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Genomic Characterization of Ciprofloxacin Resistance in a Laboratory-Derived Mutant and a Clinical Isolate of *Streptococcus pneumoniae*

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The broad-spectrum fluoroquinolone ciprofloxacin is a bactericidal antibiotic targeting DNA topoisomerase IV and DNA gyrase encoded by the *parC* and *gyrA* genes. Resistance to ciprofloxacin in *Streptococcus pneumoniae* mainly occurs through the acquisition of mutations in the quinolone resistance-determining region (QRDR) of the ParC and GyrA targets. A role in low-level ciprofloxacin resistance has also been attributed to efflux systems. To look into ciprofloxacin resistance at a genome-wide scale and to discover additional mutations implicated in resistance, we performed whole-genome sequencing of an *S. pneumoniae* isolate selected for resistance to ciprofloxacin *in vitro* (128 μg/ml) and of a clinical isolate displaying low-level ciprofloxacin resistance (2 μg/ml). Gene disruption and DNA transformation experiments with PCR fragments harboring the mutations identified in the *in vitro S. pneumoniae* mutant revealed that resistance is mainly due to QRDR mutations in *parC* and *gyrA* and to the overexpression of the ABC transporters PatA and PatB. In contrast, no QRDR mutations were identified in the genome of the *S. pneumoniae* clinical isolate with low-level resistance to ciprofloxacin. Assays performed in the presence of the efflux pump inhibitor reserpine suggested that resistance is likely mediated by efflux. Interestingly, the genome sequence of this clinical isolate also revealed mutations in the coding region of *patA* and *patB* that we implicated in resistance. Finally, a mutation in the NAD(P)H-dependent glycerol-3-phosphate dehydrogenase identified in the *S. pneumoniae* clinical strain was shown to protect against ciprofloxacin-mediated reactive oxygen species.

Streptococcus pneumoniae is a major Gram-positive pathogen responsible for pneumonia, bacteremia, otitis media, and meningitis leading to considerable morbidity and mortality among children and elderly individuals (1). Penicillin, a β-lactam antibiotic, has long been the mainstay against pneumococcal infections (2, 3), but the worldwide spread of antibiotic-resistant clones over the past decades has impaired its usefulness for dealing with *S. pneumoniae* infections (4–6). The rates of resistance against β-lactams and macrolides among *S. pneumoniae* isolates have translated into an increased usage of fluoroquinolone antibiotics in the treatment of respiratory diseases (7–10).

Fluoroquinolones are part of a class of synthetic broad-spectrum antibiotics that inhibit DNA synthesis in bacteria by targeting DNA gyrase (GyrA and -B subunits) and topoisomerase IV (ParC and -E subunits), two enzymes that are vital for DNA supercoiling and chromosome segregation, respectively (11, 12). Although the worldwide prevalence of fluoroquinolone-resistant *S. pneumoniae* remains low in relation to β-lactam resistance (≤1%) (13–15), the dissemination of successful resistant clones has nonetheless increased the prevalence in some countries (16, 17). Resistance to fluoroquinolones in *S. pneumoniae* arises in a stepwise fashion and results from alterations in the target binding site due to the acquisition of spontaneous mutations in the quinolone resistance-determining regions (QRDRs) of the topoisomerase IV and DNA gyrase genes (18, 19). Although mutations usually occur in the QRDRs of *parC* and *gyrA* (20, 21), a role for mutations in the *parE* and *gyrB* subunits in low-level resistance has been reported (22–24). *S. pneumoniae* isolates with a mutation only in ParC usually remain susceptible or display only a modest increase in resistance (19, 24), but these first-step mutants are associated with an increased risk for secondary mutations that may enhance resistance (18, 19, 24–27). Higher levels of fluoroquinolone resistance require mutations in both *parC* and *gyrA* (18, 19, 24).

Resistance to fluoroquinolones can also occur through the overexpression of efflux systems, a phenotype frequently observed among resistant clinical pneumococci (28–30). While active efflux accounts for only a moderate increase in resistance (31), efflux-positive strains exhibit an increased likelihood of acquiring QRDR mutations, and the combination of efflux and first-step mutations can lead to MICs associated with treatment failure (32). To date, two efflux systems have been found to confer resistance to fluoroquinolones on *S. pneumoniae*. The major facilitator PmrA was the first efflux system described during *in vitro* work on norfloxacin resistance in *S. pneumoniae* (33), but subsequent studies challenged the clinical significance of this transporter (34, 35). The second efflux system was revealed while characterizing 13 putative efflux systems of the ATP-binding cassette (ABC) superfamily in *S. pneumoniae* TIGR4 (36). The ABC transporters SP2075 (PatA)

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and SP2073 (PatB) were shown to act as heterodimers (37) to enable resistance to several antimicrobial agents, including ethidium bromide (EtBr), dyes, and fluoroquinolones, and the overexpression of their genes is associated with fluoroquinolone resistance in *S. pneumoniae* clinical isolates (30).

