA (Inspiralis, Norwich, UK) and reconstituted gyrase. The reaction was terminated by addition of 7.5 µl of 0.5 M EDTA (1-min incubation), and further addition of 1% SDS and 50 µg/ml proteinase K (15-min incubation at 37ºC). Samples were ethanol precipitated, suspended in electrophoresis loading buffer, and analyzed in 1% agarose gels run at 2 V/cm for 12 h. Topo IV decatenation assays (12 µl) were performed as described (18) using 0.4 µg of k-DNA from *Crithidia fasciculata* (Inspiralis, Norwich, UK), and

reconstituted topo IV. Reaction mixtures were incubated at 37ºC for 1 h and terminated by addition of 6 µl of loading buffer and 12 µl of H_2O. Samples were analyzed by electrophoresis in 0.7% agarose gels run at 3 V/cm for 1 h and then at 7 V/cm for 2 h. Relaxation assays (20 µl) were performed as described (49) using 50 ng of supercoiled pBR322 and 12.5-fold higher amount than in the decatenation assay of reconstituted topo IV. Samples were analyzed by electrophoresis in 1.4% agarose gels run at 1.8 V/cm for 22 h. After electrophoresis, gels were subjected to Southern hybridization using a 506-bp pBR322 probe obtained by amplification of plasmid DNA with 5'-byotinilated pBR-1 and pBR-2 oligonucleotides (Table 1). Southern blot and hybridization were performed following the Phototope®-Star kit (New England Biolabs) instructions. Cleavage assays were carried out either in 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 200 mM potassium glutamate and 50µg/ml BSA (for topo IV) or in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT and 0.1 mg/ml BSA (for gyrase). Reconstituted topoisomerases were incubated with 0.4 µg of supercoiled pBR322 in 25 µl reaction mixtures in the presence of different concentrations of ciprofloxacin to account for 16 × MIC for each strain (for topo IV) or for 100 x MIC (for gyrase). After 30-min incubation at 37ºC, 1 µl of 10% SDS and 2 µl of a 20 mg/ml proteinase K were added, and incubation continued for 30 min at 45ºC. Loading buffer was added to the samples, which were analysed by electrophoresis in 1% agarose gels, which were run at 2 V/cm for 12 h..

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

Two-dimensional agarose gel electrophoresis. Isolation of plasmid DNA from *S. pneumoniae* cultures grown on AGCH containing 1µg/ml of tetracycline (for selection of pLS1) was performed using a neutral method to avoid plasmid denaturation. Exponentially growing cells were harvested and lysed by treatment with lysozyme and a detergent solution of 1% Brij 58

and 0.4% sodium deoxycholate (40). Plasmid molecules were analyzed in neutral/neutral two-dimensional agarose gels. The first dimension was run in a 0.4% (w/v) agarose (Seakem; FMC Bioproducts) gel in 1× Tris-borate-EDTA (TBE) buffer containing 1 μg/ml of chloroquine (Sigma) at 1.5 V/cm at room temperature for 19 h. The second dimension was in 1% agarose gel in TBE buffer containing 2 μg/ml of chloroquine at 7.5 V/cm for 7–9 h at 4ºC. Chloroquine was added to the TBE buffer in both the agarose and the running buffer. After electrophoresis, gels were subjected to Southern hybridization using a 240-bp PCR fragment obtained from pLS1 DNA with 5´-biothinylated pLS1F and pLS1R (Table 1) as a probe on gels transferred to nylon membranes (Inmobylon NY^+, Millipore). Chemiluminiscent detection of DNA was performed with the Phototope®-Star kit (New England Biolabs). Images were captured in a VersaDoc MP400 system and analyzed with the Quantity One program (BioRad). DNA linking number (Lk) was analyzed by quantifying the amount of every given topoisomer. DNA supercoiling density (σ) was calculated with $\sigma = \Delta Lk / Lk_0$. Linking number differences (ΔLk) were determined with the equation $\Delta Lk = Lk - Lk_0$, in which $Lk_0 = N/10.5$, where N is the DNA size in bp (4408 for pLS1) and 10.5 the number of bp per one complete turn in B-DNA, the most probable helical repeat of DNA under the conditions used.

RESULTS

Mutant DNA topoisomerases are less active than wild type enzymes. Five strains (Figure 1A), which carry various fluoroquinolone-resistance mutations and *parE-parC* structures, were selected for this study among a series of isogenic R6-derived strains previously constructed (4). While strain T1, which was used as a control, carried ParCS79F as a single change, the

remaining four strains carried GyrAE85K: one as a single change (Tr7); three (T4, T9, and T14) in combination with ParCS79F. Of these last strains, while T4 carried a non-recombinant *parC* gene, T9 carried a recombinant *parC*, and T14 carried both *parE* and *parC* recombinant genes, and, accordingly, the *ant* gene in its intergenic region. Consequently, T9 and T14 carried recombinant (*rec)* topoisomerase subunits that have, in addition to changes involved in resistance, other changes (Fig. 1A) not involved in resistance (4). Changes present in *rec*ParC of strain T9 were S52G, N91D, A190T, and I201V. Strain T14 had the S52G, N91D changes, and carried in addition Y23H. The *rec*ParE subunit of strain T14 carry the G486S, I493L and F571L changes not involved in resistance (Fig. 1A). The fitness cost of these strains has been previously determined in competition experiments with the R6 strain (4). In our previous study, mixed cultures of R6 and each isogenic resistant strain were incubated in antibiotic-free medium for 6 h (c.a. 10-12 generations), diluted 1000-fold and re-growth for an additional 6 h period. The number of viable cells was determined at 0 h, at the end of the first 6 h cycle and after the second 6 h cycle. Strains were classified as high-cost when showed relative mean fitness lower than 1 both in 1-cycle (95% CI= 0.79-0.99) and in 2-cycle of competitive growth (95% CI= 0.63-0.89) experiments. Low-cost strains showed relative mean fitness lower than 1 only in the 2-cycle (95% CI= 0.81-0.99) experiments, while in the no-cost strains the 95% CI of the mean relative fitness includes the 1 value. From this classification, T1 was considered to be a low-cost strain, and all strains carrying GyrAE85K, except T14 that showed no cost, were classified as high fitness cost strains.

