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Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*

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Background: Active efflux is a common mechanism of resistance to fluoroquinolones in *Streptococcus pneumoniae*. Two efflux systems have been described so far in this species: PmrA, a member of the major facilitator superfamily; and the two ABC transporters PatA and PatB. We studied the inducibility of expression of *pmrA*, *patA* and *patB* by using subinhibitory concentrations of fluoroquinolones.

Methods: A wild-type susceptible strain, two clinical isolates resistant to fluoroquinolones and two efflux mutants selected *in vitro* after exposure to ciprofloxacin were studied. MICs were determined for these strains and their mutants in which *pmrA*, *patA* or *patB* had been disrupted. Gene expression was determined after exposure to half the MIC of norfloxacin, ciprofloxacin, levofloxacin, moxifloxacin or gemifloxacin and quantified by real-time PCR.

Results: Increased MICs of norfloxacin, ciprofloxacin and levofloxacin (to a lesser extent) and increased expression of *patA* and *patB* were seen for all resistant strains; these were reduced in *patA* or *patB* disruptants or in the presence of reserpine. Exposure to any of the five fluoroquinolones caused a reversible increase in expression of *patA* and *patB*, but not of *pmrA*. Mitomycin C, an inducer of the competence system in *S. pneumoniae*, also induced *patA* and *patB* expression in the two strains tested.

Conclusion: The ABC efflux system PatA/PatB is induced upon exposure to subinhibitory concentrations of fluoroquinolones, whether substrates of the transporter or not. This effect, possibly resulting from the activation of the competence pathway, may contribute to resistance.

Keywords: resistance, induction, ABC transporters, DNA damaging agents

Introduction

Streptococcus pneumoniae is a leading cause of respiratory tract infections, including community-acquired pneumonia (CAP).[1,2] The so-called respiratory fluoroquinolones (levofloxacin, moxifloxacin and gemifloxacin)[3] are active in the treatment of CAP.[1,2] However, the use of levofloxacin has been associated with a decrease in bacterial susceptibility and subsequent clinical failures.[4] High-level fluoroquinolone resistance is mainly due to mutations in structural genes for the GyrA subunit of DNA gyrase and for the ParC subunit of topoisomerase IV.[5] However, there is increasing evidence that active efflux can play an important role in decreasing the susceptibility of the isolates,[6–8] with ciprofloxacin and norfloxacin often being used as reporter antibiotics in this context.

PmrA, a member of the major facilitator superfamily (MFS), was the first efflux pump shown to confer resistance to norfloxacin and ciprofloxacin.[9] More recently, an efflux system belonging to the ATP binding cassette (ABC) superfamily and composed of two transporters encoded by *patA* (SP2075) and *patB* (SP2073) was identified.[10,11] Expression of these genes was increased in strains with decreased susceptibility to fluoroquinolones,[7,12,13] and induced by ciprofloxacin or norfloxacin.[10,14] These studies, carried out with a reference strain and derivative mutants, were limited to fluoroquinolones that are substrates for this efflux system. In the present study, we have

compared induction of expression of *pmrA*, *patA* and *patB* by five fluoroquinolones (putative good or poor substrates) in various strains including clinical isolates. Because fluoroquinolones and the DNA-damaging agent mitomycin C can induce a competence pathway and chromosomal transformation in *S. pneumoniae*,[15,16] we examined whether mitomycin C was able to induce *patA* and *patB* expression as part of a global stress response. In a nutshell, we report that: (i) all fluoroquinolones can induce *patA* and *patB* expression in a concentration-dependent manner; (ii) the extent of overexpression depends on the strain rather than on the inducer; and (iii) mitomycin C is able to trigger overexpression of *patA* and *patB*, confirming that this efflux system is part of a general stress response related to DNA damage.[14]

Materials and methods

Bacterial strains and growth conditions

The five strains studied were: (i) the reference *S. pneumoniae* ATCC 49619; (ii) two laboratory mutants (SP334, derived from *S. pneumoniae* ATCC 49619, and SP335, derived from the clinical isolate SP32, selected after 13 days of exposure to ciprofloxacin[12]); and (iii) two clinical isolates (SP295 and SP13) (see Table 1). Cultures were performed at 37°C in a 5% CO_2 atmosphere using Todd-Hewitt broth supplemented with 1% yeast extract (THY; BD, Franklin Lakes, NJ, USA) or Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (International Medical Products, Brussels, Belgium).

Determination of MICs

MICs of fluoroquinolones and of ethidium bromide and acriflavine (two well-known substrates for efflux pumps) were determined by the serial 2-fold macrodilution method in Mueller-Hinton agar supplemented with 5% defibrinated horse blood, with an inoculum of $\sim 10^5$ bacteria per spot.[12,17] The efflux inhibitor reserpine was used at a final concentration of 20 mg/L.[18]

DNA techniques

Chromosomal DNA was purified with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Plasmid DNA was prepared using the Plasmid Midi Preps Kit (Qiagen) and transformed into *Escherichia coli*.[19] Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA). Blunt-ending of restricted plasmid DNA was performed by the addition of 1 U of Klenow enzyme (New England Biolabs) and 33 μM deoxynucleoside triphosphates to the reaction mixture at the end of enzymatic digestion. Restriction fragments were purified from agarose gels with the QIAquick Gel Extraction Kit (Qiagen). PCR amplifications were performed according to the manufacturer's protocol for BIOTAQ Red DNA polymerase (Gentaur, Kampenhout, Belgium). The sequences of the primers used are shown in Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

Quantitative real-time PCR

S. pneumoniae was grown overnight at 37°C in a 5% CO_2 atmosphere on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Bacteria were resuspended in 15 mL of THY medium supplemented or not with inducers at an optical density (620 nm) of 0.2–0.4. For induction, bacteria were grown for up to 6 h at 37°C in 5% CO_2. For experiments examining the reversal of induction, bacteria were harvested by centrifugation (3000 **g** for 10 min) after 4 h of culture in the presence of antibiotic at half the MIC, washed once at room temperature in drug-free medium and centrifuged, and the pellet was then resuspended in THY drug-free medium and cultured for up to 5 h. Bacteria were harvested by centrifugation (5000 **g** for 5 min at 4°C) and the pellets were frozen and kept at −80°C for at least 30 min before nucleic acid extraction. Total RNA extraction and reverse transcription were performed as previously described.[12] Real-time PCR was performed in an iQ cycler (Bio-Rad Laboratories, Hercules, CA, USA) in 25 μL reaction mixtures containing 12.5 μL of iQ SYBR Green Supermix (2×), 400 nM of forward and reverse primers and 5 μL of cDNA in RNase/DNase-free water. The *rpoD* and *proC* genes were used as references to normalize transcript levels, as specified by PrimerDesign (Southampton, UK).