Studying resistance at the genome scale can reveal additional insights into mechanisms having more subtle roles, like in facilitating resistance (38, 39) or in compensating for fitness cost (40). In this study, we characterized by genome sequencing one laboratory-derived *S. pneumoniae* mutant and a derived transformant highly resistant to ciprofloxacin (CIP). We also characterized a low-level CIP-resistant transformant obtained by whole-genome DNA transformation of a clinical isolate into a susceptible strain in order gain insights into mechanisms leading to decreased CIP susceptibility in the absence of QRDR mutations. We report the functional analysis of QRDR mutations in CIP resistance, in addition to new knowledge about the role in resistance for mutations in the coding regions of *patA* and *patB* and for a mutation preventing the generation of ciprofloxacin-induced oxidants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table S1 in the supplemental material. Pneumococci were grown in brain heart infusion broth (BHI; Difco), or in blood agar containing 5% defibrinated sheep's blood. Cultures were incubated for 16 to 24 h in a 5% CO_2 atmosphere at 35°C. All strains were maintained frozen at −80°C in BHI containing 15% glycerol. The mutant R6M2B was generated from *S. pneumoniae* R6 by a stepwise fashion on plates containing increasing concentrations of CIP as described previously (39). Eight selection cycles were required to obtain R6M2B. The transformants of R6M2B (T1- to T5-R6M2B) were generated by transforming genomic DNA (gDNA) derived from R6M2B into *S. pneumoniae* R6 recipients in the presence of CIP. This gDNA transformation scheme was done four times in the presence of increasing concentrations of CIP to obtain the final transformant, T5-R6M2B. The clinical isolate 60827 was isolated from a respiratory sample collected from a 66-year-old female at Mount Sinai Hospital in Toronto, Canada. The T1-60827 transformant was produced by a single round of gDNA transformation in wild-type (WT) *S. pneumoniae* R6.

MIC determination. MICs to CIP (Sigma-Aldrich), EtBr (Fluka), and the efflux inhibitor reserpine (Sigma-Aldrich) were determined by microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). All compounds listed were prepared according to the manufacturer's instructions. The MICs for CIP and EtBr were also measured in the presence of a range of reserpine concentrations (0 to 20 μg/ml). The MICs were recorded as the lowest dilution showing no growth. All MIC measurements were done at least in triplicate.

RNA extraction and qRT-PCR. Total RNA was isolated from mid-log-phase cells grown in BHI using the Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNAs were treated with DNase I (Ambion) to avoid any DNA contamination. The quality and integrity of the RNAs were assessed using a 2100 BioAnalyzer and RNA6000 Nano chips (Agilent). The cDNAs were generated from total RNAs using Superscript II reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's instructions. Real-time quantitative reverse transcription-PCR (qRT-PCR) assays were carried out in a Bio-Rad cycler using SYBR green I (Molecular Probes). A final volume of 20 μl was used for each reaction mixture containing specific primers (see Table S2 in the supplemental material) and iQ SYBR green Supermix (Bio-Rad). All qRT-PCR data were normalized according to the amplification signals of the 16S rRNA.

Gene inactivation. The genes *patA*, *patB*, and spr0043 (coding for the ABC protein ComA) were inactivated by insertional duplication mutagenesis using either of the nonreplicative plasmids pFF3 and pFF6. The pFF3 vector is a derivative from plasmid pGEM-T Easy (Promega) from which an Eam1105I restriction site was cloned in the multicloning site and the ampicillin resistance marker was replaced by the chloramphenicol resistance marker of the pEVP3 vector (41). The pFF6 plasmid is a derivative of pFF3 in which the chloramphenicol resistance marker was replaced by the kanamycin marker of the pDL289 plasmid (42). Fragments internal to the *patA*, *patB*, and spr0043 genes were amplified from the gDNA of *S. pneumoniae* R6 and 60827 using the primers *patA*F-KO, *patA*R-KO, *patB*F-KO, *patB*R-KO, spr0043F-KO, and spr0043R-KO, respectively (see Table S2), prior to being cloned into the Eam1105I site restriction site of pFF6 or pFF3. Transformants were selected on casein tryptone medium (CAT) agar plates supplemented with 5% defibrinated sheep's blood and either 500 μg/ml of kanamycin (pFF6) or 5 μg/ml of chloramphenicol (pFF3).

DNA transformation. Genetic transformation was performed as previously described (38, 40). When needed, an *rpsL*$^+$ fragment conferring resistance to streptomycin (Lys57Thr) was cotransformed as a surrogate selection marker along with the DNA fragment of interest (38).

Whole-genome sequencing (WGS). Genomic DNA was prepared from mid-log-phase cultures of *S. pneumoniae* using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. The genomes of R6M2B, T5-R6M2B, and T1-60827 were sequenced using a 454 Life Sciences GS-FLX system (Roche). Genome sequencing, assemblies, and comparative analyses were performed at the McGill University Genome Québec Innovation Center. R6M2B, T5-R6M2B, and T1-60827 generated aggregated genome sizes of 2,016,866 bp, 2,016,652 bp, and 2,011,560 bp, respectively, at a mean 20× coverage. Whenever possible, the order and orientation of assembled contigs were done in accordance with the genome assembly of *S. pneumoniae* R6 (accession number NC_003098). Mutations deduced from massively parallel sequencing were confirmed by PCR amplification and conventional DNA sequencing using the primers listed in Table S2 in the supplemental material.