Con formato: Inglés (Estados Unidos)

A series of His-tagged topoisomerase subunits were overexpressed and purified (Fig. 1B) in order to perform enzymatic assays. All wild-type (*wt*) subunits were obtained from strain R6, GyrAE85K from T14, ParC subunits from T1 and T14, and ParE from T14. Most proteins were

obtained in soluble form with >90% homogeneity. However, additional bands of lower molecular weight were observed in GyrB and ParE of R6 (Fig. 1B). As shown below, these additional proteins did not interfere with the topoisomerase enzymatic assays used. The main gyrase activity, the ability to supercoil relaxed pBR322 in the presence of ATP was assayed. No supercoiling activity was observed when either the GyrA (Fig. 1C, lines A and AE85K) or *wt*GyrB (Figure 1C, line B) subunits alone were assayed. Supercoiling activities were only observed when both subunits were combined (Fig. 1C, lines A+B and AE85K+B). To achieve 50% of supercoiling activity, 125 fmole and 250 fmole of *wt*GyrA and GyrAE85K subunits, respectively, were required (Fig. 2A), showing that the mutant subunit is 2-fold less active than the wild-type GyrA subunit (Table 2). Two activities were assayed for topo IV: decatenation and relaxation. Decatenation, which was assayed using k-DNA as a substrate, was observed when the ParC and ParE subunits were combined (Fig. 1D, lines C+E), while no activity was observed with either the ParC or ParE (Fig. 1D, lines C and E) subunits alone. The decatenation activities of topo IV enzymes reconstituted with *wt*ParC (from R6) or ParC mutant subunits (ParCS79F from T1, and *rec*ParCS79F from T14) were compared. ParE subunits assayed were *wt* (from R6) and *rec*ParE from T14. As shown in Fig. 2, 50% decatenation activity was achieved with 50 fmole (*wt*ParC + *wt*ParE), 100 fmole (ParCS79F + *wt*ParE), 163 fmole (*rec*ParCS79F + *wt*ParE) and 127 fmole (*rec*ParCS79F + *rec*ParE) (Fig. 2B and 2C). These results showed that ParCS79F had 2-fold lower activity than *wt*ParC, and that topo IV reconstituted with *rec*ParC or *rec*ParE + *rec*ParC showed decatenation activities 3.3- and 2.5-fold lower, respectively, than *wt* topo IV (Table 2). Relaxation activity for topo IV was about 100-fold lower than that of decatenation, as it has been previously described (49), for this reason, 50 ng instead 400 ng of supercoiled

pBR322 were used as a substrate and the reaction products were detected after Southern-blot hybridization as described in material and methods. Relaxation was observed when the ParC and ParE subunits were combined (Fig. 1E, lines C+E), while no activity was observed with either the ParC or ParE (Fig. 1E, lines C and E) subunits alone. The relaxation activities of topo IV enzymes reconstituted as described above were compared. As shown in Fig. 2, 50% relaxation activity was achieved with 0.8 pmole (*wt*ParC + *wt*ParE), 1.2 pmole (ParCS79F + *wt*ParE), 1.2 pmole (*rec*ParCS79F + *wt*ParE) and 1.6 pmole (*rec*ParCS79F + *rec*ParE) (Fig. 2C). These results showed that ParCS79F and *rec*ParC had activities 1.5-fold lower than *wt*ParC, and that topo IV reconstituted with or *rec*ParE + *rec*ParC showed a relaxation activity 2.0-fold lower than *wt* topo IV (Table 2).

Since it has been suggested that fluoroquinolone action it is better assayed by stimulation of topoisomerase-mediated cleavage, this assay was performed with both gyrase and topo IV in the presence of an excess (100 × MIC and 16 × MIC, respectively) of CIP. In these assays, the enzyme is blocked in a reaction intermediary and renders linear DNA from CCC pBR322 after treatment with proteinase K and SDS, as described in material and methods. When the activities of GyrA and GyrB subunits were analyzed, linearization was only observed when the GyrA and GyrB subunits were combined (Figure 1F, lines A+B), while no activity was observed with either subunit alone. When this cleavage assay was performed using *wt*GyrA and GyrAE85K subunits, a 20% activity was achieved with 289 fmole (*wt*GyrA + GyrB) and 1035 fmole (GyrAE85K + GyrB), showing that GyrAE85K had 3.7-fold lower activity than the *wt* enzyme (Figure 3A). When the activities of the ParC and ParE subunits of topo IV were analyzed (Fig. 1G), some linearization was observed when only the E subunit was used, however, this residual activity,

which is probably due to the contaminating band observed in the purification (Fig. 1B), did not interfere with the assay. When ParC + ParE subunits were combined, a 3-fold greater activity was observed when the assay was performed in the presence of ciprofloxacin than in its absence (Fig. 1G). When this cleavage assay was used to test topo IV enzymes, a 50% activity was achieved with 282 fmole (ParC + *wt*ParE), 848 fmole (ParCS79F + *wt*ParE), 740 fmole (*rec*ParCS79F + *wt*ParE) and 963 fmole (*rec*ParCS79F + *rec*ParE) (Fig. 3B and 3C). These results showed that topo IV carrying ParCS79F, *rec*ParC, and *rec*ParC + *rec*ParE had 3.2-, 2.7- and 3.4-fold lower cleavage activity than the *wt* enzyme, respectively (Table 2).

