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Fitness of *Streptococcus pneumoniae* fluoroquinolone resistant strains with topoisomerase IV recombinant genes

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ABSTRACT

The low prevalence of *Streptococcus pneumoniae* ciprofloxacin-resistant (Cp^r) isolates carrying recombinant topoisomerase IV genes could be attributed to a fitness cost imposed by the horizontal transfer, which often implies the acquisition of larger-than normal *parEparC* intergenic regions. A study of the transcription of these genes and of the fitness

cost of 24 isogenic Cp^r strains was performed. Six first-level transformants were obtained either with PCR-products containing *parC* quinolone-resistance determinant regions (QRDRs) of *S. pneumoniae* Cp^r point mutants or with a PCR-product including *parE*QRDR-*antparC*QRDR from a Cp^r *Streptococcus mitis* isolate. The latter yielded two strains, T6 and T11, carrying *parC*-QRDR and *parE*-QRDR-*ant-parC*-QRDR, respectively. These firstlevel transformants were used as recipients in further transformations with *gyrA*-QRDRs PCR-products to obtain 18 second-level transformants. In addition, strain Tr7 (GyrAE85K change) was used. RT-PCR experiments showed that *parE* and *parC* were cotranscribed in R6, T6 and T11, and a single promoter located upstream *parE* was identified in R6 by primer extension. Fitness of transformants were estimated by pairwise competition with R6 in both 1-cycle and 2-cycle experiments. In 1-cycle experiments, only strains carrying the GyrAE85K change showed fitness cost, with the exception of recombinant T14. In 2-cycle experiments, cost was observed in first-level transformants carrying ParC changes S79F, S79Y, D83Y and GyrA E85K change, with the exception of recombinants T6 and T11. Results suggest that there is no impediment due to fitness cost for the spreading of recombinant Cp^r *S. pneumoniae* isolates, since some recombinants (T6, T11 and T14) exhibited a compensation of the cost.

Streptococcus pneumoniae (the pneumococcus) is an important cause of morbidity and mortality and a major etiological agent of community-acquired pneumonia, meningitis and acute otitis media. Pneumococcal antimicrobial resistance (including ß-lactams, macrolides, tetracyclines and co-trimoxazole) has become a worldwide problem (21). The new fluoroquinolones have been recommended for the treatment of community-acquired pneumonia in

adults (30). Although the current prevalence of fluoroquinolone resistance in pneumococci is lower than 3% (8, 12, 13, 27), values higher than 13% (10, 41) have been reported for the *viridans* streptococci of the mitis group (SMG), that are considered donors of resistance genes to pneumococci (3).

Resistance to fluoroquinolones in streptococci occurs mainly by alteration of their intracellular drug targets, the DNA topoisomerase IV (topo IV; $ParC_2ParE_2$) and DNA gyrase (gyrase; $GyrA_2GyrB_2$) essential enzymes. Resistance mutations have been identified in the quinolone-resistance determining regions (QRDRs) of ParC, ParE and GyrA, located either in the N-terminal domains of ParC and GyrA, or in the C-terminal domain of ParE. Genetic and biochemical studies have shown that for fluoroquinolones, such as ciprofloxacin (CIP) and levofloxacin (LVX), topo IV and gyrase are primary and secondary targets, respectively (15, 23, 33, 35, 43). However, for other fluoroquinolones, such as moxifloxacin and gemifloxacin, gyrase is the primary target (20). Low-level CIP-resistant (Cp^r) isolates had mutations altering the QRDRs of one of the two subunits of topo IV, while high-level Cp^r isolates had additional changes in GyrA. Clinical resistance to fluoroquinolones is only achieved with double mutants which can reach CIP MICs ≥16 µg/ml (11). Resistance can be acquired by point mutation, by intraspecific recombination (42) or by interspecific recombination with the SMG (3, 5, 11, 16, 42, 46). Although the acquisition of resistance by inter-specific recombination could be, considering the frequencies of these events in laboratory conditions, much more common than by point mutation, the frequency of *S. pneumoniae* Cp^r recombinants account for less than 11%

of the Cp^r isolates (3, 11, 42). An initial study from our group detected 2.3% Cp^r (MICs ≥4μg/ml) isolates among 3,819 pneumococci (1991-2001) collected at Bellvitge Hospital. Among 46 isolates analyzed, 86.9% and 13.1%, were point mutants and recombinants, respectively (3, 16) and unpublished results). An additional study of 2,882 pneumococci collected during 2002 at the Spanish Reference Laboratory showed 2.6% Cp^r isolates, being 93.3% and 6.7%, point mutants and recombinants, respectively (11). However, in laboratory conditions, the frequency of mutation to Cp^r is about 10^{-9} (36) while the frequencies of transformation with chromosomal DNAs from Cp^r *S. mitis* are about 10^{-3} (19, 22). Besides other factors, such as the availability of DNA in the natural environment and the competence state of the recipient cells, one cause that could account for the low frequency of the Cp^r recombinant isolates is the fitness cost imposed by the DNA interchange. It is well known that the development and dissemination of antibiotic resistance in bacteria depends on the balance between the antibiotic use and the cost that resistance imposes on bacterial fitness (1). A direct relation between consumption of fluoroquinolones and increase in the prevalence of resistance in *S. pneumoniae* has been observed (8, 27). In addition the emergence of resistance during treatment of pneumococcal pneumonia with fluoroquinolones has been described (9, 37, 38, 44). Moreover, long-term CIP therapy of *Pseudomonas aeruginosa* infections caused the emergence of Cp^r pneumococci that were colonizing a patient with bronchiectasis (12).