ROS accumulation. The intracellular accumulation of reactive oxygen species (ROS) was measured using dichlorofluorescein diacetate (DCF-DA; Invitrogen) as described previously (38). CIP was added to exponential-phase bacteria (optical density at 600 nm [OD_{600}], 0.12) according to the MIC of the tested *S. pneumoniae* strains. ROS accumulation was thus tested in the presence of 32 μg/ml of CIP for strains $R6^{parC,gyrA\text{-}R6M2B}$ and $R6^{parC,gyrA,spr1902\text{-}T5R6M2B,\ smR}$ and 128 μg/ml of CIP for strains T5-R6M2B and T5-$R6M2B^{spr0335R6,\ smR}$. One-milliliter aliquots were collected at baseline and up to 3 h after exposure to CIP, a time point at which ROS were previously shown to be induced by CIP in *S. pneumoniae* R6 (38). Aliquots were washed once with 500 μl of 1× phosphate-buffered saline (PBS) and incubated in the presence of 5 μg/ml of DCF-DA in the dark for 30 min. The cells were washed once with 1× PBS, and a 200-μl aliquot was analyzed using a Victor fluorometer (485-nm excitation/535-nm emission wavelengths) in 96-well Nunc-Immuno plates (Thermo scientific). The fluorescence signals were normalized according to the bacterial count obtained by plating on Trypticase soy agar supplemented with 5% sheep blood (BD).

Nucleotide sequence accession numbers. The sequencing data have been deposited at the EBI SRA database (http://www.ebi.ac.uk/) under study accession number ERP002062, samples accession numbers ERS199507, ERS199508, and ERS199509, corresponding to *S. pneumoniae* R6M2B, T5-R6M2B, and T1-60827, respectively.

RESULTS

Selection of *S. pneumoniae* mutants and DNA transformants resistant to ciprofloxacin. To investigate CIP resistance at the genome scale, we produced a highly resistant mutant derived from *S. pneumoniae* R6 (CIP MIC, 0.5 μg/ml) by stepwise CIP increments. The resulting *S. pneumoniae* mutant, R6M2B, had a final CIP MIC of 128 μg/ml and displayed cross-resistance to the expanded-spectrum fluoroquinolone levofloxacin (MIC = 32 μg/ml). We also studied the basis of CIP resistance in a serotype 11A

TABLE 1 Chronological appearance of the mutations functionally characterized in *S. pneumoniae* R6M2B and 60827 and their transformants

Strain	CIP MIC (μg/ml)	Mutation						
		parC	*gyrA*	*patA*[a,b]	*patB*[c]	spr0335	spr1544	spr1902
R6	0.5	WT	WT	WT	WT	WT	WT	WT
R6M2B	128	C245T	G253A	WT*	WT	WT	WT	WT
T1-R6M2B	16	C245T	WT	WT	WT	WT	A-28G	C248T
T2-R6M2B	64	C245T	G253A	WT	WT	WT	A-28G	C248T
T3-R6M2B	64	C245T	G253A	WT	WT	WT	A-28G	C248T
T4-R6M2B	64	C245T	G253A	WT	WT	WT	A-28G	C248T
T5-R6M2B	128	C245T	G253A	WT*	WT	C1246T	A-28G	C248T
T1-60827	2	WT	WT	RSS10	RSS10	WT	WT	C248T

[a] PatA mutations identified in RSS10: A-33C, G335T, T537C, A540G, and C660T. A number preceded by a hyphen indicates the position of a mutation upstream of the ATG.
[b] An asterisk indicates an increased expression of *patA* as measured by quantitative real-time PCR.
[c] PatB mutations identified in RSS10: T142G, T357C, T618C, T1260C, G1507A, and A1620G.

S. pneumoniae clinical isolate named 60827 that displayed a CIP MIC characteristic of single-step CIP-resistant mutants (2 μg/ml). *S. pneumoniae* 60827 remained susceptible to levofloxacin (MIC = 1 μg/ml). No cross-resistance to other antibiotics (erythromycin, tetracycline, tigecycline, chloramphenicol, vancomycin, penicillin, clindamycin, and amikacin) was observed in any of the strains. Owing to the extensive polymorphism occurring between *S. pneumoniae* clinical isolates (43), we reconstructed the resistance phenotype of both the *in vitro* mutant and the clinical isolate by transforming their genomic DNAs (gDNA) into the well-characterized WT *S. pneumoniae* R6 genetic background and selected transformants under CIP pressure. A total of five rounds and a single round of transformation were required to fully reconstruct the CIP resistance levels of R6M2B and 60827, the resulting T5-R6M2B and T1-60827 transformants having CIP MICs of 128 μg/ml and 2 μg/ml, respectively (Table 1).