Inactivation of patA, patB and pmrA genes

To inactivate *patA* or *patB*, the strains were transformed with genomic DNA of M246 or M240 strains, which have a *magellan2* minitransposon inserted in either *patA* or *patB*.[13] Transformants were selected on Mueller-Hinton agar containing 5% defibrinated sheep blood supplemented with 100 mg/L spectinomycin. Gene inactivation was verified by PCR.[13] To inactivate *pmrA*, a 1 kb BamHI-KpnI PCR fragment, amplified with the PmrARec-F and PmrARec-R primers (Table S1) and carrying the *pmrA* gene, was cloned in BamHI-KpnI-restricted pUC18.[20] The plasmid was then cleaved in the insert by ClaI and blunt-ended with Klenow enzyme. The *aad9* gene of *magellan2* conferring resistance to spectinomycin[21] was amplified by PCR with the Spec-1 and Spec-2 primers (Table S1), ligated with the linearized plasmid to generate pUC18Ω*pmrA*::*spt* and transformed into *S. pneumoniae* strain R6. Inactivation of *pmrA* was confirmed by PCR using the PmrA-Delta-F and PmrA-Delta-R primers.

Quinolone resistance-determining region (QRDR) sequencing

The QRDRs of *gyrA*, *parC* and *parE* were amplified and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a Genetic Analyzer 3100 (Applied Biosystems) as previously described.[12]

Antibiotics, other substrates and pump inhibitor

Levofloxacin, moxifloxacin and gemifloxacin were obtained as microbiological standards from Aventis Pharma (Antony, France), Bayer HealthCare (Leverkusen, Germany) and LG Life Sciences (Seoul, Korea), respectively. Other antibiotics, substrates or inducers were obtained as pure substances from Sigma–Aldrich (St Louis, MO, USA).

Results

Antibiotic susceptibility of the strains

Table 1 summarizes the MICs of the five fluoroquinolones and of acriflavine and ethidium bromide, determined in the absence or presence of reserpine. The MICs of norfloxacin, ciprofloxacin, acriflavine and ethidium bromide were significantly (≥2 dilutions; 1 dilution for acriflavin and SP334) higher for SP334, SP335, SP295 and SP13 than for *S. pneumoniae* ATCC 49619. The MICs of levofloxacin and gemifloxacin were significantly higher in SP334, SP335 and SP13 [reaching or exceeding the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical resistance breakpoint for levofloxacin]. Moxifloxacin MIC was increased by two dilutions only against SP335 (but remained below the EUCAST resistance breakpoint).

Table 1. Susceptibility of *S. pneumoniae* to fluoroquinolones and substrates of efflux pumps in the absence (−R) or presence (+R) of reserpine (20 mg/L)

Strains	Relevant characteristics[a]	Mutations in QRDR	MIC (mg/L) of: norfloxacin		ciprofloxacin				levofloxacin		moxifloxacin				gemifloxacin		Reference or source
			acriflavine		ethidium bromide												
−R	+R	−R	+R		−R	+R	−R	+R	−R	+R	−R	+R	−R	+R			
ATCC 49619	wild-type	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	2	1	0.5	0.125	LGC Standards
ATCC 49619*patA*	ATCC 49619 *patA*::*magellan2*, SPT^R	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.016	1	0.5	0.25	0.125	this study
ATCC 49619*patB*	ATCC 49619 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
ATCC 49619*pmrA*	ATCC 49619 *pmrA*::*magellan2*, SPT^R	none	4	2	1	0.5	0.5	0.5	0.125	0.125	0.031	0.031	4	1	1	0.125	this study
SP334	ATCC 49619 after 13 days of exposure to ciprofloxacin, CIP^R	none	**32**	4	**4**	0.5	**2**	1	0.25	0.25	**0.125**	0.031	4	1	**4**	0.125	12
SP334*patA*	SP334 *patA*::*magellan2*, SPT^R	none	4	4	1	0.5	1	1	0.125	0.125	0.063	0.031	1	1	0.25	0.125	this study
SP334*patB*	SP334 *patB*::*magellan2*, SPT^R	none	8	4	1	1	1	1	0.25	0.25	0.063	0.063	1	1	0.125	0.125	this study
SP334*pmrA*	SP334 *pmrA*::*aad9*, SPT^R	none	**32**	4	**4**	0.5	1	0.5	0.25	0.25	**0.125**	0.063	4	0.5	**2**	0.125	this study
SP335	clinical strain SP32 after 13 days of exposure to ciprofloxacin, CIP^R	ParE (Ile460Val)	**64**	8	**32**	2	**4**	2	**0.5**	0.25	**0.5**	0.125	**16**	1	**8**	0.25	12
SP335*patA*	SP335 *patA*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
SP335*patB*	SP335 *patB*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP335*pmrA*	SP335 *pmrA*::*aad9*, SPT^R	ParE (Ile460Val)	**64**	4	**8**	0.5	1	0.5	0.25	0.125	0.063	0.016	**8**	0.5	**8**	0.125	this study
SP295	clinical isolate[b]	none	**16**	2	**2**	0.5	1	0.5	0.125	0.125	0.063	0.031	**16**	1	**16**	0.25	this study
SP295*patA*	SP295 *patA*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP295*patB*	SP295 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.016	0.016	1	1	0.25	0.25	this study
SP295*pmrA*	SP295 *pmrA*::*aad9*, SPT^R	none	8	4	1	0.5	1	0.5	0.25	0.125	0.063	0.031	**8**	1	**4**	0.25	this study
SP13	clinical isolate[c]	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**256**	16	**16**	4	**2**	1	0.25	0.25	**0.25**	0.063	**16**	1	**16**	0.25	this study
SP13*patA*	SP13 *patA*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	1	1	1	0.25	0.25	0.063	0.063	1	0.5	0.25	0.125	this study
SP13*patB*	SP13 *patB*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	2	**2**	1	0.25	0.25	0.063	0.063	2	1	0.25	0.25	this study
SP13*pmrA*	SP13 *pmrA*::*aad9*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**128**	16	**16**	2	**2**	1	**0.5**	0.25	**0.125**	0.063	**8**	0.5	**8**	0.25	this study