DNA topoisomer distribution varied in isogenic strains carrying the GyrAE85K change. To estimate the supercoiling level *in vivo,* we analyzed the influence of the *parC* and *gyrA* mutations in the supercoiling level of plasmid pLS1, able to replicate in *S. pneumoniae*. This plasmid was introduced into strains R6, Tr7, T1, T4, T9 and T14 and their topoisomer distribution was analyzed by using two-dimensional agarose gel electrophoresis (Fig. 4A). Plasmid pLS1 is appropriate to study gyrase activity, given that it replicates by a rolling circle mechanism (12) and all their genes are transcribed in the same direction, avoiding problems of transcription interference during replication (47). Topoisomers were distributed in the autoradiograms in a bubble-shaped arc, negative supercoiled molecules being located to the right and positive supercoiled ones to the left (Fig. 4A). To calculate the supercoiling density (σ) values it was considered that the induced ΔLk of monomers by 2 μg/ml chloroquine in pLS1 is -14 (19). No significant difference (6.3%) in supercoiling density (σ) values were observed between the *wt*R6 (-0.063) and T1 carrying the ParCS79F change (-0.059). However σ values for strains carrying GyrAE85K, such as Tr7 that carries this single change (-0.054) and T4 that also

carries ParCS79F (-0.056) were 14.3 and 11.1% lower, respectively, than for R6. These results suggest that the GyrAE85K change causes a supercoiling level deficiency. However, two recombinant strains (T9 and T14) that also carry GyrAE85K, showed σ values that differ from the non-recombinant (Tr7 and T4) strains. While T9 (*rec*ParC) had a σ value (-0.058) 7.9% lower than R6, T14 (*rec*ParC + *rec*ParE) had a σ value (-0.062) equivalent (1.6% lower) to R6. Supercoiling densities and mean relative fitness after 12 h of competitive growth showed a good correlation (r^2= 0.75, p<0.0001) (Fig. 4B).

DISCUSSION

Under laboratory conditions, the frequencies of CIP-resistance acquisition either via inter-specific recombination or mutation are about 10^{-3} (27, 30) and 10^{-9} (54), respectively. However, the prevalence of CIP-resistant *S. pneumoniae* clinical isolates that have acquired resistance by interchange with SMG is unexpectedly low, accounting for 3- to 10% of the resistant isolates (5, 12, 63). Even when factors such as DNA availability and competence state of the recipient cells in the natural environment could affect horizontal transfer, it has been estimated that the ratio of recombination/ mutation in natural *S. pneumoniae* populations is 10:1 (17).

The fitness cost imposed by the DNA interchange could explain the low frequency of fluoroquinolone-resistant recombinant *S. pneumoniae* clinical isolates. Strain T14 had a *parE-ant-parC* structure and carries *rec*ParE + *rec*ParC subunits. Two factors could theoretically affect the fitness of this recombinant; one is a putative discoordination of the *parE-ant-parC* operon transcription, and the other the existence of recombinant topo IV subunits. With respect the first factor, we have previously shown that transcription of the operon from a promoter located

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

Con formato: Inglés (Estados Unidos)

upstream of *parE* was not affected, neither in T14 nor in other clinical isolates with small (<2 Kb) intergenic regions (4). With respect the second factor, it would be expected that a recombinant gyrase or topo IV enzyme, which has a tetrameric structure, would be a less efficient enzyme than a non-recombinant one and would cause fitness cost to the strain that carried it. However, strain T14 shows a compensation of the fitness cost imposed by the GyrAE85K change (4) maintaining its chromosome supercoiling level due to the conjunction of suboptimal activities of its recombinant topo IV and mutant gyrase enzymes. Since the strains analyzed in this study were isogenic, no alterations in topoisomerase I activity is expected. We showed that the reduced gyrase activity on supercoiling produces a decrease in bacterial fitness, which is compensated by a reduced relaxing activity of topo IV. The two effects balance to give a nearly wild type level of supercoiling and thus, of the bacterial fitness.

Gyrase enzymatic assays showed that the GyrAE85K enzyme has lower activity (Figs 2 and 3, Table 2) than the *wt* enzyme. As a consequence, the supercoiling level detected in pLS1 of strains carrying this change is affected in. Although we have not measured the supercoiling level of the bacterial chromosome, the values obtained on small plasmids provide an good estimation of chromosomal supercoiling (53). Supercoiling density values for plasmid pLS1 in strains Tr7 and T4, which carry GyrAE85K, were 14.3 and 11.1% lower, respectively than the *wt* strain (Fig. 4). However, recombinant strain showed a recovery. A full supercoiling level recovery (σ value of -0.062, equivalent to R6) was observed in strain T14 (*rec*ParC + *rec*ParE), which is in accordance with the lower activities of its topo IV enzyme. Then, the lower enzymatic activities of gyrase and topo IV of strain T14 allowed an appropriate supercoiling level *in vivo*. Likewise, partial restoration of the supercoiling level (σ value 7.9% lower than R6) was observed in T9

(*rec*ParC). Although we do not have purified topo IV of strain T9, it can be assumed that its activity would be equivalent to that of *rec*ParCS79F from T14 and *wt*ParE from R6. Then, the partial restoration in σ values could be due to a lower activity of its topo IV enzyme.