On the other hand, it has been shown that fitness depends on the specific drug and on the specific mutation (14, 17, 25, 28), and that compensatory mutations can ameliorate the fitness loss (6, 7). Although there are a few reports relating fitness cost of fluoroquinolone-resistance mutations in *S. pneumoniae* (18, 24, 39), there are no reports on the fitness cost of recombinant strains. The Cp^r pneumococcal recombinant isolates studied by our group have acquired portions of either *parE* (unpublished results), *parC* (11), or *parE* plus *parC* (3, 11, 16) from SMG. In the

latter case, given the presence of the *ant* gene in the intergenic *parE-parC* region of SMG, but not in *S. pneumoniae*, recombinants acquired an extra gene in the recombination process and consequently, had larger intergenic *parE-parC* regions (1.1 to 7.2 Kb) than that of nonrecombinant pneumococci (0.4 Kb). It is unknown how *parE* and *parC* genes are transcribed, but it could be assumed that transcription should be coordinated given the tetrameric ($ParC_2ParE_2$) structure of topo IV. If transcription of *parE-parC* occurs from a single promoter in *S. pneumoniae*, the acquisition of *ant* could affect transcription and, consequently, the fitness of recombinant isolates. Another factor that could influence fitness is the existence in recombinants of *parE* and *parC* genes of different origins (pneumococcal and SMG).

In this work we made a parallel analysis of the transcriptional characteristics and fitness cost of isogenic Cp^r, including recombinant, strains.

MATERIALS AND METHODS

MIC determination. Antimicrobial susceptibility testing was determined by the agar dilution method. MICs of CIP (Bayer, Barcelona, Spain) and LVX (Sanofi-Aventis, Barcelona, Spain), were determined according to the testing conditions proposed by the Clinical and Laboratory Standards Institute (11). Sparfloxacin (SPX, was kindly provided by Rhône-Poulec Rorer, Antony, France). Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood were used to grow the strains. *S. pneumoniae* strains ATCC 49619 and R6 were used for quality control.

Bacterial strains, growth and transformation of bacteria. *S. pneumoniae* Cp^r strains used as DNA donors in transformation experiments were: the laboratory mutant CMJ1 (31) and the Cp^r clinical isolates CipR-49, CipR-55, CipR-59, CipR-60, CipR-73 and 5237 (3, 11). The Cp^r *S. mitis* SMI181731-3 isolate (3) was also used as donor. In addition, strain Tr7, a R6 derivative carrying the GyrA E85K change (45), was kindly provided by E. Varon and L. Gutmann**.** Strain Tr7 has a CIP

MIC equal to that of R6, but its SPX MIC is 2 µg/ml, while that of R6 is 0.25 µg/ml. *S. pneumoniae* was grown in a casein hydrolysate-based medium with 0.2% sucrose (AGCH) as energy source and transformed as described previously (26). Cultures containing 9×10^6 CFU per ml of strain R6 or derivatives were treated with 0.1 µg/ml DNA for 40 min at 30ºC, then at 37ºC for 90 min, before plating on AGCH medium with 1% agar plates containing 1 µg/ml of CIP (firstlevel transformants) or 8 µg/ml of CIP (second level transformants). PCR products used in the construction of isogenic transformants were obtained either from *S. pneumoniae* with oligonucleotides parC50/ parC152R (*parC*-QRDRs), gyrA44/ gyrA170R (gyrA-QRDRs) or from *S. mitis* 181731-3 (SMI-3) with parE398/ parC152R (*parE*-QRDR-*ant*-*parC*-QRDR). In competition experiments colonies were counted after 24 h growth at 37ºC in a 5% CO_2 atmosphere on AGCH medium with 1% agar plates. The *Escherichia coli* strain used for plasmid transformation was XL1-Blue (Stratagene). *E. coli* was grown in Luria-Bertani broth and transformed as described (39).

Plasmid construction, PCR amplification and DNA sequence determination. Chromosomal DNA was obtained as described previously (19). PCR amplifications were usually performed using 1 unit of *Thermus thermophilus* DNA polymerase (Biotools, Madrid, Spain), 0.1 µg of chromosomal DNA, 1 µM (each) of the oligonucleotide primers (Table 1), 0.2 mM of each dNTP in a final volume of 50 µl, in the buffer recommended by the manufacturers. Amplification was achieved with an initial cycle of 1 min of denaturation at 94ºC and 25 cycles of 1 min at 94ºC, 45 s at 55ºC, and a polymerase extension step for 1-5 min at 72ºC, with a final extension step for 8 min at 72ºC and slow cooling at 4ºC. To amplify DNA fragments longer than 5 Kb, the "Expand Long Template PCR system" (Roche, Germany) and "Certamp long amplifications kit" (Biotools, Madrid, Spain), both able to amplify up to 20 Kb, were used following manufacturer's instructions.

PCR products were purified and sequenced with an Applied Biosystems Prism 377 DNA sequencer. Agarose gel electrophoresis of PCR products was carried out as described previously (40). Plasmids used for primer extension were

constructed as follows. To construct pLPARE, a 2528-bp PCR fragment (*parE* positions −858 to 1670) obtained with parE−853/ phosphorylated parE549R oligonucleotides from chromosomal DNA of strain R6 was cut with HindIII (a target included into parE−853) and ligated to pLS1 treated with EcoRI + T4 DNA polymerase and HindIII. To make pLPPARC, a PCR fragment of 3366-bp (positions 1612 of *parE* to 141 downstream of *parC*) amplified with phosphorylated parE538/ parCDOWN oligonucleotides from R6 was cut with HindIII and ligated to pLS1 treated as above. To construct pUPANT in *E. coli* XL1-Blue, a PCR fragment from SMI 181731-3 amplified with oligonucleotides parE583 and parCDOWN was cut with HindIII + EcoRI to obtain a 1662 pb-fragment (positions −158 to 1504 of *ant*) that was ligated to pUC18 cut with the same enzymes. *S. pneumoniae* and *E. coli* transformants were selected in 1 μg/ml tetracycline and 100 μg/ml ampicillin, respectively.

RNA extraction, primer extension and RT-PCR experiments. Total RNA of exponential cultures was extracted using the RNeasy midi kit (QIAGEN), including a DNase treatment according to the manufacturer´s instructions. *S. pneumoniae* cultures were previously lysed for 15 min at 37ºC in 10 mM Tris, 1 mM EDTA (pH 8.0), 0.1% sodium deoxycholate. Primer extension analysis was performed as described previously (2), by using 20 μg of RNA from *S. pneumoniae* R6 carrying plasmids pLPPARC or pLPPARE or from *Escherichia coli* XL1Blue carrying plasmid pUPANT. Oligonucleotides (1 pmol) used were parE26R, parC26R, and ant8R. Synthesis of cDNAs in RT-PCR experiments were carried out in 20 μl reactions containing 0.5 μg of RNA, 0.5 mM of each dNTP, 2 pM of each gene-specific primer, 40 U of the RNaseOUT ribonuclease inhibitor and 200 U of SuperScript III RNase H^- RT (Invitrogen) that can generate cDNA up to 12 Kb, in the buffer recommended by the manufacturer.