EUCAST breakpoints for resistance: ciprofloxacin and levofloxacin, >2 mg/L; and moxifloxacin, >0.5 mg/L (no values for norfloxacin and gemifloxacin). Figures in bold indicate MICs at least two dilutions higher than those of wild-type *S. pneumoniae* ATCC 49619.

[a]CIP^R, resistance to ciprofloxacin; SPT^R, resistance to spectinomycin.

[b]Isolated from blood culture (Cliniques Universitaires St Luc, Brussels).

[c]Isolated from expectoration (Universitair Ziekenhuis Brussel, Brussels).

In the presence of reserpine, the MICs of acriflavine and ethidium bromide were similar to those for the wild-type strain, suggesting an efflux mechanism in the four strains. For fluoroquinolones, restoration of wild-type MICs by reserpine was complete for SP334 and SP295, but only partial for SP335 and SP13, which have mutations in the QRDR (Table 1).

Role of PmrA, PatA and PatB in antibiotic resistance

The expression of *pmrA*, *patA* and *patB* was quantified by real-time PCR in all strains (Figure 1). As compared with *S. pneumoniae* ATCC 49619, the four resistant strains overexpressed *patA* and *patB* to levels ranging from 4.4-fold for *patA* in SP334 to 13.6-fold for *patB* in SP13. In contrast, only SP335 and SP13 showed modest overexpression of *pmrA*.

Every gene was inactivated in each of the five strains, and the MICs for the disruptants were determined (Table 1). For all strains, inactivation of either *patA* or *patB* reduced the MIC of acriflavine and ethidium bromide to a value similar to that for *S. pneumoniae* ATCC 49619 in the presence of reserpine. Likewise, the MICs of fluoroquinolones for *patA*- or *patB*-inactivated strains were reduced (±1 dilution) to those measured for the corresponding parental strain in the presence of reserpine (or even lower for SP335). In contrast, inactivation of *pmrA* did not cause a marked decrease in MICs (0 to 1 dilution).

Induction of patA, patB or pmrA expression by fluoroquinolones

The expression of these genes was then measured in bacteria grown for 4 h in the presence of fluoroquinolones at half their MIC (preliminary experiments with ciprofloxacin showed that this concentration caused the maximal effect; see Figure S1, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org). All fluoroquinolones were potent inducers of *patA* and *patB* in strains SP335 and SP295, but showed a lower effect in strains ATCC 49619 and SP334 and no effect for norfloxacin and levofloxacin in strain SP13 (Figure 2). In contrast, the expression of *pmrA* remained unaffected or even decreased upon exposure to fluoroquinolones. Specificity of induction was tested with tetracycline and chloramphenicol under the same conditions, but no change in the expression of *patA*, *patB* or *pmrA* was observed (data not shown).

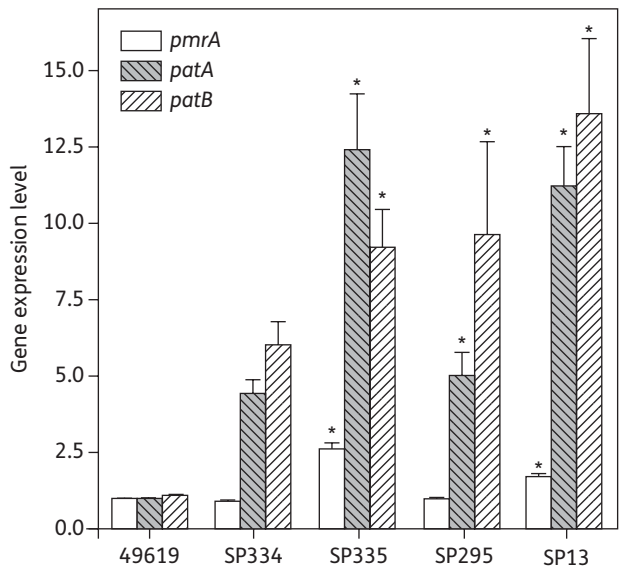

Figure 1. Expression levels of *patA*, *patB* and *pmrA* in non-induced *S. pneumoniae*. Data are expressed as the ratio to the value in *S. pneumoniae* ATCC 49619. Values are the means±SEM of duplicates from five independent experiments. *$P<0.05$ (one-way ANOVA with Dunnett's *post hoc* test for comparison with *S. pneumoniae* ATCC 49619).

Kinetics of induction

To follow the kinetics of induction of *patA* and *patB* and the time needed to revert to basal level, strains ATCC 49619 and SP335 were used as they showed a low and high basal level of *patA*/*patB* expression, respectively. Ciprofloxacin and moxifloxacin were selected as substrate and non-substrate (Figure 3). In both strains, a lag phase of ~30–40 min was observed during which no change in expression level was observed, followed by increased expression levels over time. Reduction in expression was detected as soon as the drugs were removed and reversal to original pre-exposure levels was obtained after 3–4 h. To test if changes in *patA* and *patB* expression over time did not result from growth variations, expression of the genes under non-inducing conditions in bacteria from the exponential to the stationary phase was measured and no differences were seen. Conversely, there was no change in optical density over the 6 h of induction, indicating absence of significant growth over the time frame of the experiment (see Figure S2, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org/).

