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The pneumococcal chromosome encodes about 140 transporters, many of which are predicted to be involved in efflux. In order to critically evaluate pneumococcal efflux, a series of transporter mutants were constructed, and their phenotypes were assayed by disk diffusion, microdilution drug susceptibility testing (MIC testing), growth of cultures at sub-MIC concentrations, and phenotype microarray analysis. Mutants with mutations in seven ATP binding cassette (ABC) transporters, three multiantimicrobial extrusion (MATE) family efflux pumps, and one major facilitator superfamily (MFS) transporter were obtained in *Streptococcus pneumoniae* strain DP1004. The susceptibility of these 11 mutants to over 250 different substances was compared to that of the parent strain. Of the tested transporters, only the ABC transporter PatAB (SP2073-5) presented a clear multidrug resistance (MDR) profile, as the mutant showed significantly increased susceptibility to ethidium bromide, acriflavine, and berberine. Among the other transporters analyzed, the mutants devoid of the MATE efflux pump SP2065 exhibited reduced susceptibility to novobiocin, and those with mutations of the MATE family DinF transport system (SP1939) exhibited increased susceptibility to moxifloxacin, ciprofloxacin, and levofloxacin. This change in quinolone MIC was found to be independent from the competence-mediated effect of quinolones on the *cinA-recA-dinF* operon. Furthermore, the *dinF* mutant, in contrast to the parental strain, allowed selection for quinolone-resistant mutants when exposed to moxifloxacin. These data confirm the clear MDR profile of the PatAB ABC transporter and suggest for the MATE DinF a phenotype associated with quinolone susceptibility, particularly for moxifloxacin.

Multidrug resistance efflux pumps are important components of innate and acquired bacterial resistance to antimicrobial agents (1). Many chromosome- and plasmid-encoded multidrug transporters have been identified in Gram-positive bacteria, including Bmr and Blt in *Bacillus subtilis* (2, 3), NorA and QacA in *Staphylococcus aureus* (4, 5), and LmrA and LmrP in *Lactococcus lactis* (6, 7). In *Streptococcus pneumoniae*, the initial description of a reserpine-sensitive, energy-dependent transporter which promotes efflux of ethidium bromide did not identify the transport protein associated with the efflux phenotype (8). A few years later, the PmrA transporter, belonging to the major facilitator superfamily (MFS), was described as being responsible for efflux of norfloxacin, ciprofloxacin, acriflavine, and ethidium bromide in pneumococci (9). More recently, a systematic deletion of 13 putative pneumococcal efflux transporters showed that only inactivation of the ABC transporter PatAB (SP2073 and SP2075) was associated with hypersusceptibility to norfloxacin, ciprofloxacin, acriflavine, and ethidium bromide, while no phenotype could be linked to PmrA (10). Gene expression data confirmed that PatAB was the pneumococcal multidrug resistance (MDR) efflux pump and was upregulated in clinical isolates with increased resistance to dyes and quinolones (11, 12).

Pneumococci, like other streptococci, are anaerobes metabolically. Due to this fact, the proton motive force is not available as the main mechanism to drive transport of molecules through biological membranes (13). The transporter database TransportDB reports 140 transporters, including 7 channel proteins (TC1; transporter classification 1), 25 TC2 secondary transporters, 84 TC3 primary transporters, and 22 TC4 group translocators, for *S. pneumoniae* (14–16). Following annotation of the TransportDB, we selected seven ABC transporters for analysis of efflux among the primary transporters and one MFS transporter and three multiantimicrobial extrusion (MATE) family proteins among the secondary transporters (15).