They were incubated for 60 min at 55ºC and reactions were terminated at 70ºC 15 min. Template RNA was removed by 20 min incubation at 37ºC with 20 U of RNase H (Amersham

Biosciences). The cDNAs obtained were subjected to quantitative real-time PCR (LightCycler® 2.0 Instrument) in 20 µl reactions containing 2 µl of cDNA, 2 µM of each primer, a variable amount of $MgCl_2$ (2 to 3 mM) and 2 µl of LightCycler FastStart DNA Master SYBR Green I System (Roche). Amplification was achieved with 42 cycles of a three-segment program: denaturation (10s at 95ºC); annealing (15s at 50-55ºC); elongation (6-11 s at 72ºC). To check the purity of the amplification product, a melting curve program (65-95ºC with a heating rate of 0.1ºC/s and continuous fluorescence measurement) was performed. For relative quantification of fluorescence values, a calibration curve was made with PCR products of each amplicon obtained from T11 genomic DNA. To normalize the four independent cDNA replicate samples, values were divided by those obtained of the amplification of an internal fragment of 142 bp of the *rpoB* gene with oligonucleotides rpoB428/rpoB474R.

Determination of bacterial fitness. The cost of a resistant mutation was determined by direct competition against the susceptible R6 strain. Individual strains were growth exponentially to OD_{600nm}= 0.25. Cultures were diluted 2000-fold and mixed cultures containing equivalent amounts of R6 and each resistant strain (about 5 x 10^4 CFU/ml) were incubated in antibiotic-free medium for 6 h (c.a. 10-12 generations). Mixed cultures were then diluted 1000-fold to avoid the typical lysis of *S. pneumoniae* cultures at high OD 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 (6 h) and after the second 6 h cycle (12 h) by plating aliquots of the culture on AGCH agar plates containing either 1 µg/ml CIP (0.25 µg/ml SPX when strain Tr7 was used), and no drug. The number of susceptible cells was calculated by subtracting the number of resistant cells from the total cell number revealed by the CFU counts of the plain plates. For the determination of CFU numbers, the mean of two or three plates was used. The number of generations (G) of the resistant and of the R6 strain in the mixed culture was calculated using the following formula:

$G = (\log B - \log A) / (\log 2)$.

Where A is the number of CFU/ml at time zero and B is the number of CFU/ml at the end of each cycle (6 h and 12 h). The relative fitness of each strain was determined from the ratio of the number of generations of the resistant and of the R6 strain. Means of four to nine replicate competition assays were determined. The 95% confidence intervals were calculated based on the t-distribution with N degrees of freedom, were N refers to the number of replicates. Statistical tests were performed with GraphPad Prism version 4.

RESULTS

Generation of Cp^r isogenic strains. A series of isogenic mutants derived from strain R6 carrying different combination of mutations in *parC*, *parE* and *gyrA* were obtained by transformation (Table 2). Six first-level transformants (CIP MICs of 2 to 4 µg/ml, LVX MICs of 2 µg/ml) carrying *parC* mutations were obtained. Four of them (T1, T16, T21, and T26) were obtained using *parC*-QRDRs PCR products from Cp^r *S. pneumoniae* point mutants and two (T6 and T11) with products that contained *parE*-QRDR-*ant*-*parC*-QRDR from Cp^r *S. mitis* 181731-3 (SMI-3). Sequence analysis revealed different recombination regions in T6 and T11. While T6 underwent recombination upstream *parC* (positions –84 to –59) and into *parC* (positions 625 to 641), T11 did it into *parE* (positions 1211 to 1233) and *parC* (positions 385 to 407) and acquired the *ant* gene (Fig. 1A and 1B). All six first level transformants were used as recipient in further transformation experiments with PCR products containing *gyrA*-QRDRs from genetically characterized Cp^r *S. pneumoniae* isolates. A total of 18 second-level transformants, whose CIP MICs were of 16 to 64 µg/ml, and LVX MICs of 8 to 32 µg/ml, were obtained.

Transcriptional analysis of *parE*, *ant*, and *parC*. To know if the transcription of *parE* and *parC* occurs from a single mRNA and if there is a disbalance in the amount of their respective mRNAs in the T11 strain, RT-PCR experiments were performed. cDNAs used in PCR reactions were synthesized using total RNAs from strains R6, T6 and T11 and oligonucleotide parC119R.

Three oligonucleotide pairs (Fig. 1), named 1 (parE592/ antUpR), 2 (antUP/ parC26R), and 3 (parE592/ parC26R) that would render products indicative of common mRNAs for *parE-ant*, *ant-parC*, and *parE-parC*, respectively, were used. As expected, no amplification was observed with R6 and T6 cDNAs with pairs 1 and 2 (Fig. 1C), in accordance with the absence of *ant* in these strains. cDNAs from strains T11 and SMI-3 rendered products of about 0.2 and 1 Kb with pairs 1 and 2, respectively (Fig. 1C), suggesting cotranscription for *parE* and *ant* and for *ant* and *parC*. To confirm these results and to determine if *parE* and *parC* are transcribed in a single mRNA in R6, T6, and T11, a PCR reaction with oligonucleotide pair 3 was performed. In all cDNAs used, a fragment of a size concordant with the cotranscription of *parE* and *parC* was obtained (Fig. 1C). The same RT-PCR experiments were performed with total RNA from six *S. pneumoniae* Cp^r recombinant clinical isolates (Fig. 2). PCR products were obtained with pairs 1, 2 and 3 with cDNAs from SPN 3870, CipR-73 and CipR-75, with intergenic *parE-parC* regions of 1.9, 1.2, and 1.7 Kb, respectively. No products with pairs 1 and 3 were obtained with cDNAs from SPN 3180, SPN 4391 and SPN 5237, with intergenic *parEparC* regions of 6.3, 6.2, and 6.4 Kb, respectively. However with pair 2, PCR products of about 1.9 and 2.4 Kb were obtained with cDNAs of SPN 4391 and SPN 5237 (Fig. 2), suggesting that *ant* and *parC* are cotranscribed in those strains. These results suggest that while *parE* and *parC* are cotranscribed in recombinant isolates with intergenic regions smaller than 2 Kb, this could not be the case for strains with larger intergenic regions.