The lower activities of the recombinant topo IV enzymes from T9 and T14 could be attributed in part to the presence of the ParCS79F change involved in resistance, and in part to the rest of amino acid changes not involved in resistance. Of their 3.3-fold (T9) and 2.5-fold (T14) lower activities detected in decatenation assays, only part (about 2-fold) could be attributed to the ParCS79F change of strain T1. Likewise, of the 2.0-fold lower activity detected in relaxation assays T14, only part (about 1.5-fold) could be attributed to the ParCS79F change. However, this correspondence was not total when the cleavage-complex formation activity was considered: activity decreases for ParCS79F (3.2-fold), *rec*ParCS79F (2.7-fold), *rec*ParCS79F + *rec*ParE (3.4-fold), with respect to the *wt*ParC do not perfectly fit with the estimated *in vivo* supercoiling level. These differences may be due to the *in vitro* activity conditions that do not necessarily reflect the *in vivo* conditions. Nevertheless, a good correlation was observed between σ values and mean relative fitness (Fig. 4B). These results show that the *in vivo* determinations are more accurate and sensitive than the enzymatic assays *in vitro* and suggest that the fitness cost is related to the level of supercoiling of the plasmid, and presumably, of the chromosome.

In conclusion, the presence of suboptimal topoisomerases, both gyrase that introduces negative supercoils and topo IV that relaxes DNA, allows the restoration of wild-type supercoiling levels and bacterial fitness. A similar phenomenon was noticed in *E. coli* fluoroquinolone-resistant strains where the addition of a *parC* resistance mutation to an already

low-fitness gyrase mutant caused a further reduction in drug susceptibility and an increase in relative fitness (39).

The variations in the σ values of the various strains used in our study were in the 1.6% (T14) to 14.3% (Tr7) range. These figures are considerably lower than the 23% variation that triggers a global transcriptional response in *S. pneumoniae*, which includes up-regulation of gyrase and down-regulation of relaxing topoisomerases and allows the recovery of the supercoiling levels (19). To test if an inherent transcriptional response occurred in the strains used in our study, real-time RT-PCR experiments were performed to detect changes in the expression of topoisomerase genes. No significant variations (data not shown) were detected, suggesting that either σ variations >14.3% are necessary to trigger the supercoiling homeostatic response or that real-time RT-PCR is not sensitive enough to detect the predicted low variations in transcription of the topoisomerase genes.

It is well known that the fitness cost of drug resistance can be reduced by selection of low-cost mutations (4, 30, 55) or by accumulation of secondary fitness-compensating mutations that do not reduce resistance (32, 46). During evolution, compensatory mutations, that arise spontaneously, are selected because they provide a competitive advantage. Our data show that mutations acquired by *S. pneumoniae* by interspecific recombination might be selected because they reduce the fitness costs of some fluoroquinolone-resistance mutations. Given the 10:1 prevalence of transformation versus mutation in natural *S. pneumoniae* populations (16), an increase of fluoroquinolone resistance, even in the absence of additional antibiotic exposure, is envisaged.

ACKNOWLEDGMENTS

This study was supported by grants BIO2008-02154 from Plan Nacional de I+D+I of Ministerio de Ciencia e Innovación and COMBACT-S-BIO-0260/2006 from Comunidad de Madrid. Ciber Enfermedades Respiratorias is an initiative from Instituto de Salud Carlos III. AG de la Campa is an Investigador Científico from the CSIC.

REFERENCES

1. **Adam, H. J., D. J. Hoban, A. S. Gin, and G. G. Zhanel.** 2009. Association between fluoroquinolone usage and a dramatic rise in ciprofloxacin-resistant *Streptococcus pneumoniae* in Canada, 1997-2006. Int. J. Antimicrob. Agents **34:**82-5.
2. **Andersson, D. I., and D. Hughes.** 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. **8:**260-71.
3. **Bagel, S., V. Hullen, B. Wiedemann, and P. Heisig.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob. Agents Chemother. **43:**868-875.
4. **Balsalobre, L., and A. G. de la Campa.** 2008. Fitness of *Streptococcus pneumoniae* fluoroquinolone-resistant strains with topoisomerase IV recombinant genes. Antimicrob. Agents Chemother. **52:**822-830.
5. **Balsalobre, L., M. J. Ferrándiz, J. Linares, F. Tubau, and A. G. de la Campa.** 2003. Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **47:**2072-2081.

Con formato: Inglés (Estados Unidos)

Con formato: Español (alfab. internacional)

6. **Bjorkholm, B., M. Sjolund, P. G. Falk, O. G. Berg, L. Engstrand, and D. I. Andersson.** 2001. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proc. Natl. Acad. Sci. USA **98:**14607-14612.

7. **Champoux, J. J.** 2001. DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. **70:**369-413.

8. **Chen, D. K., A. McGeer, J. C. de Azavedo, and D. E. Low.** 1999. Decreased susceptibility of *Streptococcus pneumoniae* to fluoroquinolones in Canada. N. Engl. J. Med. **341:**233-239.