The SP0972 PmrA transporter is a membrane protein of 399 amino acids with 12 transmembrane domains (9), an MFS domain at the N terminus, and a sugar transport domain at the C-terminal end (17). The gene is likely to be cotranscribed with the formamidopyrimidine-DNA glycosylase SP0970, the dephospho-coenzyme A kinase SP0971, and the 50S ribosomal protein L33, SP0973. The transporters SP1357-8 (SP1357 and SP1358), SP1434-5, SP1839-40, and SP2073-5 (PatAB) are examples of heterodimeric half-transporters, where both proteins have a transmembrane domain and an ATP binding cassette (ABC) domain, similar to the LmrCD proteins of *L. lactis* (18). In all four transport systems, the genes are not part of larger operons. The open reading frame (ORFs) of SP1357-8, SP1434-5, and SP1839-40 partially overlap, while the two ORFs SP2073-5 are spaced by 800 bp. All four transporters are classified as MDR1-like due to homology to human glycoprotein P (19). The human transporter is described as forming a heterodimer and classified as MDR1 because it confers resistance toward anticancer cytotoxic compounds (20). The

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TABLE 1 Mutants constructed

Gene(s)[a]	Name(s)	Transporter Family	ATP-binding domain(s)	Permease domain(s)	Chromosomal segment deleted[a]	Gene(s) deleted; comment	Marker	Mutant name[b]
SP1114-6		ABC	SP1114	SP1116	1,043,558–1,044,646	SP1114 deleted; downstream genes SP1115 and SP1116 probably not transcribed	*aphIII*	FP405
SP1341-2		ABC	SP1341	SP1342	1,263,347–1,265,227	SP1341-2 deleted; the probable operon SP1344-0 may have variations in transcription	*aphIII*	FP409
SP1434-5		ABC	SP1434/5	SP1434/5	1,353,894–1,354,933	SP1435 deleted; genes SP1434-8 probably not transcribed	*aphIII*	FP403
SP1918-9		ABC	SP1918	SP1919	1,828,126–1,829,290	SP1918-9 deleted; transcription of upstream SP1920-2 possibly affected	*aphIII*	FP407
SP1357-8		ABC-MDR1	SP1357/8	SP1357/8	1,278,252–1,280,257	SP1357-8; probably no other genes affected	*aphIII*	FP406
SP2073-5	*patAB*	ABC-MDR1	SP2073/5	SP2073/5	1,980,843–1,984,525	SP2073-5; probably no other genes affected	*aphIII*	FP392
SP1839-40		ABC-MDR1	SP1839/40	SP1839/40	1,749,010–1,751,780	SP1839-40; probably no other genes affected	*aphIII*	FP455
SP1166		MATE			1,101,504–1,102,688	SP1166; probably no other genes affected	*aphIII*	FP349
SP1939	*dinF*	MATE			1,842,701–1,842,702	SP1939 *mariner* transposon insertion; transcription of upstream *cin-recA* possibly affected	*aad9*	FP87
SP1939	*dinF*	MATE			1,842,701–1,842,702	SP1939 Mariner transposon insertion in strain R6 (see FP87)	*aad9*	FP501
SP2065		MATE			1,965,524–1,966,715	SP2065 deleted; SP2064 probably not transcribed	*aphIII*	FP400
SP0972	*pmrA*	MFS			918,131–919,298	SP0972 (*pmrA*); other genes probably in the same operon may be affected and include *mutM* (SP0970), *coaE* (SP0971), and *rpmG1* (SP0973)	*cat*	FP 375
SP2237	*comC*	Control			2,157,757–2,157,959	Deletion of the *comC* gene results in a competence-negative strain (40, 41)	*cat*	FP10
SP1939 + SP2237	*dinF*, *comC*	Control			1,842,701–1,842,702	SP1939 Mariner transposon insertion in strain FP10 (see FP87)	*cat*, *aad9*	FP502
SP1941 + SP2237	*cinA*, *comC*	Control			1,845,072–1,845,073	SP1941 Mariner transposon insertion in *cinA* in FP10; transcription form the *cinA* promoter abolished for *recA-dinF*	*cat*, *aad9*	FP86

[a] Numbers refer to the locus name and sequence position in TIGR4 (GenBank accession number AE005672).
[b] All mutants were constructed in Rx1 except for FP501, which was constructed in R6.

gene SP1434 is described as essential in models of lung infection in mice (21).

The ABC transporter SP1114-6 is composed of SP1114 (two ABC domains) and SP1116 (10 transmembrane segments). The two genes are in a putative operon with the putative transcriptional regulator SP1115. The proteins are described as being involved in the transport of different compounds, since both were shown to be induced by penicillin, and SP1116 was also induced by doxorubicin and nalidixic acid (11).