To confirm the RT-PCR data, the relative amounts of the mRNAs of *parE* and *parC* were quantified in real time RT-PCR experiments. cDNAs from R6, T6 and T11 were obtained with a mixture of random hexanucleotides and transcripts quantified by real-time PCR with appropriated oligonucleotides (parE214/ parE274R for *parE*, parC214/ parC278R for *parC*) to give PCR fragments of equivalent sizes (206 and 212 bp, for *parE* and *parC*, respectively).

These experiments showed that the amounts of mRNAs for *parE* and *parC* were equivalent in R6, T6 y T11 (Fig. 1D).

Primer extension experiments were performed to detect promoters and determine the initiation of transcription of *parE*, *ant*, and *parC*. Total RNA was extracted from *S. pneumoniae* R6 carrying plasmids pLPPARC, pLPPARE, or pUPANT, containing the R6 –752 to 2138 *parC* region, the R6 –858 to 1670 *parE* region, and the SMI-3 –201 to 1684 *ant* region, respectively. While no runoff products were observed for *parC* or *ant*, a 97-nt product with oligonucleotide parE26R was observed for *parE*. This result permitted to map the initiation of transcription of *parE* in R6 to the G (position –23 of *parE*) that is 8 bp downstream of a –10 sequence (Fig. 3).

Fitness assays. Using competitive growth experiments, the relative fitness of the Cp^s strain R6 was compared to that of its isogenic resistant progeny and with strain Tr7. When a single cycle (6 h in Table 3) of competitive growth was considered, no significant fitness cost was observed in most of the first-level transformants, all assays giving mean relative fitness values higher than 1 (Table 3). The only exception was strain Tr7, with a GyrAE85K change and relative fitness of 0.85. In the second-level transformants, only some strains carrying the GyrAE85K change in addition to ParC changes S79F, S79Y, D83N, and D83Y showed a

burden, with mean relative fitness of 0.92, 0.96, 0.93, and 0.92, respectively (Table 3). Although second-level transformants T9 and T14 carried the same GyrAE85K and ParCS79F changes, they showed a different fitness cost. The mean relative fitness of recombinant T9 was of 0.93, a figure compatible with that of the rest of the second-level transformants carrying the GyrAE85K change (T4, T19, T24, and T29). In contrast, recombinant T14 had a relative fitness of 1.03, showing a compensation with respect to strain T9.

When two cycles (12 h in Table 3) of competitive growth were considered, significant fitness cost was observed in 3 out of 6 of the first-level transformants, while T21, T6 and T11 did not showed fitness cost. T21 carried the ParC D83N change and showed a mean relative fitness of 0.95 (95%CI= 0.90-1.00). Strains T6 and T11 are recombinants carrying the same change (ParC S79F) of strain T1. While T1 did shown a significative cost (mean of 0.94, 95%CI=0.89-0.99), T6 (mean

of 0.97, 95%CI= 0.92-1.02) and T11 (0.97, 95%CI=0.91-1.03) did not. These results suggest the existence of compensation in the recombinants. Among the second level transformants, the only strain that did not show a fitness cost was the T14 recombinant strain (mean of 0.95, 95%CI=0.90-1.00).

DISCUSSION

The lower-than expected prevalence of recombinant *S. pneumoniae* Cp^r isolates that have acquired resistance mutations from SMG among the total of Cp^r isolates (3, 11) could be attributed, at least in part, to a lower fitness of these kind of isolates. Among the 10 recombinant clinical isolates studied by our group, 2 have acquired portions of *parC* (11), and 8 both of *parE* plus *parC* (3, 11, 16) from SMG. In the latter case, given the presence of the *ant* gene in the intergenic *parE-parC* region of SMG, but not in *S. pneumoniae*, recombinants acquired an extra gene in the recombination process and consequently, had larger intergenic *parE-parC* regions (1.2 to 6.4 Kb) than that of non-recombinant pneumococci (0.4 Kb). Two factors would influence fitness of these recombinants, one is the putative discoordination of the *parE-parC* transcription in the case of a joint transcription and acquisition of *ant*, and the other is the existence in some of the isolates of *parE* and *parC* genes of different origins. Isolates with a *parE* gene of pneumococcal origin and a *parC* gene of SMG origin are represented in this work by the strains T6 to T9, and isolates that have acquired the *ant* gene are represented by T11 to T14 strains (1.1 Kb *parE-parC* intergenic region). In addition, to discern the effect of point mutation in the fitness of the strains, a series of point mutants (Table 3) were studied.

With respect to the putative discoordination of the transcription of the *parE-parC* region, our results showed that transcription of both genes occurs from a single promoter in *S. pneumoniae* strains R6, T6 and T11 (Figs 1 and 3), allowing the synthesis of equivalent amounts of the mRNAs of both genes, as showed by real time RT-PCR (Fig. 1D), in accordance with the $ParC_2ParE_2$

293 composition of topo IV. Then, at least in the T11 strain, the presence of *ant* in the 1.1 Kb intergenic
294 *parE-parC* region does not have any effect in transcription. In isolates with intergenic regions
295 smaller than 2 Kb (SPN 3870, CipR-73 and CipR-75), co-transcription of *parE* and *parC* was
296 observed (Fig. 2). However, no co-transcription was observed in recombinant isolates with larger
297 intergenic regions (up to 7.2 Kb: SPN 3180, SPN 4391 and SPN 5237). Future work would be
298 necessary to ascertain if these kind of recombinants would have a fitness cost due to some
299 discoordination in the transcription of their topo IV genes.

300 With respect to their fitness cost, strains can be categorized as no-cost, low-cost, and high-
301 cost. High-cost strains showed relative mean fitness lower than 1 both in 1-cycle (95% CI= 0.88-
302 0.99) and in 2-cycle (95% CI= 0.63-0.89) experiments. Low-cost strains show relative mean fitness
303 lower than 1 only in the 2-cycle (95% CI= 0.81-0.99) experiments, while in the nocost strains the
304 95% CI of the mean relative fitness includes the 1 value.