Genome sequence of *S. pneumoniae* ciprofloxacin-resistant strains. The genome sequence of R6M2B revealed only four mutations that were either within the QRDRs of *parC* (S79F) and *gyrA* (E85K) or within intergenic regions upstream of spr0129 and downstream of spr0952 (Table 2). Of these, the only mutations that transferred to T5-R6M2B were those within the QRDRs of *parC* and *gyrA*. Their contribution to CIP resistance was further assessed by transforming *S. pneumoniae* R6 WT recipients with PCR products amplified from R6M2B. As CIP usually selects for mutations within *parC* prior to those in *gyrA* (24–26), *parC* was first amplified from gDNA derived from R6M2B using the primers listed in Table S2 in the supplemental material, and the PCR products were transformed into WT *S. pneumoniae* R6. The selection of transformants using a CIP concentration of 1 μg/ml enabled recovering the transformant $R6^{parC\text{-}R6M2B}$, which had a 4-fold increase in CIP resistance (2 μg/ml) compared to WT *S. pneumoniae* R6 (Table 3). PCR sequencing of *parC* confirmed that the mutation found in the mutant was transferred and selected during transformation. A second round of transformation using $R6^{parC\text{-}R6M2B}$ as recipients and PCR products covering the mutated *gyrA* of R6M2B further increased CIP resistance to a MIC of 32 μg/ml (Table 3). In contrast, no role in resistance could be attributed to the A-to-G transition observed three nucleotides upstream of spr0129 in R6M2B, as its reversion to a wild-type sequence had no effect on the CIP MIC of the mutant (Table 3). This was done by transforming a PCR fragment derived from the upstream region of spr0129 of WT *S. pneumoniae* R6 along with a PCR fragment covering the *rpsL*$^+$ allele of *S. pneumoniae* CP1296 conferring resistance to streptomycin that was used as a surrogate resistance marker (see Materials and Methods). The *rpsL*$^+$ PCR fragment had no impact on CIP susceptibility levels when transformed alone (Table 3). The lack of phenotype in CIP resistance for the mutation upstream of spr0129 is consistent with the fact that this single nucleotide polymorphism (SNP) was not transferred into T5-R6M2B, and since the 12-nucleotide deletion downstream of spr0952 in R6M2B was not transferred either (Table 2), its role in resistance was not further studied.

Despite our efforts to prevent the selection of *de novo* mutations while selecting for the T1- to T5-R6M2B transformants (see Materials and Methods), the genome sequence of T5-R6M2B revealed three additional mutations not found in the genome of its R6M2B parent mutant (Table 2). These occurred upstream of spr1544 and within the coding regions of the 6-phosphogluconate dehydrogenase gene spr0335 and the NAD(P)H-dependent glycerol-3-phosphate dehydrogenase gene spr1902 (Table 2). Although they were artifacts of transformation, we were interested in assessing the role of these mutations in resistance to ciprofloxacin, especially due to the fact that we observed an identical T83I mutation in spr1902 of the T1-60827 transformant derived from clinical isolate 60827 (Table 2; see also below). Interestingly, cotransformation (*rpsL*$^+$) of the spr1902 T83I substitution conferred a 2-fold increase in CIP resistance when transformed in the presence of both *parC* and *gyrA* mutations, but not when expressed in a WT background or only in the presence of an altered *parC* (Table 3). Similarly, the reversion of the spr0335 mutation to a wild-type allele in *S. pneumoniae* R6M2B (using a cotransformation with the *rpsL*$^+$ PCR fragment) decreased the CIP MIC of the mutant 2-fold, from 128 μg/ml to 64 μg/ml (Table 3). It should be noted that it proved impossible to transform the spr0335 mutation directly into the *S. pneumoniae* R6 WT or $R6^{parC,gyrA\text{-}R6M2B}$ background. No role in resistance could be observed for the mutation upstream of spr1544 when transformed alone (data not shown) or in the presence of QRDR mutations (Table 3).

The genome sequence of T1-60827 revealed several SNPs compared to the *S. pneumoniae* R6 WT reference. We found a total of 7 stretches of SNPs that clustered in neighbor genes and that we named multigene recombination sequence segments (RSSs) (Table 2 and Fig. S1). Multigene RSSs were comprised of 2 to 4 genes. An additional eight RSSs were also transferred from strain 60827, but these were restricted to single genes (Table 2; see also Fig. S1 in the supplemental material). None of the RSSs covered the QRDR of *parC* or *gyrA* (Tables 1 and 2), which is consistent with the low

TABLE 2 List of mutations identified by WGS in ciprofloxacin-resistant *S. pneumoniae*

Gene ID[a]	Gene function	Mutation[b,c,d]		
		R6M2B	T5-R6M2B	T1-60827[e]
spr0084	Conserved hypothetical protein			RSS1
spr0085	Hypothetical protein			RSS1
spr0086	Hypothetical protein			RSS1
spr0129	Hypothetical protein	**G-3T**		
spr0291	Phosphotransferase system (PTS system) IIA component			RSS2
spr0309	Hypothetical protein			RSS3
spr0310	Alpha-1-6-glucosidase			RSS3
spr0335	6-Phosphogluconate dehydrogenase		**G1246C** ***V416L***	
spr0757	DNA topoisomerase IV subunit A	**C245T** ***S79F***	**C245T** ***S79F***	
spr0931	Conserved hypothetical protein			RSS4
spr0952	Hypothetical protein	12-nucleotide deletion downstream		
spr0974	Phosphoenolpyruvate carboxylase			RSS5
spr0981	Glycosyltransferase			RSS6
spr1039	Second subunit of major exonuclease			RSS7
spr1099	DNA gyrase subunit A	**G247A** ***E85K***	**G247A** ***E85K***	
spr1248	Conserved hypothetical protein			RSS8
spr1249	Alpha-acetolactate decarboxylase			RSS8
spr1250	Conserved hypothetical protein			RSS8
spr1251	ABC transporter substrate-binding protein; glutamine-binding protein			RSS8
spr1544	Preprotein translocase subunit SecA		**A-28G**	
spr1545	Hypothetical protein			RSS9
spr1885	ABC transporter ATP-binding/membrane-spanning protein—unknown substrate			**RSS10**
spr1886	Degenerate transposase			**RSS10**
spr1887	ABC transporter ATP-binding/membrane-spanning protein—unknown substrate			**RSS10**
spr1902	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase		**C248T** ***T83I***	**RSS11**
spr1919	ABC transporter membrane-spanning permease—maltose/maltodextrin			RSS12
spr1945	Choline-binding protein			RSS13
spr1946	Degenerate transposase (orf1)			RSS13
spr1979	D-Alanine transfer from undecaprenol-phosphate to the poly(glycerophosphate) chain			RSS14
spr1981	D-Alanine transfer from Dcp to undecaprenol-phosphate			RSS14
spr1982	D-Alanine-D-alanyl carrier protein ligase			RSS14
spr1983	Hypothetical protein			RSS14
spr1988	Glycerol uptake facilitator protein			RSS15
spr1989	Glycerol-3-phosphate dehydrogenase, truncation			RSS15
spr1990	Glycerol-3-phosphate dehydrogenase, truncation			RSS15

