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Antimicrob Agents Chemother. 2003 Apr;47(4):1257-61.

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AAC 1022 -02 Revised

Generation of Antibiotic-Resistant Strains of *Streptococcus pneumoniae* with High Efficiency by Using PCR and Transformation

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Running title: Generation of *S. pneumoniae* mutants

ABSTRACT

We designed a method to generate antibiotic-resistant strains of *Streptococcus pneumoniae* at frequencies 4 orders of magnitude greater than the spontaneous mutation rate. The method is based on the natural ability of this organism to be genetically transformed with PCR products carrying sequences homologous to its chromosome. The genes encoding the targets of ciprofloxacin (*parC*, encoding the ParC subunit of DNA topoisomerase IV), rifampin (*rpoB*, encoding the β subunit of RNA polymerase) and streptomycin (*rpsL*, encoding the S12 ribosomal protein) from the susceptible laboratory strain R6 were amplified by PCR and used to transform the same strain. Resistant mutants were obtained with a frequency of 10^{-4} to 10^{-5}, depending on the fidelity of the DNA polymerase employed in PCR amplifications. Ciprofloxacin-resistant mutants, with MICs 4-to-8-fold higher than that of R6, carried single mutations at residues of the quinolone-resistance determining-region: S79 (change to A, F or Y) or D83 (change to N or V). Rifampin-resistant strains, with MICs at least 133-fold higher than that of R6, contained single mutations within the cluster I of *rpoB*: S482 (change to P), Q486 (change to L), D489 (change to V) or H499 (change to L or Y). Streptomycin-resistant mutants, with MICs at least 64-fold higher than that of R6, carried mutations at either K56 (change to I, R or T) or K101 (change to E). PCR products obtained from the mutants were able to transform R6 to resistance with high efficiency (>10^4). This method could be used to efficiently obtain resistant mutants for any drug whose target is known.

Streptococcus pneumoniae, the pneumococcus, is the human pathogen responsible for most community-acquired pneumonia, meningitis and otitis media, causing about three million deaths annually in children in the developing world (15). Since the 1990's, the number of pneumococcal clinical isolates resistant to the major therapeutic drugs, including new ones such as the fluoroquinolones (5, 27) has been increasing worldwide (9, 18, 33) and is becoming a major problem for public health. In this scenario, studies on the mechanisms involved in antibiotic resistance are of primary importance. These studies rely mainly on the identification of the antibiotic targets by locating the mutations involved in resistance and on biochemical studies of inhibition mechanisms. A significant progress in this direction has been the determination of the complete genome sequences of the laboratory R6 pneumococcal strain (19), of a serotype 4 isolate (40), and most of a serotype 19F isolate (7). Deciphering the role of these genomic sequences implies the generation of large theoretical information that must be corroborated experimentally by molecular biology. However molecular methods for *S. pneumoniae* are still limited (22) despite its high clinical importance. On the other hand, it is well known that *S. pneumoniae* is a natural-competent bacterium and methods for its transformation under laboratory conditions have been developed. Competence state is a process dependent on cell density triggered by the accumulation in the medium of the competence-stimulating peptide that signals the two-component ComD - ComE system (16, 35), which results in the transcriptional activation of a competence-specific sigma factor (26). This factor enables transcription of the late competence genes that encode enzymes for the binding, uptake, and recombination of the donor DNA with the chromosome (3, 24, 36).

We have recently obtained several mefloquine-resistant pneumococcal mutants by using PCR amplification of fragments of the genes *atpC* and *atpA* encoding the *c* and *a* subunits of the F_0F_1 ATPase, respectively (10, 29). Transformation with these PCR products

obtained from strain R6 and selection of transformants in inhibitory mefloquine concentrations rendered mutants at a frequency several orders of magnitude greater than the spontaneous mutation rate. The information provided by the new mutants has significantly contributed = to our understanding of the arrangement of the F_0F_1 ATPase (28). It was proposed that those mutants were originated as a result of the error rate of the DNA polymerase used in the PCR amplifications (28). In this work, we present evidence supporting this hypothesis and that the method is useful for obtaining *S. pneumoniae* mutants at high frequency in, at least, three genes, *parC*, *rpoB*, and *rpsL* known to be targets of ciprofloxacin (CIP) (20, 30, 34, 39), rifampin (RIF) (8, 32) and streptomycin (STR) (37), respectively.