305 The high-cost strains included the first-level transformant Tr7 and second-level
306 transformants (T4, T19, T24, T29, and T9), which carried the GyrAE85K change. The E85 GyrA
307 residue is located within the QRDR, close to the catalytic cleavage residue Y120 (equivalent to
308 Y122 of *E. coli* GyrA). The three-dimensional structure of the breakage-reunion domain of *E. coli*
309 GyrA reveals that the active-site tyrosines (Y122) are on loops at either end of the dimer interface
310 and sit at the ends of strongly basic grooves created by the dimer-related monomers for binding
311 the G segment (32). Residues S83 and D87 (equivalent to S81 and E85, respectively, of *S.*
312 *pneumoniae* GyrA) are solvent exposed and probably interact with the C-8 group of the
313 fluoroquinolone. We have found that strains with substitutions in GyrA S81 did not show fitness
314 cost in 1-cycle experiments (Table 3), but the E85K substitutions imposed a burden. In accordance,
315 a Ser83A substitution has not effect on *E. coli* gyrase activity, however, a D87A substitution causes
316 a 60% reduction in supercoiling activity, probably due to stabilization of enzyme-DNA interactions
317 (4). It is tempting to speculate that the burden imposed by the E85K substitution could be also

attributed to a stronger interaction of gyrase with DNA due to the more positive charge of the K residue. Given that topo IV and gyrase have complementary activities (34), compensation by the recombinant T14 topo IV enzyme (*parC* and *parE* recombinant genes) of the fitness cost imposed by the GyrAE85K change (Table 3), could be attributed to a mechanism of bypass (29) due to the putatively more efficient T14 topo IV enzyme.

The low-cost strains include single ParC mutants carrying S79F, S79Y, D83Y changes, all double mutants carrying a ParC change plus a GyrA change (except E85K, Table 3) and recombinant strains T7, T8, T12 and T13. Some of these changes have been reported by other authors to confer a fitness cost by using 1-cycle experiments (18, 24, 39). However, in general, there is not agreement among results from different laboratories, which suggest that 1-cycle experiments are not enough to detect a low cost, which would be masked by experimental errors, being necessary to perform 2-cycle experiments to improve the accuracy of results.

The no-cost strains include the single ParC mutant carrying D83N and recombinant strains T6 and T11. Although the last strains carried *parE* and *parC* genes of different species (*S. pneumoniae* or SMG), this feature was not associated with a fitness cost in spite that the ParE changes of strain T11 mapped in the C-terminus region that is involved in the interaction of ParE with ParC. Nevertheless, T6 and T11 showed a compensation of the fitness cost imposed by the ParC S79F change, which could be attributed to the presence in these strains of a more efficient topo IV. However, when this feature was associated with mutations in *gyrA*, fitness cost was detected in the 2-cycle experiments (Table 3), with exception of T14 (see above).

From the results showed here it could be assumed that there is not impediment for the spreading of recombinant isolates that had acquired Cp^r resistance mutations from SMG streptococci due to fitness cost, since recombination will compensate the fitness cost imposed by specific mutations.

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FlGURE LEGENDS

FIG. 1. Transcriptional analysis of *parE* and *parC* genes in *S. pneumoniae* R6, T6 and T11 strains**.** (A) Genetic structure of the *parE-parC* region. SPN, *S. pneumoniae*; SMI, *S. mitis*. Oligonucleotides are indicated by black arrows (not drawn to scale). (B) Nucleotide sequences of the regions surrounding the recombination points in strains T6 (a and b) and T11 (c and d). (C) RT-PCR analysis. cDNAs (synthesized using total RNAs from R6, T6 and T11 and oligonucleotide parC119R) and control RNAs (cDNAs prepared in the absence of RT) were subjected to PCR reactions with three pairs of oligonucleotides named 1 (parE592/ antUpR), 2 (antUP/ parC26R), and 3 (parE592/ parC26R) that would render products indicative of common mRNAs for *parE-ant*, *ant-parC*, and *parE-parC*, respectively. Products were run in 1% agarose gels and stained with 0.5 μg/ml ethidium bromide. Mw, molecular weight standard, HindIII + EcoRI cut lambda DNA. (D) Quantitative real-time RT-PCR. The results (mean ± SEM, standard error of the mean) of four independent replicates are represented.

FIG. 2. Transcriptional analysis of *parE* and *parC* genes in *S. pneumoniae* recombinant clinical isolates**.** (A) Genetic structure of the *parE-parC* region. SPN, *S. pneumoniae*. Oligonucleotides are indicated by black arrows (not drawn to scale). cDNAs (synthesized using total RNAs from indicated isolates with oligonucleotide parC119R) and control RNAs (cDNAs prepared in the absence of RT) were subjected to PCR reactions with three pairs of oligonucleotides named 1 (parE592/ antUpR), 2 (antUP/ parC26R), and 3 (parE592/ parC26R) that would render products indicative of common mRNAs for *parE-ant*, *ant-parC*, and *parE-parC*, respectively. Products were run in 1% agarose gels and stained with 0.5 μg/ml ethidium bromide. Mw, molecular weight standard, Biotools DNA marker 1-Kb ladder.

FIG. 3. Localization of the transcription initiation site of *parE*. Sequenase reactions employing pLPARE plasmid as template and parE26R as primer provided a reference sequence ladder: G, A, T, and C indicate the dideoxynucleotides used. For primer extension experiments, 20 μg of RNAs obtained from *S. pneumoniae* R6 carrying pLPPARE (line 1) were used. The double strand DNA sequence of the 5´*parE* region is showed with their principal features: the −35 and −10 regions, the first nucleotide of the mRNA (+1), the putative ribosome-binding site (RBS), and the start codon of *parE*.