9. **Choi, E. H., S. H. Kim, B. W. Eun, S. J. Kim, N. H. Kim, J. Lee, and H. J. Lee.** 2008. *Streptococcus pneumoniae* serotype 19A in children, South Korea. Emerg. Infect. Dis. **14:**275-281.

10. d**e la Campa, A. G., C. Ardanuy, L. Balsalobre, E. Pérez-Trallero, J. M. Marimon, A. Fenoll, and J. Liñares.** 2009. Changes in fluoroquinolone-resistant *Streptococcus pneumoniae* after 7-valent conjugate vaccination, Spain. Emerg. Infect. Dis. **15:**905-911.

11. **de la Campa, A. G., L. Balsalobre, C. Ardanuy, A. Fenoll, E. Pérez-Trallero, and J. Liñares.** 2004. Fluoroquinolone resistance in penicillin-resistant *Streptococcus pneumoniae* clones, Spain. Emerg Infect Dis **10:**1751-179.

12. **de la Campa, A. G., G. H. del_Solar, and M. Espinosa.** 1990. Initiation of replication of plasmid pLS1. The initiator protein RepB acts on two distant DNA regions. J. Mol. Biol. **213:**247-62.

13. **Dorman, C. J.** 1991. DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. **59:**745-749.

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

14. **Drlica, K., M. Malik, R. J. Kerns, and X. Zhao.** 2008. Quinolone-mediated bacterial death. Antimicrob. Agents Chemother. **52:**385-392.

15. **Ender, M., N. McCallum, R. Adhikari, and B. Berger-Bachi.** 2004. Fitness cost of SCCmec and methicillin resistance levels in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **48:**2295-2297.

16. **Feil, E. J., E. C. Holmes, D. E. Bessen, M. S. Chan, N. P. Day, M. C. Enright, R. Goldstein, D. W. Hood, A. Kalia, C. E. Moore, J. Zhou, and B. G. Spratt.** 2001. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. Proc. Natl. Acad. Sci. USA **98:**182-187.

17. **Fenoll, A., J. J. Granizo, L. Aguilar, M. J. Gimenez, L. Aragoneses-Fenoll, G. Hanquet, J. Casal, and D. Tarrago.** 2009. Temporal trends of invasive *Streptococcus pneumoniae* serotypes and antimicrobial resistance patterns in Spain from 1979 to 2007. J. Clin. Microbiol. **47:**1012-1020.

18. **Fernández-Moreira, E., D. Balas, I. González, and A. G. de la Campa.** 2000. Fluoroquinolones inhibit preferentially *Streptococcus pneumoniae* DNA topoisomerase IV than DNA gyrase native proteins. Microb. Drug Resist. **6:**259-267.

19. **Ferrándiz, M. J., A. J. Martín-Galiano, J. B. Schvartzman, and A. G. de la Campa.** 2010. The Genome of *Streptococcus pneumoniae* is organized in topology-reacting gene clusters. Nucl. Acids Res. **38**: 3570-3581.

20. **Ferrándiz, M. J., A. Fenoll, J. Liñares, and A. G. de la Campa.** 2000. Horizontal transfer of *parC* and *gyrA* in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **44:**840-847.

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

21. **Fuller, J. D., and D. E. Low.** 2005. A review of *Streptococcus pneumoniae* infection treatment failures associated with fluoroquinolone resistance. Clin. Infect. Dis. **41:**118-121.

22. **Gagneux, S., C. D. Long, P. M. Small, T. Van, G. K. Schoolnik, and B. J. Bohannan.** 2006. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science **312:**1944-1946.

23. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872-3876.

24. **Gillespie, S. H., L. L. Voelker, J. E. Ambler, C. Traini, and A. Dickens.** 2003. Fluoroquinolone resistance in *Streptococcus pneumoniae*: evidence that *gyrA* mutations arise at a lower rate and that mutation in *gyrA* or *parC* predisposes to further mutation. Microb. Drug Resist. **9:**17-24.

25. **Gmüender, H., K. Kuratli, K. Di Padova, C. P. Gray, W. Keck, and S. Evers.** 2001. Gene expression changes triggered by exposure of *Haemophilus influenzae* to novobiocin or ciprofloxacin: combined transcription and translation analysis. Genome Res. **11:**28-42.

26. **Houssaye, S., L. Gutmann, and E. Varon.** 2002. Topoisomerase mutations associated with in vitro selection of resistance to moxifloxacin in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **46:**2712-2715.

27. **Jacobs, M. R., D. Felmingham, P. C. Appelbaum, R. N. Grüneberg, and the Alexander Project Group.** 2003. The Alexander project 1998-2000: susceptibility of pathogens isolated from community-acquired respiratory tract infection to commonly used antimicrobial agents. J. Antimicrob. Chemother. **52:**229-246.

28. **Janoir, C., V. Zeller, M.-D. Kitzis, N. J. Moreau, and L. Gutmann.** 1996. High-level fluoroquinolone resistance in *Streptococcus pneumoniae* requires mutations in *parC* and *gyrA*. Antimicrob. Agents Chemother. **40:**2760-2764.

29. **Jeong, K. S., Y. Xie, H. Hiasa, and A. B. Khodursky.** 2006. Analysis of pleiotropic transcriptional profiles: a case study of DNA gyrase inhibition. PLoS Genet **2:**e152.

30. **Johnson, C. N., D. E. Briles, W. H. Benjamin, S. K. Hollingshead, and K. B. Waites.** 2005. Relative fitness of fluoroquinolone-resistant *Streptococcus pneumoniae*. Emerg. Infect. Dis. **11:**814-820.