While this manuscript was in preparation, a PCR-based approach to drug target identification in *S. pneumoniae* was published (2). Although PCR methodology and the natural transformability of the pneumococcus are the bases of both studies, our work has been focused on the generation and characterization of the antibiotic-resistant pneumococcal transformants.

MATERIALS AND METHODS

Bacterial strains, growth and transformation of bacteria. *S. pneumoniae* strains used were the laboratory strain R6, ATCC 49619 strain, the STR-resistant (Str^R) strain 533 (*str-41, sul, nov-1, ery*) and the CIP-resistant (Cip^R) clinical isolate 4114. *S. pneumoniae* was grown in a casein hydrolysate-based medium with 0.2% sucrose (AGCH) as energy source and transformed as described (23). Strain R6 was used as recipient in transformation experiments. Cultures containing 9×10^6 CFU per ml were treated with DNA at 0.15 μg/ml for 40 min at 30ºC, then at 37ºC for 90 min, before plating on media plates containing 2 μg/ml of CIP, 1 μg/ml of RIF, or 100 μg/ml of STR. Colonies were counted after 24 h growth at 37 ºC in a 5% CO_2 atmosphere in AGCH medium with 1% agar. Rates of spontaneous mutation to drug resistance were

estimated by plating 2×10^{10} cells in 1 μg/ml RIF or 100 μg/ml STR.

DNA techniques. *S. pneumoniae* chromosomal DNA was prepared as previously described (14). Synthetic oligonucleotide primers used in PCR amplifications and in sequencing reactions are listed in Table 1 and were designed based on the previously published sequences of the corresponding genes of strain R6 (11, 19, 30). Amplifications were performed with 1 U of *Thermus thermophilus* (Tth) thermostable DNA polymerase (Biotools) or 2,5 units of a proof-reading enzyme, Expand High Fidelity PCR (Hf) system (Boehringer Manheim), 1 μg of genomic DNA, 0.4 μM (each) of the corresponding synthetic oligonucleotide primers, 0.2 mM of each dNTP, and 2 mM $MgCl_2$ in a final volume of 50 μl. Amplification was carry out with an initial cycle of 1 min denaturation at 94ºC, 30 cycles of 30 s at 94ºC, 90 s at 55ºC and a 75 s polymerase extension step at 72ºC, and a final 8-min step followed by slow cooling to 4ºC. The remaining dNTPs and primers were removed from PCR products using HR S-400 columns (Amersham) prior to sequencing or transformation. Sequencing was done on both DNA strands according to manufacturers protocols using an Applied Biosystems Prism 377 DNA sequencer

Minimal inhibitory concentration (MIC) determination. MICs were determined by the microdilution method, using cation-adjusted Mueller-Hinton broth (Difco) supplemented with 2.5% lysed horse blood as recommended by the National Committee for Clinical Laboratory Standards (31). Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood were used to grow the strains overnight. The inoculum was prepared by suspension of several colonies in Mueller-Hinton broth and adjusting the turbidity to the 0.5 McFarland standard (ca. 10^8 CFU/ml). The suspension was further diluted to provide a final bacterial concentration of 10^4 CFU/ml in each well of the microdilution trays. Plates were covered with plastic tapes and incubated in ambient atmosphere at 37ºC for 20-24 h. MIC was defined as the lowest concentration of drug that inhibited visible growth. The *S.*

pneumoniae strains ATCC 49619 and R6 were used for quality control. CIP was kindly provided by Bayer (Barcelona, Spain) whereas RIF and STR were purchased from Sigma.