Induction of patA/patB and of the competence regulon by mitomycin C

DNA-damaging agents or antibiotics are capable of inducing the SOS response,[22] or a competence pathway in bacteria devoid of an SOS system, such as *S. pneumoniae*.[15,16,23] We therefore examined whether mitomycin C, a DNA-damaging agent known to induce competence in *S. pneumoniae*,[15,16] was also able to induce expression of *patA* and *patB*. In parallel, we quantified the expression levels of two genes involved in competence via the *com* regulon,[16] namely *recA*[24] and *ssbB*.[25,26] The expression of these genes upon induction by ciprofloxacin or mitomycin C was largely parallel to that of *patA* and *patB*, with a correlation coefficient of 0.879 and 0.897 for *ssbB* and *recA* versus *patA* and *patB*, respectively (see Figure S3, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org/).

Discussion

Two important observations were made. Our study shows that PatA and PatB play a major role in fluoroquinolone resistance in the two clinical isolates and the two *in vitro* mutants examined here, while PmrA does not, confirming the data of Piddock *et al.*[27] and Garvey and Piddock.[13] Inactivation of either *patA* or *patB* restored full susceptibility to ethidium bromide or acriflavine in the four strains, or to fluoroquinolones in those strains that did not harbour mutations in the genes encoding the target proteins.

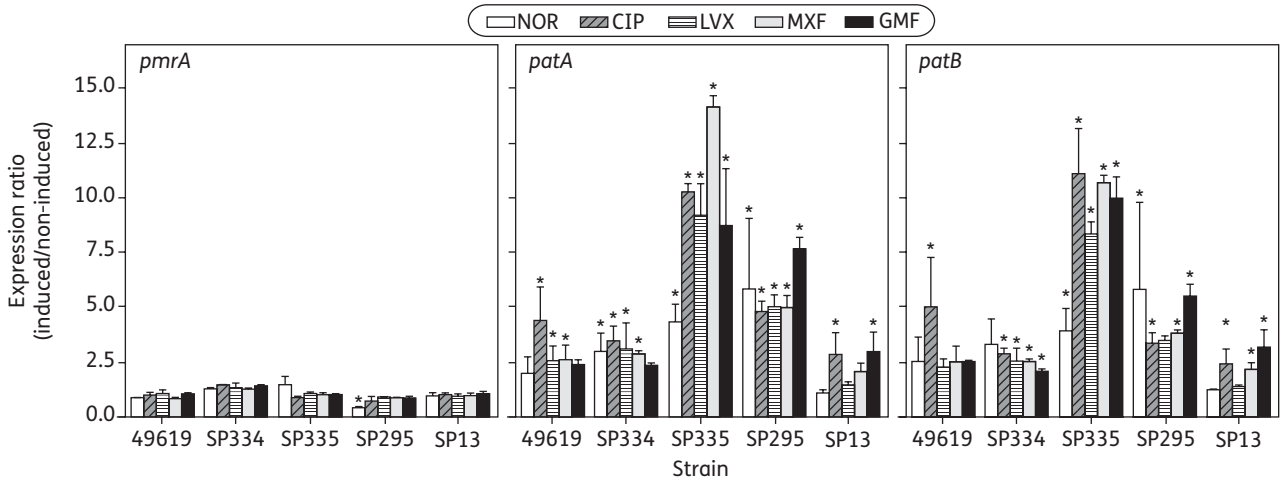

Figure 2. Induction of *pmrA*, *patA* and *patB* expression in *S. pneumoniae* exposed for 4 h to half the MIC of various fluoroquinolones. Data are presented as ratios of expression measured under induced and non-induced conditions. Values are the means ± SEM of duplicates from two independent experiments. *$P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test for comparison with the non-induced condition). NOR, norfloxacin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GMF, gemifloxacin.