2 TABLE 1. Oligonucleotides used in this work.

Oligonucleotide	Sequence (5´-3´)[a]	Nucleotide positions[b]
AntUP	GCTGTCGCCATGTCTGGTTCACG	79 to 101 of *ant*
AntUPR	CGTGAACCAGACATGGCGACAGC	Complementary to 79 to 101 of *ant*
ant8R	CATCGAGCATTTCTGTTTCA	Complementary to 24 to 43 of *ant*
gyrA44	CCGTCGCATTCTCTACGGAATGAATGAATT	129 to 158 of *gyrA*
gyrA170R	GCTCCATTAACCAAAAGGTTTGGAA	Complementary to 482 to 506 of *gyrA*
parC26R	GAATATCTGGCAAAGCCCGGTCTTG	Complementary to 76 to 100 of *parC*
parC50	AAGGATAGCAATACTTT	148 to 164 of *parC*
parC152R	TGGTTCTTTCTCCGTATCG	Complementary to 438 to 456 of *parC*
parC119R	GTATAACGCATAGCCGCAGG	Complementary to 337 to 356 of *parC*
parC214	CCTACAGGGGCTATTATTCAG	640 to 660 of *parC*
parC278R	ATCCCAGCTACCTTGTTATT	Complementary to 832 to 851 of *parC*
parCDOWN	CGTTACTGTCATATTCCACTCC	Complementary to −120 to −141 of *parC*
parE26R	GGACCGCATCCAACCC	Complementary to 61 to 76 of *parE*
parE214	AAGCGAACAGATGAAGCGATTGAG	640 to 663 of *parE*
parE274R	TCCTTGGTGCGAACGTTATTGACA	Complementary to 822 to 845 of *parE*
parE398	AAGGCGCGTGATGAGAGC	1180 to 1197 of *pare*
parE483R	GTCTGCTCCAACACCCGCA	Complementary to 142 to 1470 of *parE*
parE538	GCCCTCCCACCTCTTTACAAG	1612 to 1632 of *parE*
parE549R	TAGGCCACTTCTTCTTTCTTGCCTTT	Complementary to 1645 to 1670 of *parE*
parE583	gcgcaagCTTGGTGAGATGAATGCGGACC	1747 to 1768 of *parE*
parE592	GGGAAACAACCATGAACCCA	1775 to 1794 of *parE*
parE−853	gcgcaagCTTAGCCCTGTGCAATACAG	−858 to −839 of *parE*
rpoB428	CGGTTGGTGAATTGCTTGCCAACC	1283 to 1306 of *rpoB*

rpoB468R	ACTGCAGCTGTTACAGGACGG	Complementary to 1404 to 1424 of *rpoB*

3

[a] The 5´ends of some of the primers contained a sequence including a HindIII restriction site, which is underlined. Bases not present in *S. pneumoniae* R6 are lowercased. [b] Nucleotide numbering refers to the genes and proteins of the *S. pneumoniae* R6 sequence, except for those of the *ant* gene that refer to *S. pneumoniae* 3870 (antUP and antUPR) or to *S. mitis* 181731-3 (ant8R), with the first nucleotide being at position 1.

TABLE 2. Generation of Cp^r mutants derived of strain R6 by genetic transformation.

Transformant strain	Receptor strain	Donor DNA[a]			MIC (μg/ml)	
		Strain		PCR fragment	CIP	LVX
		Name	Gene	Oligonucleotides used		
	R6	□	□	□	0.5	0.25
T1	R6	CMJ1	*parC*	parC50/ parC152R	4	2
T2	T1	CipR73	*gyrA*	gyrA44/ gyrA170R	32	16
T3	T1	5237	*gyrA*	gyrA44/ gyrA170R	32	16
T4	T1	CipR49	*gyrA*	gyrA44/ gyrA170R	32	16
T16	R6	CipR55	*parC*	parC50/ parC152R	2	2
T20	T16	CipR73	*gyrA*	gyrA44/ gyrA170R	32	16
T18	T16	5237	*gyrA*	gyrA44/ gyrA170R	64	32
T19	T16	CipR49	*gyrA*	gyrA44/ gyrA170R	32	32
T21	R6	CipR59	*parC*	parC50/ parC152R	2	2
T25	T21	CipR73	*gyrA*	gyrA44/ gyrA170R	16	8
T23	T21	5237	*gyrA*	gyrA44/ gyrA170R	16	8
T24	T21	CipR49	*gyrA*	gyrA44/ gyrA170R	16	8
T26	R6	CipR60	*parC*	parC50/ parC152R	4	2
T30	T26	CipR73	*gyrA*	gyrA44/ gyrA170R	32	8
T28	T26	5237	*gyrA*	gyrA44/ gyrA170R	32	8
T29	T26	CipR49	*gyrA*	gyrA44/ gyrA170R	32	8

T6	R6	SMI181731-3	*parC*	parE398/ parC152	2	2
T7	T6	CipR73	*gyrA*	gyrA44/ gyrA170R	32	16
T8	T6	5237	*gyrA*	gyrA44/ gyrA170R	32	16
T9	T6	CipR49	*gyrA*	gyrA44/ gyrA170R	32	16
T11	R6	SMI181731-3	*parE-ant-parC*	parE398/ parC152R	2	2
T12	T11	CipR73	*gyrA*	gyrA44/ gyrA170R	32	16
T13	T11	5237	*gyrA*	gyrA44/ gyrA170R	32	16
T14	T11	CipR49	*gyrA*	gyrA44/ gyrA170R	32	16

[a] *S. pneumoniae* Cp[r] strains CMJ1, CipR-49, CipR-55, CipR-59, CipR-60, CipR-73 and 5237 carried a single nucleotide change in the amplified fragment. The Cp[r] *S. mitis* SMI181731-3 isolate carried, besides the mutation involved in resistance, additional changes.