The ABC transporters SP1341-2 and SP1918-9 are homodimeric transporters with one protein coding for a permease and one for the ABC domain. SP1918-9 appears not to be part of an operon. The SP1341-2 transporter is located in a putative operon also containing the prolyl oligopeptidase family protein SP1343, a putative serine/threonine kinase, SP1342, and the hypothetical protein SP1340. Deletion of SP1341-2 conferred slightly decreased susceptibility to vancoresmycin and bacitracin, but no molecular mechanism for this phenotype was described (22), and gene expression analysis showed induction of SP1341-2 by penicillin, nalidixic acid, and valinomycin (11).

The three transporters SP1166 (NorM), SP1939 (DinF), and SP2065 are proteins with two MATE domains and 10 to 12 predicted transmembrane segments. DinF has been assigned to one of the competence-induced operons, which includes the competence-induced protein A gene (*cinA*) and *recA* (23). SP2065 is cotranscribed with a putative haloacid dehalogenase-like hydrolase, SP2064. Tiling array data assign SP1166 to an operon that includes uracil-DNA glycosylase *ung* protein SP1169, the mutator *mutT* protein SP1168, the dihydroorotase *pyrC* protein SP1167, and a noncoding small RNA (24).

In this work, we performed a through and detailed analysis of efflux in pneumococci. A large number of transporters was analyzed by employing different types of assays for testing the phenotypes and assessing efflux for a large number of compounds.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and mutant construction. All pneumococcal strains were constructed in the genetic background of the unencapsulated D39 derivative DP1004 (25). Pneumococci were grown in liquid in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and plated on tryptic soy agar (TSA; Liofilchem, Teramo, Italy) with 3% defibrinated horse blood (Biotec, Grosseto, Italy). Mutants were constructed as previously described, by direct transformation of PCR fragments containing an antibiotic resistance gene ligated during PCR to chromosomal segments flanking the gene to be deleted (Table 1) (25, 26). Mutants in the *cinA-recA-dinF* locus were generated by *mariner* mutagenesis (27). In the latter case, insertions were verified by sequencing. The primers used in this work are shown in Table S1 in the supplemental material.

Susceptibility testing. Disk diffusion susceptibility testing and MIC determinations in microdilution were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (28), except for the use of Todd-Hewitt broth (Difco, Becton Dickinson, Sparks, MD) plus yeast extract (Difco, Becton Dickinson) as the MIC assay medium (10). Antibiotic disks were from Oxoid (Difco, Becton Dickinson, Sparks, MD), and disks used for other compounds were prepared by adding 30 μg of the compound to empty disks. All compounds were from Sigma (Sigma-Aldrich, Milano, Italy), except for ciprofloxacin (Bayer, Milano, It-

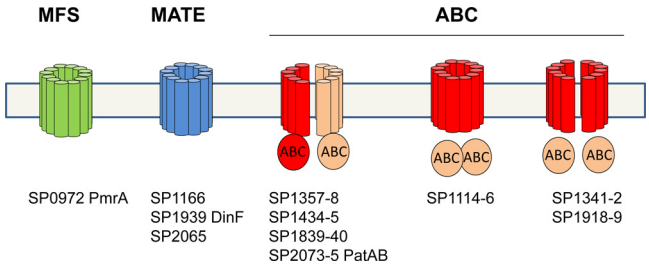

FIG 1 Schematic representation of the putative efflux transporters deleted in *S. pneumoniae*. The MFS transporter SP0972 is represented as a 12-transmembrane-domain protein (green). The three MATE transporters SP1166, SP1939 (DinF), and SP2065 are shown as blue 12-transmembrane-domain proteins, while the ABC transporters are depicted in red. In the case of the ABC heterodimeric half-transporters SP1357-8, SP1434-5, SP1839-40, and SP2073-5, the two proteins forming the heterodimer are differently shaded. The transporter SP1114-6 is formed by an ATPase protein with two ABC domains and a 12-transmembrane-domain permease. The ABC transporters SP1341-2 and SP1918-9 are predicted to be formed by a homodimer consisting of both the ABC protein and the permease. Numbering refers to the TIGR4 genome (GenBank accession number AE005672).