RESULTS

Construction of resistant strains by PCR and transformation. Fragments of about 1,600 bp were amplified by PCR from *S. pneumoniae* R6 by using specific oligonucleotides: parCUP and parC503R for *parC*; rpoB227 and rpoB773R for *rpoB*; rpsLUP and rpsLDOWN for *rpsL* (Table 1, Fig.1). The 1,629-bp *parC* PCR fragment has the sequence encoding the first 508 amino acid (aa) residues of the 824-residue-ParC subunit of the DNA topoisomerase IV, and includes the quinolone-resistance determining region (QRDR, 30). The 1,641-bp *rpoB* PCR fragment codes for 547 residues (residues 227 to 554) of the central region of the β subunit of the RNA polymerase. The 1,615-bp *rpsL* PCR fragment includes the coding region for the first 320 residues of Spr0247, a putative alkaline amylopullulanase, the *rpsL* gene that encodes the 137-residues-long 30S ribosomal protein S12, and most (149 residues out of 156) of the 30S ribosomal protein S7 encoded by the *rpsG* gene. These R6-PCR derived fragments were used to transform competent R6 cells and transformants were selected on CIP at 2 μg/ml (4-fold the MIC of R6), RIF at 1 μg/ml (higher than 33-fold the MIC of R6), or STR at 100 μg/ml (32-fold the MIC of R6). These antibiotic concentrations were chosen taking into account the levels of resistance to these drugs achieved by single mutations in *parC* (30), *rpoB* (8, 32) and *rpsL* (37). The frequency of resistant mutants obtained by transformation was of $1-12.4 \times 10^{-5}$ and $0.4-6.4 \times 10^{-5}$ when using PCR products amplified with Tth or Hf, respectively (Table 2). In consequence, 2−4 fold more transformants appeared with the Tth enzyme, consistent with the error-rate differences (3-fold) of these polymerases reported by the manufacturers. Transformants resistant to a particular antimicrobial agent appeared only when the corresponding target gene was present in the PCR product used as donor DNA,

whereas no colonies were detected when other antimicrobial agents were used for selection (Table 2). In this way, transformants resistant to either CIP, RIF, or STR appeared when the PCR products contained *parC*, *rpoB*, or *rpsL*, respectively. The MICs for the mutant strains showed increments in resistance of 4−8 fold for CIP, at least 133-fold for RIF, and at least 64-fold for STR (Table 3).

Characterization of antibiotic-resistant strains. Ten resistant mutants for each antibiotic were chosen and pertinent regions (Fig. 1) of the *parC*, *rpoB* and *rpsL* genes were sequenced. A region of 310-bp encoding ParC residues 50 to 172 was amplified and sequenced with oligonucleotides parC50 and parC152 (Table 1). The 10 Cip^R strains carried single mutations affecting residues S79 or D83 of the ParC QRDR (Table 3). A 380-bp region of *rpoB* coding for residues 427-to-554 was amplified and sequenced with oligonucleotides rpoB427 and rpoB554R. The RIF-resistant (Rif^R) strains carried mutations affecting residues S482, Q486, D489 or H499. A 378-bp fragment of *rpsL* encoding residues 6-to-131 of the S12 ribosomal protein from the Str^R strains was also amplified and sequenced with oligonucleotides rpsL6 and rpsL131R, showing mutations that would produce changes at K56 or K101.

Genetic evidence demonstrating that the mutations carried by the resistant strains were indeed involved in resistance was obtained by genetic transformation. PCR products of about 1,600 bp amplified from the Cip^R, Rif^R, and Str^R strains described above were able to transform strain R6 with high efficiency (0.2−to−14 × 10^5 transformants/ ml) to resistance (Table 3). Two independent colonies from each of these transformation experiments were selected and analyzed. Their MICs and mutations were identical to that of the parental Cip^R, Rif^R or Str^R strain (not shown). These results confirmed the relationship between aa change and resistance phenotype.