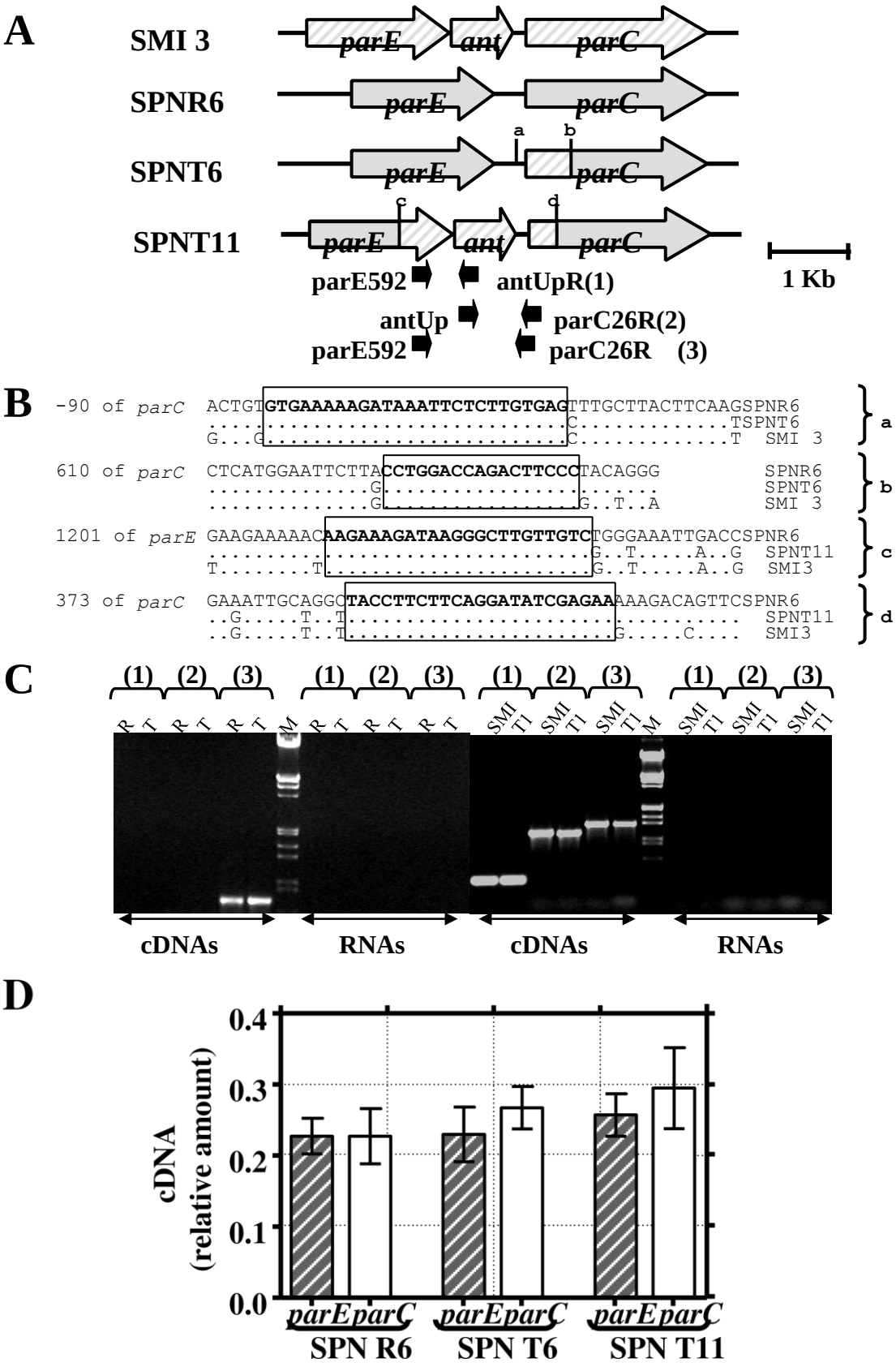
2 TABLE 3. Competitive fitness of fluoroquinolone-resistant mutants of *S. pneumoniae*
3

Strain	Amino acid change in[a]:												Relative Fitness[b]							
	ParE			ParC							GyrA		Time 6 h				Time 12 h			
	G486	I493	F571	Y23	S52	**S79**	**D83**	N91	A190	I201	**S81**	**E85**	Mean	SD	(95%CI)	N	Mean	SD	(95%CI)	N
Tr7												**K**	0.85	0.05	0.79-0.91	5	0.76	0.05	0.68-0.84	4
T1						**F**							1.03	0.04	1.00-1.06	9	0.94	0.03	0.89-0.99	4
T2						**F**					**F**		1.04	0.03	1.02-1.06	9	0.84	0.02	0.81-0.87	4
T3						**F**					**Y**		1.04	0.04	1.01-1.07	9	0.90	0.01	0.88-0.92	4
T4						**F**						**K**	0.92	0.05	0.88-0.96	9	0.80	0.02	0.77-0.83	4
T16						**Y**							1.07	0.05	1.03-1.11	9	0.97	0.01	0.95-0.99	4
T20						**Y**					**F**		1.09	0.04	1.06-1.12	9	0.93	0.02	0.90-0.96	4
T18						**Y**					**Y**		1.03	0.03	1.01-1.05	9	0.94	0.01	0.92-0.96	4
T19						**Y**						**K**	0.96	0.04	0.93-0.99	10	0.82	0.04	0.76-0.88	4
T21							**N**						1.04	0.04	1.01-1.07	10	0.95	0.03	0.90-1.00	4
T25							**N**				**F**		1.04	0.04	1.01-1.07	9	0.92	0.02	0.89-0.95	4
T23							**N**				**Y**		1.08	0.05	1.04-1.12	8	0.91	0.02	0.88-0.94	4
T24							**N**					**K**	0.93	0.03	0.91-0.95	9	0.77	0.01	0.75-0.79	4
T26							**Y**						1.04	0.04	1.01-1.07	9	0.92	0.02	0.89-0.95	4
T30							**Y**				**F**		1.08	0.04	1.05-1.11	8	0.91	0.02	0.88-0.94	4
T28							**Y**				**Y**		1.08	0.04	1.05-1.11	8	0.89	0.03	0.84-0.94	4
T29							**Y**					**K**	0.92	0.05	0.88-0.96	9	0.69	0.04	0.63-0.75	4
T6					G	**F**		D	T	V			1.04	0.04	1.01-1.07	8	0.97	0.03	0.92-1.02	4
T7					G	**F**		D	T	V	**F**		1.05	0.05	1.01-1.09	8	0.87	0.03	0.82-0.92	4
T8					G	**F**		D	T	V	**Y**		1.05	0.04	1.02-1.08	9	0.94	0.01	0.92-0.96	4

T9	□	□	□	□		G	**F**	□	D	T	V	□	**K**	0.93	0.04	0.90-0.96	9	0.83 0.04	0.77-0.89	4
T11	S	L	L	H	G	**F**	□	D	□	□	□	□	1.05	0.03	1.02-1.08	8	0.97	0.04	0.91-1.03	4
T12	S	L	L	H	G	**F**	□	D	□	□	**F**	□	1.07	0.05	1.03-1.11	8	0.92	0.01	0.90-0.94	4
T13	S	L	L	H	G	**F**	□	D	□	□	**Y**	□	1.06	0.04	1.03-1.09	8	0.92	0.02	0.89-0.95	4
T14	S	L	L	H	G	**F**	□	D	□	□	□	**K**	1.03	0.05	0.99-1.08	9	0.95	0.03	0.90-1.00	4

[a] Residues involved in resistance are showed in boldface an underlined.