31. **Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *E. coli.* Cell **63:**393-404.

32. **Komp Lindgren, P., L. L. Marcusson, D. Sandvang, N. Frimodt-Moller, and D. Hughes.** 2005. Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections. Antimicrob. Agents Chemother. **49:**2343-2351.

33. **Kusuma, C., A. Jadanova, T. Chanturiya, and J. F. Kokai-Kun.** 2007. Lysostaphin-resistant variants of *Staphylococcus aureus* demonstrate reduced fitness in vitro and in vivo. Antimicrob. Agents Chemother. **51:**475-482.

34. **Kyaw, M. H., R. Lynfield, W. Schaffner, A. S. Craig, J. Hadler, A. Reingold, A. R. Thomas, L. H. Harrison, N. M. Bennett, M. M. Farley, R. R. Facklam, J. H. Jorgensen, J. Besser, E. R. Zell, A. Schuchat, and C. G. Whitney.** 2006. Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae*. N. Engl. J. Med. **354:**1455-1463.

35. **Lacks, S. A., P. López, B. Greenberg, and M. Espinosa.** 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. **192:**753-765.

36. **Liñares, J., A. G. de la Campa, and R. Pallarés.** 1999. Fluoroquinolone resistance in *Streptococcus pneumoniae*. N. Engl. J. Med. **341:**1546-1547.

37. **Luo, N., S. Pereira, O. Sahin, J. Lin, S. Huang, L. Michel, and Q. Zhang.** 2005. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc. Natl. Acad. Sci. USA **102:**541-546.

38. **Mandell, L. A., R. G. Wunderink, A. Anzueto, J. G. Bartlett, G. D. Campbell, N. C. Dean, S. F. Dowell, T. M. File, Jr., D. M. Musher, M. S. Niederman, A. Torres, and C. G. Whitney.** 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin. Infect. Dis. **44 Suppl 2:**S27-72.

39. **Marcusson, L. L., N. Frimodt-Moller, and D. Hughes.** 2009. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. PLoS Pathog **5:**e1000541.

40. **Martín-Parras, L., I. Lucas, M. L. Martínez-Robles, P. Hernández, D. B. Krimer, O. Hyrien, and J. B. Schvartzman.** 1998. Topological complexity of different populations of pBR322 as visualized by two-dimensional agarose gel electrophoresis. Nucl. Acids Res. **26:**3424-3432.

41. **Menzel, R., and M. Gellert.** 1987. Fusions of the *Escherichia coli gyrA* and *gyrB* control regions to the galactokinase gene are inducible by coumermycin treatment. J. Bacteriol. **169:**1272-1278.

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

42. **Menzel, R., and M. Gellert.** 1987. Modulation of transcription by DNA supercoiling: a deletion analysis of the *Escherichia coli gyrA* and *gyrB* promoters. Proc. Natl. Acad. Sci. USA **84:**4185-4189.

43. **Menzel, R., and M. Gellert.** 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell **34:**105-113.

44. **Moore, M. R., R. E. Gertz, Jr., R. L. Woodbury, G. A. Barkocy-Gallagher, W. Schaffner, C. Lexau, K. Gershman, A. Reingold, M. Farley, L. H. Harrison, J. L. Hadler, N. M. Bennett, A. R. Thomas, L. McGee, T. Pilishvili, A. B. Brueggemann, C. G. Whitney, J. H. Jorgensen, and B. Beall.** 2008. Population snapshot of emergent Streptococcus pneumoniae serotype 19A in the United States, 2005. J. Infect. Dis. **197:**1016-1027.

45. **Muñoz, R., and A. G. de la Campa.** 1996. ParC subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase A subunit in forming resistance phenotype. Antimicrob. Agents Chemother. **40:**2252-2257.

46. **Nagaev, I., J. Bjorkman, D. I. Andersson, and D. Hughes.** 2001. Biological cost and compensatory evolution in fusidic acid-resistant *Staphylococcus aureus*. Mol. Microbiol. **40:**433-4389.

47. **Olavarrieta, L., P. Hernández, D. B. Krimer, and J. B. Schvartzman.** 2002. DNA knotting caused by head-on collision of transcription and replication. J. Mol. Biol. **322:**1-6.

Con formato: Español (alfab. internacional)

48. **Pan, X.-S., J. Ambler, S. Mehtar, and L. M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **40:**2321-2326.

49. **Pan, X. S., and L. M. Fisher.** 1999. *Streptococcus pneumoniae* DNA gyrase and topoisomerase IV: overexpression, purification, and differential inhibition by fluoroquinolones. Antimicrob. Agents Chemother. **43:**1129-1136.

50. **Perichon, B., J. Tankovic, and P. Courvalin.** 1997. Characterization of a mutation in the *parE* gene that confers fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents. Chemother. **41:**166-167.

51. **Peter, B. J., J. Arsuaga, A. M. Breier, A. B. Khodursky, P. O. Brown, and N. R. Cozzarelli.** 2004. Genomic transcriptional response to loss of chromosomal supercoiling in *Escherichia coli*. Genome Biol. **5:**R87.

52. **Pilishvili, T., C. Lexau, M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, A. Reingold, A. Thomas, W. Schaffner, A. S. Craig, P. J. Smith, B. W. Beall, C. G. Whitney, and M. R. Moore.** 2010. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J. Infect. Dis. **201:**32-41.