DISCUSION

In the present study we describe a simple method to obtain antibiotic-resistant strains of *S. pneumoniae* taking advantage of the PCR methodology, the error rate of the DNA polymerases used in the amplifications and of the natural transformation ability of *S. pneumoniae*. The appearance of resistant colonies upon transformation with the 1,600 bp PCR products carrying the appropriate R6 genes could be attributed to the error rate of the polymerase. This rate is of 1 error/10 kb, therefore 1.6 errors would be expected for 10 molecules of 1,600 bp. Since 4.5×10^5 competent cells (5% of 9×10^6 CFU) could be transformed with chromosomal DNA in our = experiments, the total number of putative mutants would be of about 7.2×10^4. From our results, of the putative nucleotide changes that would occur in the *parC*-PCR fragment (encoding 508 residues of ParC) and at the *rpsL*-PCR fragment (encoding 320 residues of Spr0247, 137 of RpsL, and 149 of RpsG), only changes at 2 residue positions conferred CIP-resistance (0.4%) or STR-resistance (0.3%). However, of the putative nucleotide changes that would occur in the *rpoB* PCR fragment (encoding 547 residues of the β subunit of RNA polymerase) changes at 4 residue positions conferred RIF-resistance (0.7%) (Table 3). If we introduce these corrections, among 7.2×10^4 putative mutants, the expected number of resistant clones would be approximately 3×10^2 for Cip^R, 2×10^2 for Str^R and 5×10^2 for Rif^R. These figures are consistent with the number of drug-resistant clones = (0.9×10^2 of Cip^R, 3×10^2 of Str^R and 1×10^3 of Rif^R) obtained, and are also in line with those previously reported for RIF and STR (2) and with the frequencies reported for mefloquine-resistant mutants (28).

The method allowed obtaining mutants with frequencies several orders of magnitude higher than that of spontaneous mutation. The frequency of mutation to Cip^R, Rif^R and Str^R in *S. pneumoniae* has been shown to be in the range 10^{-8}–10^{-9} (2, 34, our own results) whereas the transformation frequencies to Cip^R, Rif^R and Str^R with the corresponding PCR products

were about 10^{-5}, 10^{-4}, and 10^{-5}, respectively (Table 3).

Among the 10 resistant mutants for each antibiotic sequenced, five Cip^R, five Rif^R and four Str^R different mutations were obtained. All ParC QRDR mutations found in the Cip^R strains obtained in this work had been previously described in laboratory or clinical isolates (1, 6, 20, 30, 34, 39). Although S79F and S79Y have been shown to be involved in resistance by transformation (20, 30, 39), the results presented in this work represent the first evidence that the S79A, D83N and D83V changes are involved in low-level CIP-resistance.

All pneumococcal Rif^R strains obtained in this work had mutations at cluster I of *rpoB* (R6 residue positions 478 to 510), a conserved region where most of bacterial Rif^R mutations map (4, and references cited therein) and also where Rif^R mutations have been characterized in *S. pneumoniae* clinical isolates (8, 32). The Rif^R mutations found in this work were at residues S482, Q486, D489 and H499 (Table 3, Fig. 1). Structural and biochemical studies of *Thermus aquaticus* core RNA polymerase have revealed that RIF binds to a pocket of the RNA polymerase β subunit deep within the DNA/RNA channel and blocks the path of the elongating RNA when the transcript becomes 2 to 3 nt in length. Ten residues of cluster I are directly implicated in the interaction with RIF (4, 41). These residues are identical between *Escherichia coli*, *Mycobacterium tuberculosis*, and *S. pneumoniae* (6 of them are shadowed in Fig. 1). Three of them are the residues equivalent to those found mutated in Rif^R *S. pneumoniae* that established hydrogen bonds with the antibiotic: Q486, D489 and H499. These results suggest that the binding of RIF to *S. pneumoniae* RNA polymerase is similar in all bacterial enzymes. Accordingly, mutations altering D489 and H499 have been found in Rif^R *S. pneumoniae* clinical isolates (8, 32) and those altering the Q residue equivalent to *S. pneumoniae* R6 Q486 have been shown to be involved in Rif^R in *E. coli* (21, 38) and *M. tuberculosis* (17). However, no mutations at the residue equivalent to S482 of *S. pneumoniae* have been previously reported in other Rif^R bacteria (4, and references cited therein). This