[b] Relative competitive fitness is the ratio of the number of generations of the resistant and susceptible R6 strains. Mixed cultures with equal amounts of susceptible and resistant strains (c.a, 5 x 10^4 CFU/ml each) were incubated in antibiotic-free medium for 6 h, diluted 1000-fold and re-growth for an additional 6 h period. The number of viable cells was determined at 0 h, 6 h and 12 h by plating on media with either 1 μg/ml CIP (or 0.25 μg/ml sparfloxacin for strain Tr7), or no drug. CI, confidence interval. N, number of replicates. SD, standard deviation. CI values shadowed in grey corresponded to strains with a fitness cost.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T.....TG..T.....A..G SMI3
373 of parC GAAATTGCAGGCTACCTTCTTCAGGATATCGAGAAAAAGACAGTTCSPNR6
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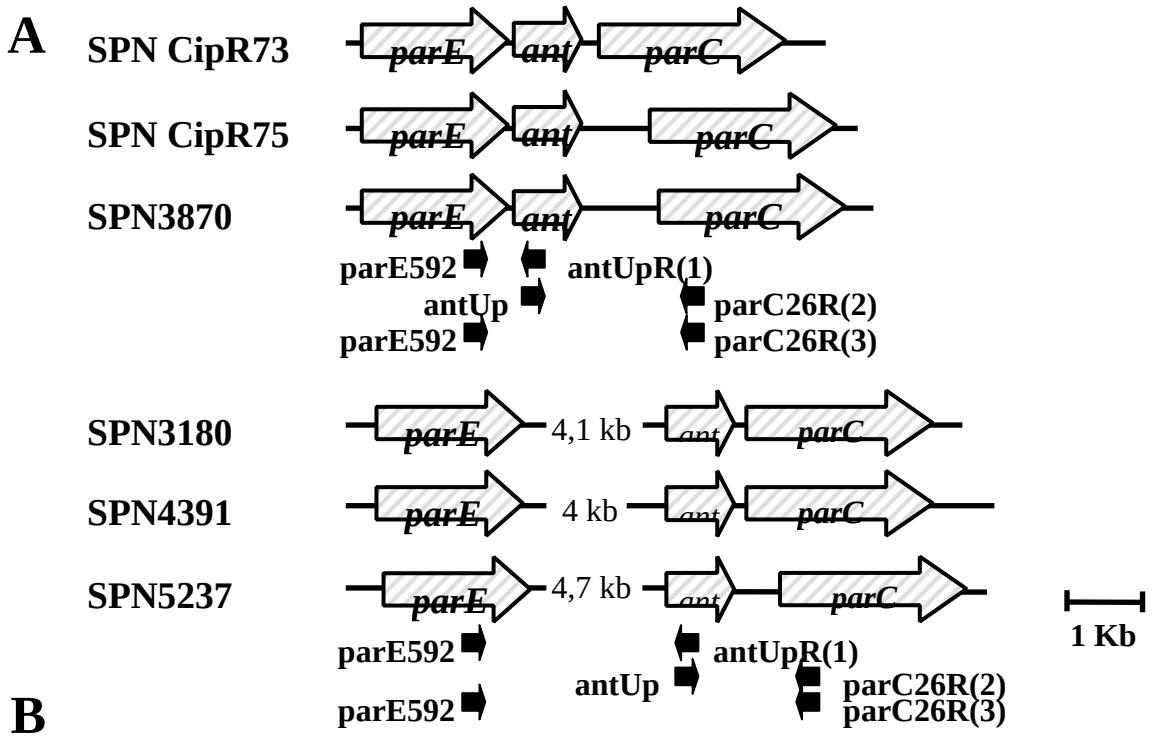
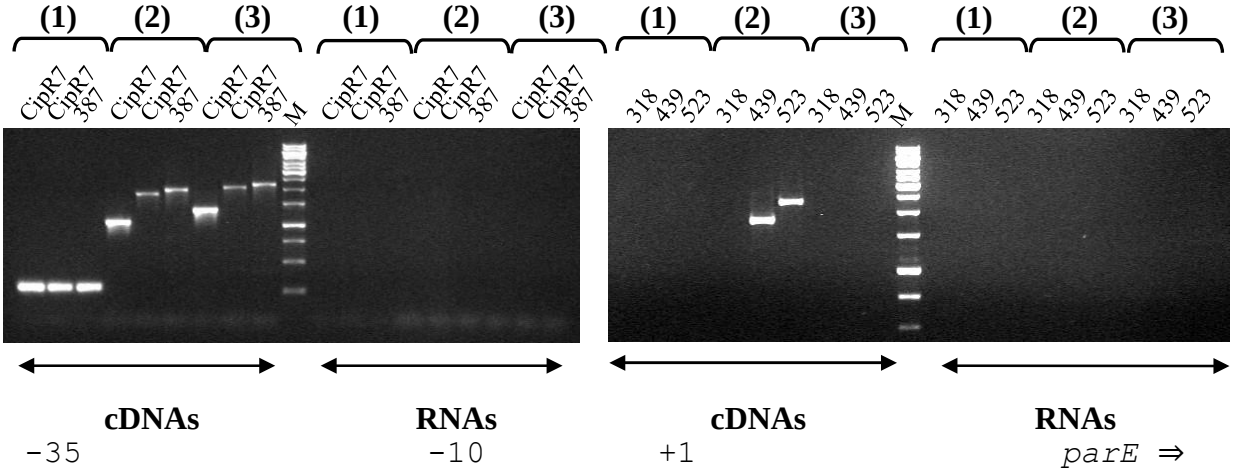
SPN T6
SPN T11





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