53. **Pruss, G., S. Manes, and K. Drlica.** 1982. *Escherichia coli* DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell. **31:**35-42.

54. **Riedel, S., S. E. Beekmann, K. P. Heilmann, S. S. Richter, J. Garcia-de-Lomas, M. Ferech, H. Goosens, and G. V. Doern.** 2007. Antimicrobial use in Europe and antimicrobial resistance in *Streptococcus pneumoniae*. Eur. J. Clin. Microbiol. Infect. Dis. **26:**485-490.

55. **Rozen, D. E., L. McGee, B. R. Levin, and K. P. Klugman.** 2007. Fitness costs of fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **51:**412-416.

56. **Rui, S., and Y. C. Tse-Dhin.** 2003. Topoisomerase function during bacterial responses to environmental challenge. Front. Biosci. **8:**d256-d263.

57. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor laboratory Press, Cold Spring Harbor, N. Y.

58. **Stanhope, M. J., S. L. Walsh, J. A. Becker, M. J. Italia, K. A. Ingraham, M. N. Gwynn, T. Mathie, J. A. Poupard, L. A. Miller, J. R. Brown, and H. Amrine-Madsen.** 2005. Molecular evolution perspectives on intraspecific lateral DNA transfer of topoisomerase and gyrase loci in *Streptococcus pneumoniae*, with implications for fluoroquinolone resistance development and spread. Antimicrob. Agents Chemother. **49:**4315-4326.

59. **Tankovic, J., B. Perichon, J. Duval, and P. Courvalin.** 1996. Contribution of mutations in *gyrA* and *parC* genes to fluoroquinolone resistance of mutants of *Streptococcus pneumoniae* obtained in vivo and in vitro. Antimicrob. Agents Chemother. **40:**2505-2510.

60. **Thiara, A. S., and E. Cundliffe.** 1993. Expression and analysis of two *gyrB* genes from the novobiocin producer, *Streptomyces sphaeroides*. Mol. Microbiol. **8:**495-506.

61. **Travers, A., and G. Muskhelishvili.** 2005. DNA supercoiling - a global transcriptional regulator for enterobacterial growth? Nat. Rev. Microbiol. **3:**157-169.

62. **Tse-Dinh, Y. C.** 1985. Regulation of the *Escherichia coli* DNA topoisomerase I gene by DNA supercoiling. Nucl. Acids Res. **13:**4751-4763.

63. **Unniraman, S., M. Chatterji & V. Nagaraja.** 2002. DNA gyrase genes in *Mycobacterium tuberculosis*: a single operon driven by multiple promoters. *J. Bacteriol* **184:**5449-5456.

64. **Whitney, C. G., M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, R. Lynfield, A. Reingold, P. R. Cieslak, T. Pilishvili, D. Jackson, R. R. Facklam, J. H. Jorgensen, and A. Schuchat.** 2003. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N. Engl. J. Med. **348:**1737-1746.

65. **World and Health Organization.** 2007. Pneumococcal conjugate vaccine for childhood immunization—WHO position paper. Wkly Epidemiol. Rec. **82:**93-104.

FIGURE LEGENDS

FIG. 1. Characteristics of the strains used and of their gyrase and topo IV enzymes. (A) Amino acids that change in strains Tr7, T1, T4, T9 and T14 with respect to R6 are indicated and those involved in fluoroquinolone resistance are showed in bold-face and underlined. For each strain is shown their ciprofloxacin (CIP) MIC, mean relative fitness and 95% CI calculated in competition experiments with R6 (4). Fitness cost categorized as high is shown in bold-face and underlined. (B) Purified topoisomerase subunits revealed by staining with Coomassie blue. His-tagged proteins were overexpressed in *E. coli*, purified by nickel resin chromatography, and examined on an SDS-12% polyacrylamide gel. Mw, molecular weight marker (sizes in kDa). Approximately 0.5 µg of each protein was loaded per well. (C) Supercoiling activity of gyrase subunits. Reactions contained no enzyme (lane 0), 142.5 fmol of GyrA (lane A) or GyrAE85K (lane AE85K), 541.5 fmol of GyrB (lane B) or reconstituted wild type (lane A+B) and mutant (lane E85K+B) gyrase. N, nicked; R, relaxed; S, negatively supercoiled pBR322. (D) Decatenation activity of topo IV. Standard reaction mixtures containing either no enzyme (lane 0), 16 fmol of ParC (lane C), 61 fmol of ParE (lane E), or reconstituted topo IV (lane C+E). kDNA, concatenated kinetoplast DNA; m, monomers. (E) Relaxation activity of topo IV. Standard reaction mixtures containing either no enzyme (lane 0), 2.5 pmol of ParC (lane C), 9.3 pmol of ParE (lane E), or reconstituted topo IV (lane C+E). (F) Stimulation of gyrase-mediated cleavage activity by ciprofloxacin. Reactions contained either no enzyme (lanes 0), 1.1 pmol of GyrA (lane A), 5.9 pmol of GyrB (lane B) or reconstituted gyrase (lanes A+B). (G) Stimulation of topo IV-mediated cleavage activity by ciprofloxacin. Reactions contained either no enzyme (lanes 0), 2.3 pmol of ParC (lane C), 8.7 pmol of ParE (lane E) or reconstituted topo IV (lanes

C+E). The different forms of plasmid pBR322 are indicated: OC, open circle; L, linear; S, negatively supercoiled.