aly). Minimum bactericidal concentrations (MBCs) were determined by subculturing 10 μl from each well without visible bacterial growth on Mueller-Hinton blood agar plates (Difco, Becton Dickinson, Sparks, MD). After 24 h of incubation at 37°C, the dilution yielding three colonies or less was considered the MBC (29). Sensitivity to chemical compounds was tested by phenotype microarray (Biolog, Hayward, CA) by incubating pneumococci in TSB with catalase (200 U/ml; Sigma-Aldrich) and the tetrazolium dye at a final concentration of 1% as previously described (14). All strains were incubated in Biolog plates PM11 to PM20. This allowed assessment of the sensitivity to 240 chemical agents at four concentrations of each agent. Susceptibility data are reported as IC_5s, which are expressed in well units and defined as the well or fraction of a well at which a particular per-well parameter is at half its maximal value over a concentration series (30). Growth at sub-MIC concentrations was performed in 96-well microtiter plates sealed with gas-permeable sealing membranes (Breath-Easy; Diversified Biotech, Boston, MA) and incubated at 37°C for 24 h in a thermostatic kinetic microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA). The optical density at 590 nm (OD_{590}) was measured every 10 min. Prior to each reading, plates were gently shaken for 2 s (14).

Selection of fluoroquinolone mutants. The FP87 *dinF* (SP1939) mutant and the wild-type strain DP1004 were grown to late stationary phase, and approximately 10^{10} CFU was plated on TSA blood agar plates containing 0.5 mg/liter moxifloxacin, 1 mg/liter levofloxacin, or 2 mg/liter ciprofloxacin. The mutation selection frequencies were calculated as the ratio of the number of colonies grown on antibiotic-containing plates to the number of colonies grown on plates without antibiotic (31).

RESULTS AND DISCUSSION

Eleven *S. pneumoniae* mutants were constructed in putative efflux transporters, seven of which were ABC transporters, one was annotated as an MFS transporter, and three were annotated as MATE (Fig. 1). The group of transporters included six of those analyzed previously (10), including SP1435, which was reported to be essential for growth. As described previously (10), our mutants also showed no differences in growth profile with respect to their parent strain (data not shown). To test the substrate specificity of the transporters and assess whether the mutations conferred increased susceptibility to a series of compounds, the mutants were compared in four different assays, including disk diffusion, MIC, growth curves at sub-MICs, and phenotype microarray (Biolog) (Table 2). Disk diffusion of 28 compounds showed increased susceptibility for a few compounds in 6 of 11 mutants, including those with mutations in SP2073-5 (berberine and ethidium bromide), SP1114-6 (novobiocin, ofloxacin, and oxacillin), SP1341-2 (ofloxacin and oxacillin), SP1434-5 (ethidium bromide), SP1918-9 (cotrimoxazole, novobiocin, and oxacillin), and SP0972 (fusidic acid and SDS) (see Table S2 in the supplemental material).

The phenotype microarray was used to expand the number of substrates assayed to 240 different chemical compounds. The presence or absence of metabolic activity in the four wells with different drug concentrations was assessed, and the IC_{50} data for

TABLE 2 Phenotypes of transporter mutants

ORF[a]	Name	Class[b]	Compound(s) for which indicated assay increased susceptibility			
			Disk diffusion	Phenotype microarray	Microdilution MIC	Sub-MIC growth
SP1357-8		MDR ABC				
SP1839-40		MDR ABC				
SP2073-5	PatAB	MDR ABC	Berberine, ethidium bromide	Acriflavine, thioctic acid, rifampin, oxytetracycline, rolitetracycline, chelerythrine	Acriflavine, berberine, ethidium bromide	Ethidium bromide
SP1114-6		ABC	Novobiocin, ofloxacin, oxacillin	Chlortetracycline		
SP1341-2		ABC	Ofloxacin oxacillin			
SP1434-5		ABC	Ethidium bromide	Dichlofluanid		
SP1918-9		ABC	Cotrimoxazole, novobiocin, oxacillin	Oxytetracycline		
SP0972	PmrA	MFS	Fusidic acid, SDS			
SP1166		MATE				
SP1939	DinF	MATE		Cefotaxime	Ciprofloxacin, levofloxacin, moxifloxacin	Moxifloxacin
SP2065		MATE		Rifampin, sodium salicylate	Novobiocin	

[a] Numbers refer to the locus names in TIGR4 (GenBank accession AE005672).
[b] Transporters are classified according to TransportDB classification (15).