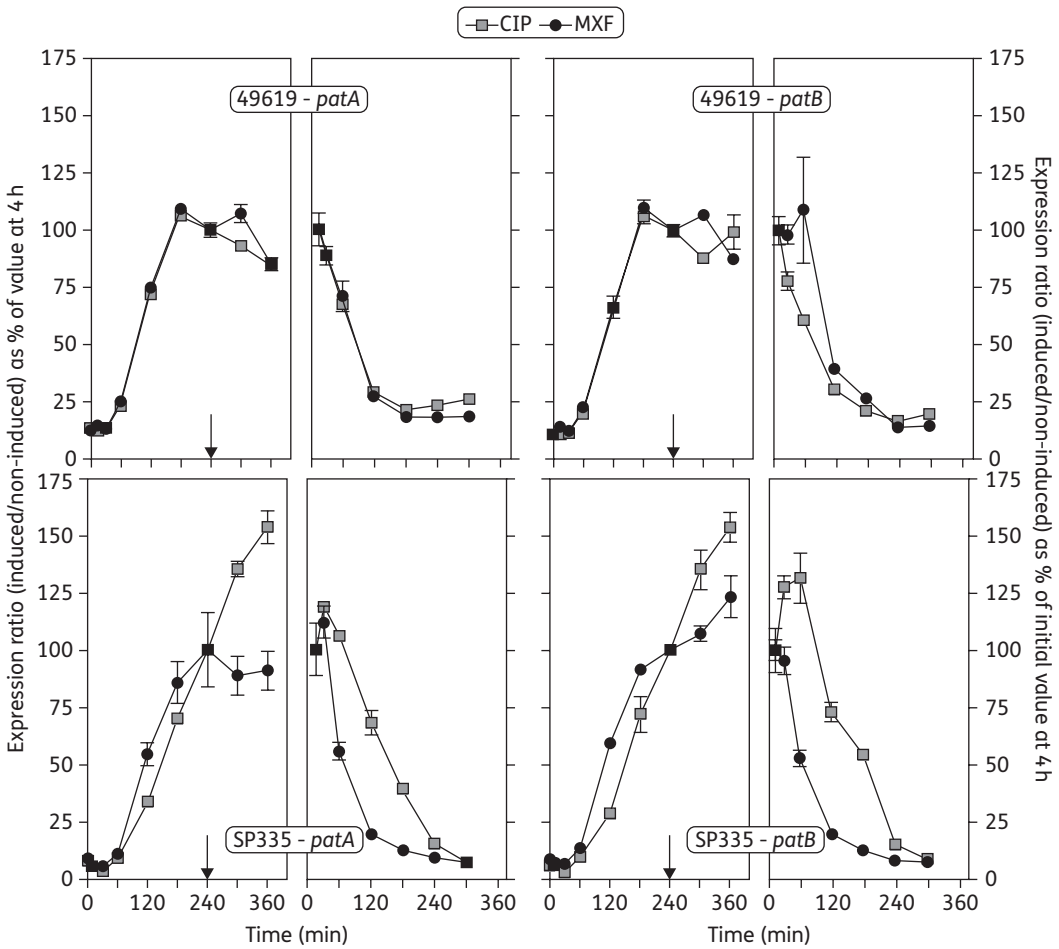

Figure 3. Kinetics of induction and deinduction of *patA* and *patB* expression by ciprofloxacin and moxifloxacin in *S. pneumoniae* ATCC 49619 (top) and SP335 (bottom). Bacteria were induced by exposure to half the MIC of ciprofloxacin or moxifloxacin over 6 h. For reversion, bacteria induced for 4 h were harvested and regrown in broth without antibiotic for 5 h. Data are presented as ratios of *patA* (left) or *patB* (right) expression measured under induced and non-induced conditions for each strain as a percentage of the value at 4 h (staring point of reversion, as indicated by the arrows). Values are the means ± SEM of duplicates from two independent experiments. CIP, ciprofloxacin; MXF, moxifloxacin.

In contrast, *pmrA* inactivation had only a marginal effect, in agreement with other studies.[7,11,28]

First, resistance mediated by PatA/PatB did not affect all fluoroquinolones to the same extent, with norfloxacin being the most affected, followed by ciprofloxacin, gemifloxacin, levofloxacin and finally moxifloxacin. This is in agreement with a previous ranking established for fluoroquinolone susceptibility to efflux in *S. pneumoniae*[6,29,30] or in *S. aureus*,[31] suggesting that hydrophilic molecules are better substrates. We extend here this observation to other fluoroquinolones, but show that moxifloxacin, the most lipophilic molecule among those tested here, was little affected by the overexpression of PatA/PatB. Interestingly this ranking seems to apply to efflux pumps of the ABC superfamily (like PatA/PatB) as well as to those of the MFS superfamily (such as NorA in *S. aureus*). This observation may suggest that common molecular or physicochemical determinants in substrates are recognized by non-phylogenetically related transporters.

Second, inactivation of either *patA* or *patB* is sufficient to restore full susceptibility to fluoroquinolones and no phenotypic discrepancies are observed between *patA* and *patB* knockouts with respect to fluoroquinolone, acriflavine or ethidium susceptibility. Together with the facts that (i) homologues of PatA and PatB appear as pairs of proteins working together[13] and (ii) predictions of topologies for PatA and PatB propose four to seven transmembrane segments for each of these proteins [using either TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) or SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui)], these observations suggest that the two proteins may constitute a heterodimeric ABC-type multidrug transporter[13,32] or, at least, a need to interact to confer fluoroquinolone resistance.[10]

When bacteria are exposed to antibiotics, dyes, solvents or detergents, they can adapt by inducing the expression of efflux systems.[33,34] It has been shown that *patA* and *patB* expression of a wild-type *S. pneumoniae* and of an *in vitro* resistant mutant thereof is inducible upon exposure to norfloxacin or ciprofloxacin.[10,14] This observation is extended here by showing that induction is obtained (i) for all fluoroquinolones tested, whether substrates of PatA/PatB or not, and (ii) not only in a wild-type strain, but also in *in vitro* mutants and in clinical isolates overexpressing *patA* and *patB* under non-inducing conditions. Increase in expression develops rapidly, irrespective of the fluoroquinolone used, and is fully reversible. Because induction seems specific to fluoroquinolones, is observed even in strains with pre-existing high basal efflux expression and is observed with inducers that are or are not substrates, it is tempting to speculate that overexpression is the consequence of a change in global regulatory responses induced by fluoroquinolones.

Regulation of ABC-type efflux transporters involves local regulators, repressors or activators, as well as global transcriptional regulators.[32–34] Yet the regulators of *patA* and *patB* expression are unknown. A microarray analysis showed that exposure of *S. pneumoniae* to ciprofloxacin induces the expression of genes involved in the competence pathway, mismatch repair system or replication.[14] We found here a coexpression of *patA* and *patB* and of two genes of the competence pathway[15,16,23] upon exposure to ciprofloxacin or the DNA-damaging agent mitomycin C. This strongly suggests that the overexpression of *patA* and *patB* observed upon induction by fluoroquinolones is not only dependent upon local regulators, but is also part of a global response related to the stress imposed by their interaction with DNA.[22,35]

The data presented here may have important implications for the clinical use of fluoroquinolones. Induction of *patA* and *patB* expression by subinhibitory concentrations of any fluoroquinolone may contribute to increased levels of resistance to the molecules of the class that are substrates for efflux. As MICs may remain below or at the limit of the susceptibility breakpoint for the more potent fluoroquinolones, this highlights the usefulness of antibiotics like norfloxacin or ciprofloxacin in laboratory screens and/or for identifying resistance mechanisms at the molecular level. This inducible character also compromises the potential importance of efflux inhibitors that would act as competitive substrates, as illustrated by the cross-resistance to reserpine observed in a strain overexpressing *patA*.[13]

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1–S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Table S1. Primers used in this study