FIG. 2. The supercoiling and decatenation activities of mutant topoisomerases are lower than those of wild-type enzyme**.** (A) Gyrase supercoiling. Subunits *wt*GyrA or GyrAE85K (lane AE85K) were reconstituted with *wt*GyrB in a 1:3.8 proportion and used to supercoil relaxed plasmid pBR322 (0.4 μg) with various amounts of reconstituted gyrase (31 to 500 GyrA fmol). Lane 0, relaxed pBR322 sustrate. (B) Decatenation activity of topo IV (lanes E+C) and its ParCS79F mutant (lanes E+CS79F) reconstituted with *wt*ParE, *rec*ParC (lanes E+C*S79F) or in *rec*ParE+*rec*ParC (lanes E*+C*S79F) in a 3.8:1 proportion (12.5 to 200 ParC fmol). Enzymes were incubated with k-DNA (0.4 μg). (C) Relaxation activity of topo IV and its ParCS79F mutant reconstituted with *wt*ParE, *rec*ParC or *rec*ParE+*rec*ParC in a 3.8:1 proportion (0.3 to 2.5 ParC pmol). Enzymes were incubated with 50 ng of supercoiled plasmid pBR322.

FIG. 3. Mutant topoisomerases showed lower ciprofloxacin-promoted DNA cleavage activities than wild-type enzyme**.** (A) Ciprofloxacin-promoted DNA cleavage of gyrase. Plasmid pBR322 DNA (0.4 μg) was incubated with different amounts of reconstituted gyrase (71 to 1140 GyrA fmol). (B) Ciprofloxacin-promoted DNA cleavage with different amounts of reconstituted topo IV (lanes E+C) and its ParCS79F mutant (lanes E+CS79F), using 125 to 1000 ParC fmol. (C) Ciprofloxacin-promoted DNA cleavage by topo IV with *rec*ParC (lanes E+C*S79F) or *rec*ParE + *rec*ParC (lanes E*+C*S79F).

FIG. 4. Supercoiling levels correlate with bacterial fitness. (A) Distribution of pLS1 topoisomers of the indicated isogenic strains after two-dimensional agarose gel electrophoresis run in the presence of 1 and 2 μg/ml chloroquine in the first and second dimensions, respectively. An empty

arrowhead indicates the most abundant topoisomer. Supercoiling density (σ) values are indicated below the image of one characteristic gel of each strain as average ± SD, number of determinations (**B**) Correlation between supercoiling density and fitness at 12 h of growth in competition with R6. A black square indicates those strains carrying the GyrAE85K change.

TABLE 1. Oligonucleotides used in this work

Oligonucleotide[a]	Sequence (5´-3´)
pLS1F	GTGCCGAGTGCCAAAATCAA
pLS1R	TTTCAAGTACCGATTCACTTAATG
pBR-1	CGGTGAAAACCTCTGACACA
pBR-2	CGCCACCTCTGACTTGAGC
gyrAF	ATGCAGGATAAAAATTTAGTG
gyrAR	gcgcgcatgcGCCAGTGACAGTAATATCAGAAATCCTGC
gyrBF	ATGACAGAAGAAATCAAAAATCTGC
gyrBR	gcgcgcatgcGACCAAGGGAACTACTTCTCCC
parCF	ATGTCTAACATTCAAAACATGTCCC
parCR	gcgcgcatgcCCTCCAATAAAAACCATC
parEF	GTGTCAAAAAAGGAAATC
parER	gcgcgcatgcCATAGTCATTCACATCCGACTC
T11parER	gcgcgcatgcCCGTGAACCAGACATGGCCACAGCCG

[a] F, forward; R, reverse. The 5´ends of some of the primers contained a sequence including a *Sph*I restriction site, which is underlined. Bases not present in the R6 strain sequence are lowercased.

Con formato: Inglés (Estados Unidos)

TABLE 2. Enzymatic activities of type II DNA topoisomerases

Subunit purified from strain:				Gyrase specific activity[a]		Topo IV specific activity [a]		
ParC	ParE	GyrA	GyrB	Supercoiling [b]	Cleavage[c]	Decatenation[d]	Relaxation[e]	Cleavage[f]
–	–	R6	R6	8.6	3.7	–		–
–	–	T14	R6	4.3 (2.0)	1.0 (3.7)	–		–
R6	R6	–	–	–	–	21.2	1.4	3.8
T1	R6	–	–	–	–	10.6 (2.0)	0.9 (1.5)	1.2 (3.2)
T14	R6	–	–	–	–	6.5 (3.3)	0.9 (1.5)	1.4 (2.7)
T14	T14	–	–	–	–	8.3 (2.5)	0.7 (2.0)	1.1 (3.4)

[a] Activities are in units/ mg $\times 10^4$, numbers in parentheses are the fold-decrease in activity with respect to the R6 enzyme.

[b] One unit defined as the amount of enzyme required to convert 50% of 0.4 µg of relaxed pBR322 to the supercoiled form in 1 h at 37ºC.

[c] One unit defined as the amount of enzyme required to linearize 20% of 0.4 µg of CCC pBR322 in 30 min at 37ºC.

[d] One unit defined as the amount of enzyme required to decatenate 50% of 0.4 µg of kDNA in 1 h at 37ºC

[e] One unit defined as the amount of enzyme required to convert 50% of 50 ng of CCC pBR322 to the OC form in 1 h at 37ºC.

[f] One unit defined as the amount of enzyme required to linearize 50% of 0.4 µg of CCC pBR322 kDNA in 30 min at 37ºC.