residue does not make a direct interaction with RIF, although is conserved among bacterial β subunits and in close proximity to the RIF binding pocket (4). The change of S482 by proline conferred low-level RIF resistance (CMI = 4 μg/ml) to the RMJ4 strain (Table 3) and might affect the folding or packing of the protein in the local vicinity of this residue, causing distortions of the RIF binding pocket, as has been proposed for other Rif^R mutations that also map at residues surrounding this pocket (4).

With respect to the Str^R strains, mutations were found at two lysine residues, K56 and K101 (equivalent to K42 and K87 of *E. coli*). These two residues have been show to be involved in Str resistance in *E. coli* (13) and *M. tuberculosis* (12, and references cited therein), and the K56T change has been shown to be responsible for the Str^R phenotype of *S. pneumoniae* 533 (37).

In summary, with the method described in this work, it was possible to construct Cip^R, Rif^R and Str^R strains carrying mutations at specific gene regions. The same method might be used to construct all possible resistant mutants to other drugs. It would be also possible to make double mutants by sequential PCR and transformation cycles. Strains resistant to two (o more) antibiotics of the same family can be obtained in this way. The activity of the various antibiotics could be tested in the mutants obtained. This information would be useful to select the more adequate therapy, ideally, antibiotics not showing cross-resistance.

ACKNOWLEDGMENTS

We thank E. García for critical reading of the manuscript. The technical assistance of Amaya Aguirre is acknowledged. A. J. M-G is the recipient fellowship from Comunidad Autónoma de Madrid. This study was supported by Grant 1274/01 from Instituto de Salud Carlos III, Grant 00/0258 from Fondo de Investigación Sanitaria and by Grant BIO2002-01398 from Ministerio de Ciencia y Tecnología.

REFERENCES

1. **Bast, D. J., D. E. Low, C. Duncan, L. Kilburn, L. A. Mandell, R. J. Davidson, and J. C. S. de Azevedo.** 2000. Fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*: contributions of type II topoisomerase mutations and efflux to levels of resistance. Antimicrob. Agents Chemother. **44:**3049-3054.
2. **Belanger, A. E., A. Lai, M. A. Brackman, and D. J. LeBlanc.** 2002. PCR-based ordered genomic libraries: a new approach to drug target identification for *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **46:**2507-2512.
3. **Campbell, E. A., S. Y. Choi, and H. R. Masure.** 1998. A competence regulon in *Streptococcus pneumoniae* revealed by genome analysis. Mol. Microbiol. **27:**929-939.
4. **Campbell, E. A., N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb, and S. A. Darst.** 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerases. Cell **23:**901-912.
5. **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.
6. **Davies, T. A., G. A. Pankuch, B. E. Dewasse, M. R. Jacobs, and P. C. Appelbaum.** 1999. In vitro development of resistance to five quinolones and amoxicillin-clavulanate in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **43:**1177-1182.
7. **Dopazo, J., A. Mendoza, J. Herrero, F. Caldara, Y. Humbert, L. Friedli, M. Guerrier, E. Grand-Schenk, C. Gandin, M. de Francesco, A. Polissi, G. Buell, G. Feger, E. García, M. Peitsch, and J. F. García-Bustos.** 2001. Annotated draft genomic sequence from a *Streptococcus pneumoniae* type 19F clinical isolate. Microb. Drug Resist. **7:**99-125.
8. **Enright, M., P. Zawadski, P. Pickerill, and C. G. Dowson.** 1998. Molecular evolution of rifampicin resistance in *Streptococcus pneumoniae*. Microb. Drug Resist. **4:**65-70.