Primers	Sequence (5'-3') (endonuclease)[a]	Source or reference
Gene expression		
rpoD-F	CAGGTAGCAGAATTTATCCGTAATC	PrimerDesign Ltd[b]
rpoD-R	CCCATCAGCGTCCAAGGTA	PrimerDesign Ltd[b]
proC-F	TTATCCCAAGTCAACACCGAAT	PrimerDesign Ltd[b]
proC-R	GCAATTAGGAGACAAGGCATAAC	PrimerDesign Ltd[b]
pmrA-S	TCCAGTATGGGCTTTTCCAG	[1]
pmrA-AS	CCAATCCAAAGAGGAAACGA	[1]
patA-F	TCCTGATGACAGGCTTGATG	This study
patA-R	TGCGAGGACAACATTGAGTC	This study
patB-F	ATGGCAAAGCCTATCAGGAA	This study
patB-R	AGGATATCGCCATCTTGTCG	This study
recA-2-F	CTCATCATACGAGCCTGCAA	This study
recA-2-R	GTCTTGAGATTGCGGGAAAA	This study
ssbB-2-F	AAAGACCAAAACGGTGAACG	This study
ssbB-2-R	TACGCAATTCTCCATCAACG	This study
Sequencing		
PNC10	TGGGTTGAAGCCGGTTCA	[2]
PNC11	CAAGACCGTTGGTTCTTTC	[2]
SPPARE7	CCAATCTAAGAATCCTG	[3]
SPPARE8	GCAATATAGACATGACC	[3]
gyrA-S	CCTGTTCACCGTCGCATTCT	[1]
gyrA-AS	AGTTGCTCCATTAACCA	[1]
Gene inactivation		
PmrARec-F	CTCGGATCCGCATTGCCTGGTTTGGTAAT (BamHI)	This study
PmrARec-R	CTCGGTACCCACAAAGGCTTGTCGCATAA (KpnI)	This study
Spec-1	CTCGCGGCCGCCCCCGGTCTGACACATAGAT (NotI)	This study
Spec-2	CTCAGATCTTCCCCGGATCTAACAAAGAA (BglII)	This study
PmrA-Delta-F	CCTTCTTGAGGGAGGTAGGC	This study
PmrA-Delta-R	TGGATTGGTTTTTGGTTGGT	This study

[a] Restriction sites introduced in primers are underlined and the corresponding endonuclease indicated in parentheses. Amplification reactions were conducted at 61°C, 50°C, 54°C for gene inactivation, sequencing experiments and gene expression experiments, respectively.

[b] primers designed by this company (http://www.primerdesign.co.uk/research_with_integrity.html)

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Figure S1. Induction of *pmrA*, *patA,* and *patB* expression in *S. pneumoniae* exposed for 4 h to increasing concentrations of ciprofloxacin. Data are presented as the ratios of gene expression in every strain grown with and without inducer. Values are the mean ± SEM of duplicates from 2 independent experiments. Statistical analysis for the global effect of concentration on gene expression levels (Friedman test, one-way paired ANOVA, with Dunnett's post-hoc test for comparison with non-induced condition): p-value = 0.012 for *patA* and 0.001 p*atB*, and 0.2096 (NS) for *pmrA*, with p<0.05 for 1/4 and 1/2 MIC for *patA* and for 1/2 MIC for *patB* vs. non induced conditions.

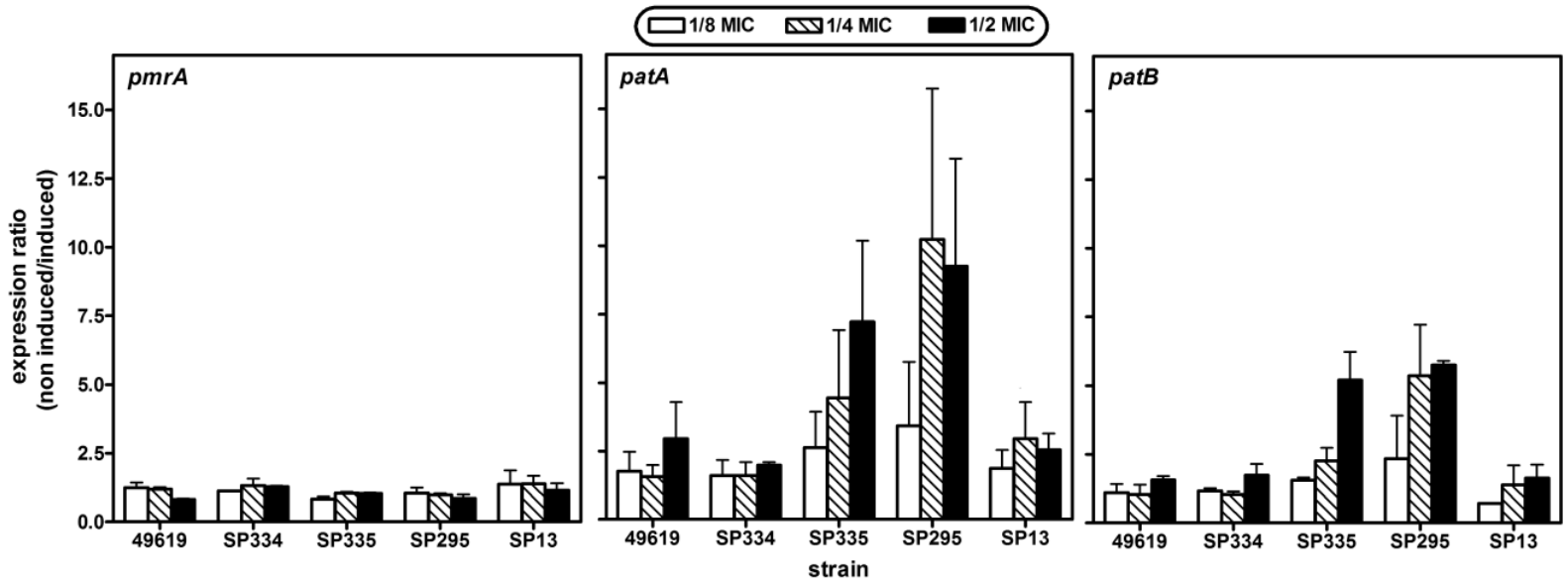


Figure S2. Evolution over time of $OD_{620\ nm}$ (left axes) and of *patA* and *patB* expression (right axes) in non induced (left panels) and induced (right panels; 1/2 x MIC of ciprofloxacin) *S. pneumoniae* ATCC49619 (top) and SP335 (bottom). Values are the means ± SEM of duplicates from 2 independent experiments.