TABLE 3 Quinolone MICs of the *dinF* mutant and related mutants

Strain	Mutated gene(s)	Moxifloxacin[b]		Levofloxacin[b]		Ciprofloxacin[b]	
		MIC	MBC	MIC	MBC	MIC	MBC
Rx1		0.125	0.25	1	2	1	1
Rx1	*dinF*	0.06	0.125	0.5	1	0.5[c]	1
Rx1	*comC*	0.125	ND	1	ND	1	ND
Rx1	*comC dinF*	0.06	ND	0.5	ND	0.5[c]	ND
Rx1	*comC cinA*	0.125	ND	1	ND	1	ND
R6		0.125	0.125	1	1	1	1
R6	*dinF*	0.06	0.06	0.5	ND	0.5–1[c]	1
Breakpoint[a]							
Susceptibility		≤0.5		≤2		≤0.125	
Resistance		>0.5		>2		>2	
ECOFF		≤0.5		≤2		≤2	

[a] Reference values are according to EUCAST guidelines. ECOFF, epidemiological cutoff value.
[b] All concentrations are expressed as mg/liter.
[c] Not all MIC assays performed evidenced reduction of the MIC from 1 to 0.5 mg/liter of ciprofloxacin.

all compounds are reported in Table S3 in the supplemental material (30). In particular, decreases in metabolic activity in the presence of single compounds were observed for SP1114-6 (chlortetracycline), SP1434-5 (dichlofluanid), SP1918-9 (oxytetracycline), SP1939 (DinF) (cefotaxime), SP2065 (rifampin and sodium salicylate), and SP2073-5 PatAB (acriflavine, thioctic acid, rifampin, oxytetracycline, rolitetracycline, and chelerythrine) mutants. For a few compounds, the phenotype microarray indicated increased metabolic activity (see Table S3). A microdilution MIC assay was used as a quantitative method to confirm variations in susceptibility detected by disk diffusion and phenotype microarray (see Table S4 in the supplemental material). The SP2073-5 mutant showed increased susceptibility to acriflavine (1-fold-decreased MIC), berberine, and ethidium bromide (2-fold decreases). The SP2065 mutant showed a 1-fold increase in susceptibility to novobiocin, and the SP1939 mutant a 1-fold increase for moxifloxacin, levofloxacin, and ciprofloxacin (Table 3; also see Table S4). For moxifloxacin and levofloxacin, the 1-dilution change was consistently detected, while not all assays evidenced the 1-dilution variation in MIC for ciprofloxacin. When the MBC of quinolones was assayed, a decrease could be observed only for the moxifloxacin MBC (Table 3). Analysis of the growth profiles at sub-MICs showed a reduced growth slope for the SP2073-5 mutant in ethidium bromide (0.5 mg/liter) (not shown) and for the SP1939 mutant in moxifloxacin (0.03 mg/liter) (Fig. 2A).

The use of several assays allowed us to identify a consistent phenotype for the SP2073-5 and SP1939 mutants, namely, a classical MDR resistance profile conferred by PatAB SP2073-5 (10) and a variation in susceptibility to quinolones conferred by DinF SP1939. The MDR phenotype for PatAB, together with the absence of detectable phenotypes for most other transporters, is in accordance with the data of Robertson et al. (10). Our data assign a lower number of phenotypes to mutants than previously published work (9–11), probably due to the fact that in this work, a phenotype was considered significant if detected by more than one type of assay. All of the apparent inconsistencies between phenotypes identified in the different assays and reported in Table 2 were reconfirmed (Table 2). This indicates that it may well be possible that some transporters confer significant phenotypes only under specific experimental conditions.