[a] ID, identity. Nomenclature according to the genome annotation of *S. pneumoniae* R6.
[b] When mutations are within coding regions, the change in amino acids is also indicated in italics.
[c] In noncoding sequence, the number preceded by a hyphen indicates the position upstream of the ATG.
[d] Mutations in bold were further studied to determine their potential role in CIP resistance.
[e] Mutations in bold RSSs have been confirmed in the parental isolate 60827.

level of resistance of strain 60827 and with the absence of cross-resistance to levofloxacin (data not shown). Targeted sequencing of *parC* and *gyrA* in the 60827 clinical isolates also revealed the absence of mutations (data not shown).

ABC transporters and ciprofloxacin resistance. While the T5-R6M2B transformant is resistant to CIP similarly to its parent R6M2B mutant, the introduction of the mutations found in the latter by transformation of PCR fragments failed to fully reconstruct resistance. Resistance to the fluoroquinolones CIP and norfloxacin (but not to newer fluoroquinolones) is often associated with a reserpine-sensitive efflux mediated by the ABC proteins PatA and PatB (30, 36, 44), whose expression is frequently increased in strains showing a reserpine-sensitive phenotype in fluoroquinolone resistance (30). The CIP MIC of the *S. pneumoniae* R6M2B and 60827 mutants and of their transformants was thus measured in the presence and absence of the efflux pump inhibitor reserpine to assess the role of efflux in resistance. The resistance of R6M2B and T5-R6M2B was partly reverted in the pres-

TABLE 3 Functional analysis of mutations found by whole-genome sequencing in R6M2B, T5-R6M2B, and T1-60827

Strain[a]	Mutation								CIP MIC (μg/ml)[b]
	parC	*gyrA*	spr0129	spr0335	spr1544	spr1902	*patA*	*patB*	
R6	WT	WT	WT	WT	WT	WT	WT	WT	0.5
R6^{smR}	WT	WT	WT	WT	WT	WT	WT	WT	0.5
R6$^{spr1902\text{-}T5R6M2B,\ smR}$	WT	WT	WT	WT	WT	T5-R6M2B	WT	WT	0.5
R6$^{parC\text{-}R6M2B}$	R6M2B	WT	WT	WT	WT	WT	WT	WT	**2**
R6$^{parC,spr1902\text{-}T5R6M2B,\ smR}$	R6M2B	WT	WT	WT	WT	T5-R6M2B	WT	WT	2
R6$^{parC,gyrA\text{-}R6M2B}$	R6M2B	R6M2B	WT	WT	WT	WT	WT	WT	**32**
R6$^{parC,gyrA,spr1544\text{-}T5R6M2B,smR}$	R6M2B	R6M2B	WT	WT	T5-R6M2B	WT	WT	WT	32
R6$^{parC,gyrA,spr1902\text{-}T5R6M2B,smR}$	R6M2B	R6M2B	WT	WT	WT	T5-R6M2B	WT	WT	**64**
R6$^{patA,T1\text{-}60827,smR}$	WT	WT	WT	WT	WT	WT	T1-60827	WT	**1**
R6$^{patB,T1\text{-}60827,smR}$	WT	WT	WT	WT	WT	WT	WT	T1-60827	**1**
T1-60827	WT	WT	WT	WT	WT	WT	T1-60827	T1-60827	2
T5-R6M2B	R6M2B	R6M2B	WT	T5-R6M2B	T5-R6M2B	T5-R6M2B	WT	WT	128
T5-R6M2B^{spr0335R6,smR}	R6M2B	R6M2B	WT	WT	R6M2B	R6M2B	WT	WT	**64**
R6M2B	R6M2B	R6M2B	R6M2B	WT	WT	WT	WT	WT	128
R6M2B^{spr0129R6,smR}	R6M2B	R6M2B	WT	WT	WT	WT	WT	WT	128

[a] "smR" indicates that an *rpsL*$^+$ allele conferring resistance to streptomycin was cotransformed along with the PCR fragment of interest for selection purposes. These strains are resistant to streptomycin.
[b] MICs in bold are significantly different from the parent strain without the mutation.