9. **Fenoll, A., I. Jado, D. Vicioso, A. Pérez, and J. Casal.** 1998. Evolution of *Streptococcus pneumoniae* serotypes and antibiotic resistance in Spain: update (1990-1996). J. Clin. Microbiol. **36:**3447-3454.

10. **Fenoll, A., R. Muñoz, E. García, and A. G. de la Campa.** 1994. Molecular basis of the optochin-sensitive phenotype of pneumococcus: characterization of the genes encoding the F_0 complex of the *Streptococcus pneumoniae* and *Streptococcus oralis* H^+-ATPases. Mol. Microbiol. **12:**587-598.

11. **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.

12. **Finken, M., P. Kirschner, A. Meier, A. Wrede, and E. C. Böttger.** 1993. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of ribosomal protein S12 and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol. Microbiol. **9:**1239-1246.

13. **Funatsu, G., and H. G. Wittmann.** 1972. Location of amino acid replacements inprotein S12 isolated from *Escherichia coli* mutants resistant to streptomycin. J. Mol. Biol. **68:**547-550.

14. **González, I., M. Georgiou, F. Alcaide, D. Balas, J. Liñares, and A. G. de la Campa.** 1998. Fluoroquinolone resistance mutations in the *parC*, *parE*, and *gyrA* genes of clinical isolates of viridans group streptococci. Antimicrob. Agents Chemother. **42:**2792-2798.

15. **Greenwood, B.** 1999. The epidemiology of pneumococcal infection in children in the developing world. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **354:**777-785.

16. **Håvarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **92:**11140-11144.

17. **Heep, M., B. Brandstätter, U. Rieger, N. Lehn, E. Richter, S. Rüsch-Gerdes, and S. Nieman.** 2001. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. J. Clin. Microbiol. **39:**107-110.

18. **Hoffman, J., M. S. Cetron, M. M. Farley, W. S. Baughman, R. R. Facklam, J. A. Elliot, K. A. Deaver, and R. F. Breiman.** 1995. The prevalence of drug-resistant *Streptococcus pneumoniae* in Atlanta. N. Engl. J. Med. **333:**481-486.

19. **Hoskins, J., W. E. Alborn, J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, P. L. Skatrud, and J. I. Glass.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709-17.

20. **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.

21. **Jin, D. J., and C. A. Gross.** 1988. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. J. Mol. Biol. **202:**45-58.

22. **Lacks, S.** 2000. Cloning and expression of pneumococcal genes in *Streptococcus pneumoniae*. *In* A. Tomasz (ed.), *Streptococcus pneumoniae*: molecular biology & mechanism of disease. Mary Ann Liebert, Inc., Larchmont, N. Y.

23. **Lacks, S. A.** 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53:**207-235.

24. **Lacks, S. A., S. Ayalew, A. G. de la Campa, and B. Greenberg.** 2000. Regulation of competence for genetic transformation in *Streptococcus pneumoniae*: expression of *dpnA*, a late competence gene encoding a DNA methyltransferase of the *Dpn*II restriction system. Mol. Microbiol. **35:**1089-1098.

25. **Lacks, S. A., and B. Greenberg.** 1975. A deoxyribonuclease of *Diplococcus pneumoniae* specific for methylated DNA. J. Biol. Chem. **250:**4060-4066.

26. **Lee, M. S., and D. A. Morrison.** 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J. Bacteriol. **181:**5004-5016.

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

28. **Martín-Galiano, A. J., B. Gorgojo, C. M. Kunin, and A. G. de la Campa.** 2002. Mefloquine and new related compounds target the F_0 complex of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **46:**1680-1687.

29. **Martín-Galiano, A. J., M. J. Ferrándiz, and A. G. de la Campa.** 2001. The promoter of the operon encoding the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae* is inducible by pH. Mol. Microbiol. **6:**1327-1338.

30. **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.

31. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa.