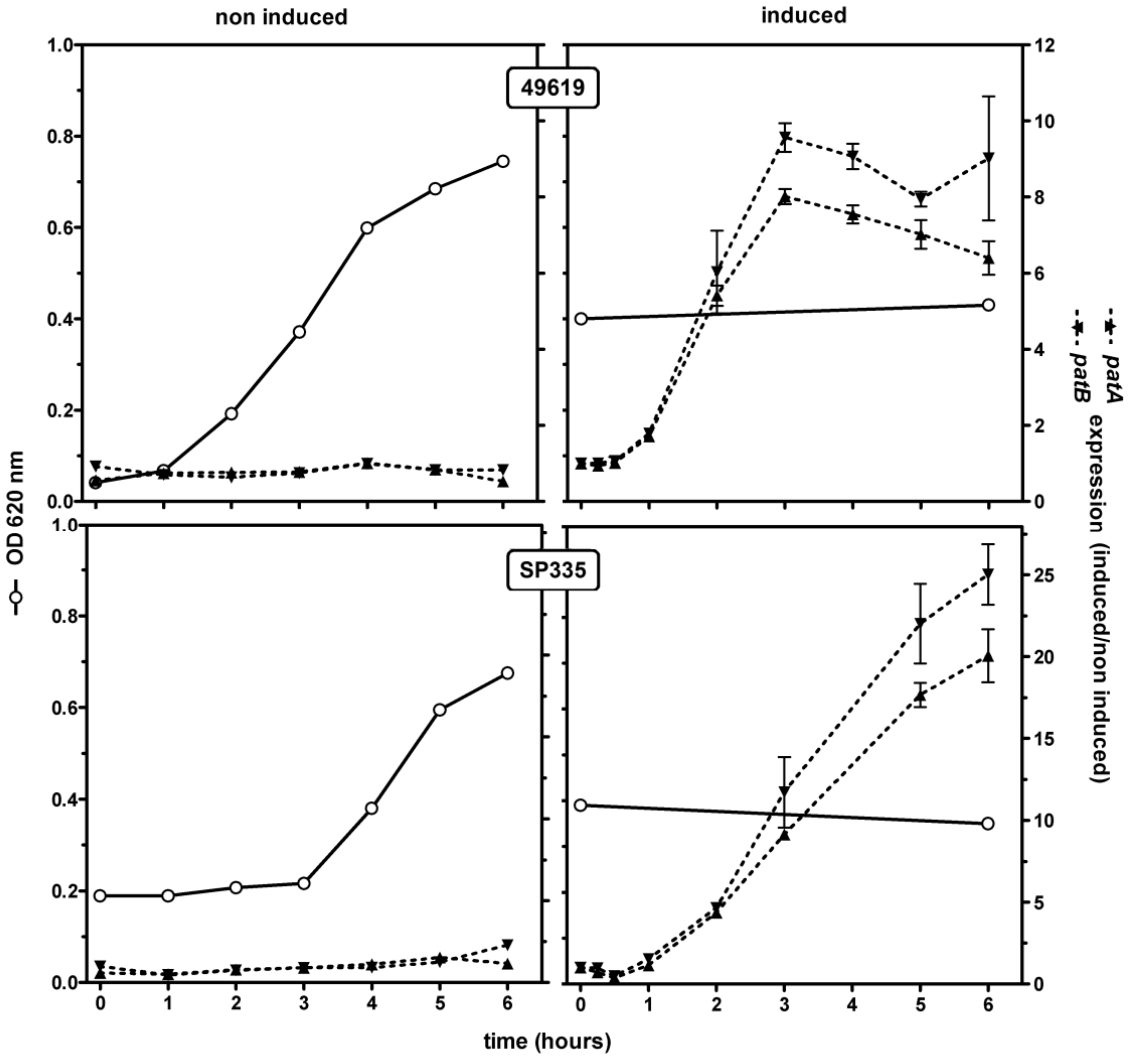
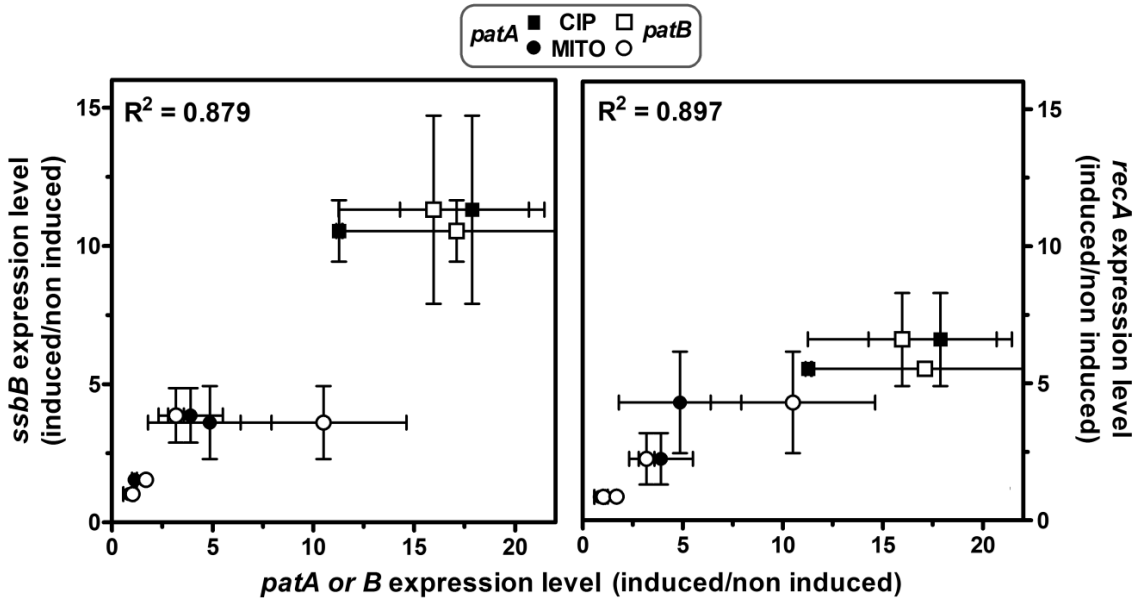