ence of reserpine (Table 4; see also Table S3 in the supplemental material) to a level attributable to the mutations in QRDRs (32 μg/ml) (Table 3). The inactivation of either *patA* or *patB* mirrored the phenotype conferred by reserpine (Table 4) and confirmed that these were fully responsible for the reserpine-sensitive CIP resistance of R6M2B and T5-R6M2B. In R6M2B, a massive overexpression of *patA* was confirmed by quantitative RT-PCR (Table 4), a phenotype specifically transferred to its last-level transformant (Table 1). This was also true for a panel of clinical isolates with various levels of susceptibility to ciprofloxacin for which a higher expression level of *patA* is correlated with resistance (see Table S4 in the supplemental material). The high expression of *patA* in R6M2B and T5-R6M2B could not be correlated with a point mutation in the promoter region of the *patA-patB* locus (Table 2). Overexpression was a prerequisite for *patA* to confer resistance, since neither reserpine nor gene inactivation impacted

TABLE 4 ABC transporters and ciprofloxacin resistance

Genetic background	QRDR mutation[a]		*patA* expression[b]	Gene inactivated	MIC (μg/ml)[c]			
	gyrA	*parC*			CIP	CIP + R	EtBr	EtBr + R
R6	WT	WT	1.0	None	0.5	0.5	2	0.25
	WT	WT		*patB*	0.5	0.5	**1**	0.25
	WT	WT		*patA*	0.5	0.5	**1**	0.25
	WT	WT		spr0043	0.5	0.5	2	0.25
R6M2B	E85K	S79F	66 ± 12.44	None	128	32	16	0.25
	E85K	S79F		*patB*	**32**	32	**4**	0.25
	E85K	S79F		*patA*	**32**	32	**4**	0.25
	E85K	S79F		spr0043	**64**	32	16	0.25
T5-R6M2B	E85K	S79F	170 ± 9.25	None	128	32	16	0.25
	E85K	S79F		*patB*	**32**	32	**4**	0.25
	E85K	S79F		*patA*	**32**	32	**1**	0.25
	E85K	S79F		spr0043	128	32	16	0.25
T1-60827	WT	WT	1.82 ± 0.28	None	2	0.5	16	0.25
	WT	WT		*patB*	**1**	0.5	**8**	0.25
	WT	WT		*patA*	2	0.5	**8**	0.25
	WT	WT		spr0043	2	0.5	16	0.25
60827	WT	WT	4.78 ± 0.88	None	2	0.5	16	0.25
	WT	WT		*patB*	2	**1**	16	0.25
	WT	WT		*patA*	2	0.5	16	0.25
	WT	WT		spr0043	2	0.5	16	0.25

[a] *gyrA*, DNA gyrase gene; *parC*, topoisomerase IV gene.
[b] As measured by qRT-PCR.
[c] Gene inactivations conferring a ≥2-fold-change in MIC compared to that of the parental strain are shown in bold. R, reserpine (20 μg/ml).

the CIP susceptibility of WT *S. pneumoniae* R6 (Table 4). Reserpine also altered the susceptibility levels to EtBr of WT *S. pneumoniae* R6, R6M2B, and T5-R6M2B, a phenotype that could be attributed only in part to PatA and PatB (Table 4). The gene spr0043 codes for the ATP-binding protein ComA, an ABC transporter unrelated to resistance, and was used as a negative control for ABC gene inactivation (Table 4).

Consistent with previous reports about the role of efflux in low-level CIP resistance in the absence of QRDR mutations, the resistance of T1-60827 and of its parent mutant was almost exclusively the result of reserpine-sensitive efflux (Table 4; see also Table S3 in the supplemental material). The expression of *patA* was only slightly increased in the 60827 mutant and its T1-60827 transformant compared to an *S. pneumoniae* R6 reference (Table 4). The role of *patA* and *patB* in resistance was less clear, with only the inactivation of *patB* having a minor effect on the level of CIP resistance in T1-60827 (Table 4). This lack of a clear phenotype might be explainable by the fact that overexpression of *patA* seems primordial for resistance (see above) and by the probable presence of another reserpine-sensitive efflux system (Table 4). Notwithstanding, the genome sequence of T1-60827 revealed that multigene RRS10 transferred from isolate 60827 conveyed nonsynonymous mutations in the coding regions of *patA* (spr1887) and *patB* (spr1885) in addition to an A-to-C transversion 33 nucleotides upstream of *patA* (Tables 1 and 2). To assess the role of these mutations in CIP resistance, PCR fragments of *patA* and *patB* derived from T1-60827 were cotransformed in WT *S. pneumoniae* R6 along with an *rpsL*$^+$ fragment for the selection of recombinant clones. The resulting *S. pneumoniae* transformants, R6$^{patA,T1\text{-}60827,\ smR}$ and R6$^{patB,T1\text{-}60827,\ smR}$, displayed a 2-fold increase in CIP MIC compared to that of WT *S. pneumoniae* R6 and confirmed the role for the *patA* and *patB* mutations in resistance (Table 3). While mutations in the promoter region of *patA* and *patB* were previously shown to be responsible for their increased expression in linezolid-resistant *S. pneumoniae* (40), we could not attribute a phenotype to the mutation upstream of the *patA* loci in T1-60827 by targeted transformation.