32. **Padayachee, T., and K. Klugman.** 1999. Molecular basis of rifampin resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **43:**2361-2365.

33. **Pallarés, R., J. Liñares, M. Vadillo, C. Cabellos, F. Manresa, P. F. Viladrich, R. Martin, and F. Gudiol.** 1995. Resistance to penicillin and cephalosporins and mortality from severe pneumococcal pneumonia in Barcelona, Spain. N. Engl. J. Med. **333:**474-480.

34. **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.

35. **Pestova, E. V., L. S. Håvarstein, and D. A. Morrison.** 1996. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol. Microbiol. **21:**853-862.

36. **Pestova, E. V., and A. D. Morrison.** 1998. Isolation and characterization of three *Streptococcus pneumoniae* transformation-specific loci by use of a *lacZ* reporter insertion vector. J. Bacteriol. **180:**2701-2710.

37. **Salles, C., L. Créancier, J.-P. Claverys, and V. Méjean.** 1992. The high level streptomycin resistance gene from *Streptococcus pneumoniae* is a homologue of the ribosomal protein S12 gene from *Escherichia coli*. Nucl. Acids Res. **20:**6103.

38. **Severinov, K., M. Soushko, A. Goldfarb, and V. Nikiforov.** 1993. Rifampicin region revisited. New rifampicin-resistant and streptolydigin-resistant mutants in the β subunit of *Escherichia coli* RNA polymerase. J. Biol. Chem. **268:**14820-14826.

39. **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.

40. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science. **293:**498-506.

41. **Zhang, G., E. A. Campbell, L. Minakhin, C. Ritcher, K. Severinov, and S. A. Darst.** 1999. Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Å resolution. Cell **98:**811-824.

FIGURE LEGENDS

FIG. 1. Location of the PCR products employed in this work, of regions sequenced, and of the mutations present in the Cip^R (A), Rif^R (B) and Str^R (C) strains. Black arrows (not draw to scale) indicate the oligonucleotides used to amplify the fragments of about 1,600 bp used in PCR experiments. Hatched rectangles correspond to the regions that have been sequenced to identify the mutations. AA substitutions present in the resistant strains are shown below the wild-type residue position (in boldface and underlined).

TABLE 1. Oligonucleotides used in this work

Name	Relevant features	
	Sequence (5'-3')	Nt positions (aa positions)[a]
parCUP	GAACACGCCCTAGATACTGTG	−103 to −83 of *parC*
parC50	AAGGATAGCAATACTTT	147−163 of *parC* (^{50}KDSNTF^{55})
parC152	GTTGGTTCTTTCTCCGTATCG	Complementary to 456−438 of *parC* (^{147}DTEKEP^{152})
parC503R	GCCTTGGTCACGCTGACGTAGG	Complementary to 1526−1505 of *parC* (^{502}TYVSVTKA^{509})
rpoB227	GCGAATTGGTTCGCAACACTG	680−700 of *rpoB* (^{227}ELVRNT^{233})
rpoB427	CGGTTGGTGAATTGCTTGCCAACC	1282−1306 of *rpoB* (^{427}AVGELLAN^{435})
rpoB554R	CAAGTGTCCGTAAGATGCAAG	Complementary to 1641−1662 of *rpoB* (^{548}LSSYGHL^{554})
rpoB773R	GTCATGTAGGCAACGATTGGG	Complementary to 2322−2301 of *rpoB* (^{768}PIVAYMT^{774})
rpsLUP	GGGCTAGTAGAAGTAGTTGGC	320−300 of spr0247 (^{101}PTTSTSP^{107})
rpsL6	CCAATTGGTTCGCAAACCGCG	15−35 of *rpsL* (^{6}QLVRKPR^{12})
rpsL131R	CCGTATTTAGAACGGCCTTG	Complementary to 392−373 of *rpsL* (^{125}QGRSKYG^{131})
rpsLDOWN	CGGAAGTGTGCGAATGCACGG	Complementary to 443−426 of *rpsG* (^{143}RMAEANR^{149})

[a] Nt and AA numbering refers to the genes/proteins obtained from the *S. pneumoniae* R6 sequence, taken the first Nt or AA as position 1.