Figure S3. Relation between induction of *patA* and *patB* and of *ssbB* (left panel) and *recA* (right panel) in *S. pneumoniae* SP335 exposed for 4 h to ciprofloxacin (0.5 or 1x MIC ; higher concentrations could not be tested because of an intense bactericidal activity) or mitomycin C (0.5, 1, 10, or 100 x MIC). The data are presented as the ratios of expression measured for each strain grown in induced and non-induced conditions. Values are the means ± SEM of duplicates from 2 independent experiments. No change in the expression of the housekeeping genes was noticed, excluding a non specific effect. Correlation coefficients are calculated from linear regressions of the data.



A similar experiment performed with *S. pneumoniae* ATCC49619 (mitomycin C MIC = 0.015 mg/L) produced essentially the same results, but with lower levels of over-expression (data not shown).

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Erratum

Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*

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The column headings in Table 1 of this article were misaligned in the published version. The corrected version of Table 1 is reproduced on the next page. The publisher apologizes for this error.

Table 1. Susceptibility of *S. pneumoniae* to fluoroquinolones and substrates of efflux pumps in the absence (−R) or presence (+R) of reserpine (20 mg/L)

			MIC (mg/L) of:														
			norfloxacin		ciprofloxacin		levofloxacin		moxifloxacin		gemifloxacin		acriflavine		ethidium bromide		
Strains	Relevant characteristics[a]	Mutations in QRDR	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	Reference or source
ATCC 49619	wild-type	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	2	1	0.5	0.125	LGC Standards
ATCC 49619*patA*	ATCC 49619 *patA*::*magellan2*, SPT^R	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.016	1	0.5	0.25	0.125	this study
ATCC 49619*patB*	ATCC 49619 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
ATCC 49619*pmrA*	ATCC 49619 *pmrA*::*magellan2*, SPT^R	none	4	2	1	0.5	0.5	0.5	0.125	0.125	0.031	0.031	4	1	1	0.125	this study
SP334	ATCC 49619 after 13 days of exposure to ciprofloxacin, CIP^R	none	**32**	4	**4**	0.5	**2**	1	0.25	0.25	**0.125**	0.031	4	1	**4**	0.125	[12]
SP334*patA*	SP334 *patA*::*magellan2*, SPT^R	none	4	4	1	0.5	1	1	0.125	0.125	0.063	0.031	1	1	0.25	0.125	this study
SP334*patB*	SP334 *patB*::*magellan2*, SPT^R	none	8	4	1	1	1	1	0.25	0.25	0.063	0.063	1	1	0.125	0.125	this study
SP334*pmrA*	SP334 *pmrA*::*aad9*, SPT^R	none	**32**	4	**4**	0.5	1	0.5	0.25	0.25	**0.125**	0.063	4	0.5	**2**	0.125	this study
SP335	clinical strain SP32 after 13 days of exposure to ciprofloxacin, CIP^R	ParE (Ile460Val)	**64**	8	**32**	2	**4**	2	**0.5**	0.25	**0.5**	0.125	**16**	1	**8**	0.25	[12]
SP335*patA*	SP335 *patA*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
SP335*patB*	SP335 *patB*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP335*pmrA*	SP335 *pmrA*::*aad9*, SPT^R	ParE (Ile460Val)	**64**	4	**8**	0.5	1	0.5	0.25	0.125	0.063	0.016	**8**	0.5	**8**	0.125	this study
SP295	clinical isolate[b]	none	**16**	2	**2**	0.5	1	0.5	0.125	0.125	0.063	0.031	**16**	1	**16**	0.25	this study
SP295*patA*	SP295 *patA*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP295*patB*	SP295 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.016	0.016	1	1	0.25	0.25	this study
SP295*pmrA*	SP295 *pmrA*::*aad9*, SPT^R	none	8	4	1	0.5	1	0.5	0.25	0.125	0.063	0.031	**8**	1	**4**	0.25	this study
SP13	clinical isolate[c]	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**256**	16	**16**	4	**2**	1	0.25	0.25	**0.25**	0.063	**16**	1	**16**	0.25	this study
SP13*patA*	SP13 *patA*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	1	1	1	0.25	0.25	0.063	0.063	1	0.5	0.25	0.125	this study
SP13*patB*	SP13 *patB*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	2	**2**	1	0.25	0.25	0.063	0.063	2	1	0.25	0.25	this study
SP13*pmrA*	SP13 *pmrA*::*aad9*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**128**	16	**16**	2	**2**	1	**0.5**	0.25	**0.125**	0.063	**8**	0.5	**8**	0.25	this study

EUCAST breakpoints for resistance: ciprofloxacin and levofloxacin, >2 mg/L; and moxifloxacin, >0.5 mg/L (no values for norfloxacin and gemifloxacin). Figures in bold indicate MICs at least two dilutions higher than those of wild-type *S. pneumoniae* ATCC 49619.

[a]CIP^R, resistance to ciprofloxacin; SPT^R, resistance to spectinomycin.

[b]Isolated from blood culture (Cliniques Universitaires St Luc, Brussels).

[c]Isolated from expectoration (Universitair Ziekenhuis Brussel, Brussels).