Ciprofloxacin resistance and protection against reactive oxygen species. As previously mentioned, we were intrigued that a mutation identical to the one found at position 248 of spr1902 in T5-R6M2B was also acquired by T1-60827 (Table 1) as part of RSS11 (Table 2). The same mutation could also be found in the parent clinical isolate 60827 (data not shown). The spr1902 gene product corresponds to a glycerol 3-phosphate dehydrogenase which participates in the maintenance of the intracellular redox potential by reducing NADP$^+$ to NADPH while catalyzing the conversion of sn-glycerol 3-phosphate to glycerone phosphate (45). Given that the mode of action of bactericidal antibiotics such as CIP was proposed to involve the production of reactive oxygen species (ROS) (46), we hypothesized that the basis for the selection of the mutation in spr1902 in both T5-R6M2B and strain 60827 involved protection against reactive oxygen species, hence contributing to resistance. The accumulation of ROS upon exposure to CIP according to the status of spr1902 was analyzed in an *S. pneumoniae* background of altered *parC* and *gyrA* (R6$^{parC,gyrA\text{-}R6M2B}$ and R6$^{parC,gyrA,spr1902\text{-}T5R6M2B,\ smR}$) using DCF-DA, a dye whose fluorescence intensity is indicative of the intracellular ROS levels. *S. pneumoniae* R6$^{parC,gyrA\text{-}R6M2B}$ cells subjected to 32 μg/ml of CIP (the MIC of R6$^{parC,gyrA\text{-}R6M2B}$) demonstrated a time-dependent increase in DCF-DA fluorescence signals (Fig. 1), confirming an increase in ROS production upon exposure to inhibitory CIP concentrations. In contrast, the same treatment failed to induce the accumulation of oxidants in the *S. pneumoniae* R6$^{parC,gyrA,spr1902\text{-}T5R6M2B,\ smR}$ transformant harboring the T83I substitution of T5-R6M2B or T1-60827 in spr1902 (Fig. 1). While NADPH is also produced by 6-phosphogluconate dehydrogenase (corresponding to spr0335), an enzyme catalyzing the decarboxylation of 6-phosphogluconate into ribulose 5-phosphate in the presence of NADP during the third step of the pentose phosphate pathway, the role in resistance for the mutation identified in T5-R6M2B (Table 3) could not be linked to ROS protection (data not shown).

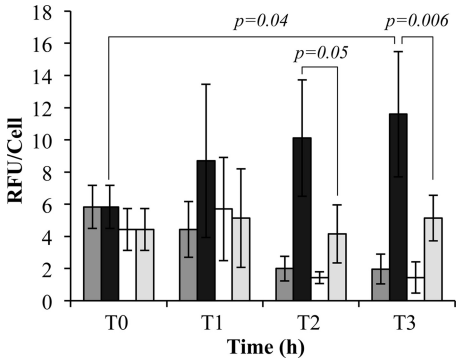

FIG 1 Mutation in spr1902 and ciprofloxacin-induced production of reactive oxygen species. Intracellular levels of reactive oxygen species in the absence of ciprofloxacin were monitored in *S. pneumoniae* R6$^{parC,gyrA\text{-}R6M2B}$ (dark gray bars) and R6$^{parC,gyrA,spr1902\text{-}T5R6M2B,\ smR}$ (white bars) by measuring DCF-DA fluorescence signals for 3 h. Ciprofloxacin-induced reactive oxygen species were monitored in *S. pneumoniae* R6$^{parC,gyrA\text{-}R6M2B}$ (black bars) and R6$^{parC,gyrA,spr1902\text{-}T5R6M2B,\ smR}$ (light gray bars) at baseline and up to 3 h after exposure to 32 μg/ml of ciprofloxacin. Results are the averages of three independent experiments. The significance of the differences in DCF-DA signal between conditions was confirmed by Student's *t* test. The level of significance was set to a *P* value of ≤0.05.

DISCUSSION

Fluoroquinolone antibiotics act by interfering with DNA topoisomerase and DNA gyrase, which ultimately leads to inhibition of DNA replication and to bacterial death (11, 12). Prior work on *S. pneumoniae* laboratory-derived CIP-resistant mutants and unsusceptible clinical isolates has underlined the major role that QRDR mutations have in resistance in addition to highlighting efflux proteins as contributors to resistance (21, 28). Genetic analyses of the basis of resistance found that conventional QRDR mutations usually target positions 79 and 83 of ParC and positions 81 and 85 of GyrA (47–49). Nonetheless, resistance appears to be heterogeneous, since mutations at other QRDR sites also decreased susceptibility to fluoroquinolones (22, 50). The effect of specific mutations on the level of resistance depends on the fluoroquinolone molecule, however, and the combination of amino acid changes within the four QRDR genes probably has more impact than the number of mutations (22). In this study, the genome sequences of T5-R6M2B and of its parent mutant revealed mutations in QRDRs of both *parC* and *gyrA*. The Ser79Phe substitution in ParC was transferred early into the WT *S. pneumoniae* R6 recipients, at the first step of transformation, while the Glu85Lys change in GyrA occurred during the second round of transformation (Table 1). This is consistent with mutations in *parC* usually preceding those in *gyrA* during the selection for CIP resistance (25). Through

targeted transformation of mutated PCR fragments, a role in CIP resistance was further confirmed for both mutations, the ParC Ser79Phe conferring low-level resistance (CIP MIC, 2 μg/ml), while the GyrA Glu85Lys led to higher resistance levels in the presence of an altered ParC (CIP MIC, 32 μg/ml). This is in agreement with previous work having shown that mutations in both genes were necessary for higher levels of fluoroquinolone resistance (18, 19, 24). Mutations affecting the Glu85 of GyrA have previously been observed in *S. pneumoniae* isolates unsusceptible to CIP and levofloxacin from the United States and Canada (22, 29, 48, 49, 51) and were associated with a decreased susceptibility to fluoroquinolones in the presence of other QRDR mutations (52).