TABLE 2. Antibiotic-resistant R6 transformants obtained with R6-PCR products

Donor DNA	Enzyme[b]	Transformants/ml (Transformation frequency × 10^{-5}) selected on [a]		
		CIP	RIF	STR
parC	Tth	94 ± 21 (1.0)	None	None
	Hf	33 ± 22 (0.4)	None	None
rpoB	Tth	None	1,119 ± 319 (12.4)	None
	Hf	None	580 ± 193 (6.4)	None
rpsL	Tth	None	None	299 ± 88 (3.2)
	Hf	None	None	72 ± 28 (0.8)

[a] 0.15 μg of PCR products carrying the genes indicated were used to transform 1 ml (9×10^6 CFU) of a competent R6 culture. Values (mean ± standard deviation) of three independent experiments are represented. Transformation frequency: number of transformants/total number of cells. None, no transformants were observed when 300 μl of the transformation mix were plated on selective plates, which gave a frequency $< 4 \times 10^{-7}$. PCR products used as controls were: a *parC* PCR product from a *S. pneumoniae* 4114 carrying a S79F change that yielded $2.0 \pm 1.5 \times 10^4$ transformants/ml, and a *rpsL* PCR product from *S. pneumoniae* 533 (25) that carry a K56R (37) change that yielded $5.8 \pm 2.8 \times 10^5$ transformants/ml.

[b] Tth: *Thermus thermophilus* thermostable DNA polymerase (Biotools); Hf: Expand High Fidelity PCR system (Boehringer Manheim).

TABLE 3. Characteristics of the resistant strains

Drug	Strain (number of clones)	Gene	AA change (codon change)[a]	MIC (μg/ml) (increase)[b]	Transformants/ml ($\times 10^{-4}$)[c]
CIP	CMJ1 (4)	*parC*	S79F (TCT→T<u>T</u>T)	4 (8×)	4
	CMJ2 (1)		D83N (GAT→<u>A</u>AT)	2 (4×)	6
	CMJ3 (1)		S79A (TCT→<u>G</u>CT)	2 (4×)	10
	CMJ4 (2)		S79Y (TCT→T<u>A</u>T)	4 (8 ×)	27
	CMJ10 (1)		D83V (GAT→G<u>T</u>T)	2 (4×)	2
RIF	RMJ1 (5)	*rpoB*	Q486L (CAG→C<u>T</u>G)	16 (> 533×)	140
	RMJ3 (1)		H499Y (CAC→<u>T</u>AC)	8 (> 266×)	13
	RMJ4 (1)		H499L (CAC→C<u>T</u>C)	16 (> 533×)	43
	RMJ5 (2)		S482P (TCA→<u>C</u>CA)	4 (>133×)	14
	RMJ7 (1)		D489V (GAC→G<u>T</u>C)	16 (> 533×)	48
STR	SMJ1 (2)	*rpsL*	K56I (AAA→A<u>T</u>A)	>800 (>256×)	16
	SMJ2 (6)		K56R (AAA→A<u>G</u>A)	>800 (>256×)	7
	SMJ4 (1)		K56T (AAA→A<u>C</u>A)	>800 (>256×)	27
	SMJ6 (1)		K101E (AAA→<u>G</u>AA)	200 (64×)	43

[a] AA positions of the genes indicated are according to the *S. pneumoniae* R6 genomic sequence.

[b] MICs are the average of four independent determinations. Values in parentheses are the MIC value for the resistant strain divided by the MIC value for R6 (0.5 μg/ml CIP, <0.03 μg/ml RIF, and 3.12 μg/ml STR).

[c] PCR products carrying parts of the indicated genes from resistant strains were used to transform R6 competent cells. PCR products used as controls were those indicated in Table 2.