CIP resistance in the *S. pneumoniae* R6M2B mutant and in the clinical isolate 60827 was reversible by reserpine, suggesting that efflux may be implicated. The gene coding for the ABC transporter PatA is overexpressed in R6M2B and its transformant (Table 4). No mutations in the promoter region of *patA-patB* could explain this overexpression, and the mechanism of overexpression remains unknown. Whatever the mechanism, it is intriguing that it is transferable by transformation, and work is now ongoing to look into this. It is salient to point out that this phenomenon is likely to have clinical implications, given that a similar lack of mutations upstream of *patA* or its *patB* partner was reported for *S. pneumoniae* clinical isolates resistant to fluoroquinolones with overexpression of *patA* and *patB* (30). Inactivation of *patA* and *patB* in R6M2B fully accounted for the reserpine-reversible CIP resistance. The inactivation of *patA* and *patB* also confirmed their role in resistance to EtBr, although in this case an additional reserpine-sensitive efflux pump is also likely to intervene, since the sensitization to EtBr conferred by reserpine was higher than that observed in the absence of functional *patA* or *patB*. The T1-60827 transformant acquired a multigene RSS derived from clinical isolate 60827 that conveyed nonsynonymous mutations on the *patA* and *patB* loci, and a 2-fold increase in resistance to CIP was conferred by these mutations when transformed into a WT background (Table 3). While increased expression of *patA* and *patB* has been shown to lead to resistance, this is the first example that point mutations can also lead to CIP resistance. The inactivation of *patA* and *patB* had only a minor effect on the level of CIP resistance in strain 60827, however, and susceptibility assays in the presence of reserpine revealed that another efflux system may also be implicated in resistance (Table 4). This efflux system was probably transferred from the clinical isolate 60827 to the transformants during the DNA transformation process, which suggested that it may be encoded by one of the 15 RSSs present in T1-60827. While clearly enhancing resistance to CIP, it is possible that the contribution of the mutations in the coding regions of *patA* and *patB* is masked in the clinical isolate by the presence of this additional reserpine efflux system. The single mutation in PatA (R112L) is predicted to be located in the first cytoplasmic loop located between transmembrane helices 2 and 3. The crystal structure of the related Sav1866 ABC protein purified from *Staphylococcus aureus* indicated that the first intracellular loop is important for the activity of the protein by participating in conformational changes upon ATP binding and hydrolysis that are transmitted from the nucleotide-binding domain to the transmembrane domain through noncovalent interactions at the shared interface (53). For PatB, the S48A substitution is predicted to occur in the first transmembrane helix, while the A503T change is located in the C-terminal hydrophilic domain of the protein. Unfortunately, the lack of crystal structures for PatA and PatB precludes the precise determination of the role of the PatA and PatB mutations in resistance.

Bactericidal antibiotics, regardless of their primary targets, are thought to kill bacteria by inducing alterations in iron homeostasis which ultimately lead to the accumulation of hydroxyl radicals through the Fenton reaction (46). In that context, nonenzymatic antioxidant molecules such as NADPH can help maintain a reduced intracellular environment by scavenging ROS (54). Two of the enzymes mutated in our mutants or transformants (corresponding to spr0335 and spr1902) are responsible for the reduction of $NADP^+$ and should affect the pool of intracellular NADPH (45). Although the effect of the mutation on the activity of the protein remains unclear, ROS accumulation assay indicated that the T83I substitution in the glycerol-3-phosphate dehydrogenase gene spr1902 prevents the accumulation of ROS after exposure to CIP. Similarly to a previously described mutation in a putative iron importer that protected penicillin-resistant *S. pneumoniae* against the antibiotic-induced production of ROS (38), the T83I substitution in the spr1902 product appeared early during the acquisition of CIP resistance. This suggests that monitoring for similar mutations in *S. pneumoniae* clinical isolates might become warranted since these may possibly favor the acquisition of additional mutations (e.g., in QRDRs) by providing an early survival benefit against one of the main modes of action of bactericidal antibiotics. In contrast, the role of the spr0335 product V416L substitution in CIP resistance appears to be unrelated to ROS.

In conclusion, resistance reconstruction by whole genomic DNA transformation combined with WGS had previously proved useful for pinpointing known and new mutations implicated in resistance to β-lactams (38) and linezolid (40) in *S. pneumoniae*, and we have now applied this approach to CIP resistance. In addition to confirming the role of efflux and of QRDR mutations in resistance, we provide new knowledge into the more subtle but relevant roles for mutations in drug transporters and in redox enzymes. As these could prove important in facilitating resistance, it will be interesting to assess their prevalence in additional *S. pneumoniae* isolates resistant to fluoroquinolones.

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