In pneumococci, the *cinA-recA-dinF* operon is induced by quinolones through an SOS-like stress mechanism via the competence system, and such induction is lost in competence mutants (23, 32). Likewise, in *Escherichia coli*, the MATE DinF is associated with the SOS response and the MATE NorM with protection from reactive oxygen species (33, 34). To demonstrate that the *dinF* phenotypes were specific to inactivation of the transporter and competence independent, we constructed a competence system mutant (*comC* mutant) and a double mutant with mutations in *comC* and *cinA* (the first gene of the *cinA-recA-dinF* operon). Neither of the two mutants showed any change in quinolone MIC, indicating that the abolishment of quinolone-induced transcription of *cinA-recA-dinF* is not relevant for the *dinF*-dependent phenotype. The specificity of our observation was confirmed by transfer of the *dinF* mutation into another strain (R6) and in a competence-negative background (*comC* negative). In both cases, we recreated the quinolone susceptibility phenotype initially observed. In addition to quinolones, kanamycin had also been shown to induce transcription of the *cinA-recA-dinF* operon (32). None of our *cinA*, *dinF*, and *comC* mutants showed any change in kanamycin MIC (data not shown). This further indicates that the *dinF* phenotype is independent from "stress induction" of the *cinA-recA-dinF*, to a good extent ruling out the possibility that our *dinF* phenotype is due to a polar effect on *recA*.

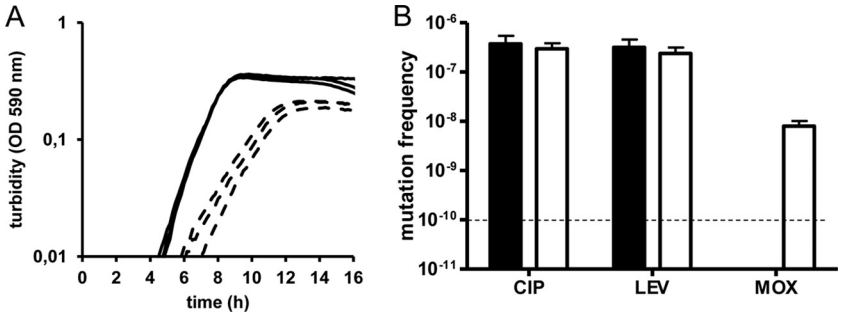

FIG 2 Phenotypes of the *dinF* (SP1939) mutant. (A) Growth profiles of the parental DP1004 (continuous line) and the *dinF* (SP1939) mutant (dashed line) in triplicate in TSB medium with 200 U/ml of catalase and 0.03 mg/liter of moxifloxacin. The slopes of the growth curves are 0.79 for the parent and 0.56 for the *dinF* mutant. (B) Mutation frequency of the parental DP1004 (closed bars) and the *dinF* SP1939 mutant (open bars) for resistance to three quinolones. The mutation frequency of the parental strain for moxifloxacin resistance was below 10^{-10} (dashed line). Error bars show standard deviations.

In order to further investigate the novel association of the MATE DinF to quinolone susceptibility, we investigated the mutation frequency of moxifloxacin, which had been reported to be 1,000-fold lower than for other fluoroquinolones, such as ciprofloxacin or levofloxacin (35–37). The MIC of moxifloxacin for *S. pneumoniae* DP1004 is 0.125 mg/liter, while it was one dilution lower (0.06 μg/ml) for the *dinF* (SP1939) mutant. The mutation frequency of both DP1004 and the *dinF* mutant did not change for resistance to ciprofloxacin and levofloxacin. In contrast to the parental strain, however, it was also possible to recover moxifloxacin mutants in the *dinF* (SP1939)-deficient strain (Fig. 2B). The moxifloxacin mutant recovered in the *dinF* strain showed a MIC of 0.25 mg/liter for moxifloxacin and a MIC of 4 mg/liter for both levofloxacin and ciprofloxacin and a classical point mutation in *parC* (Ser-79-Tyr [TCT-TAT]) (38). Albeit *recA* expression could be influenced by the *dinF* deletion and DinF orthologs in *E. coli* have been linked to quinolones, SOS response, and export of radicals (32–34), we favor the hypothesis that it is essentially the lower moxifloxacin MIC of the *dinF* strain which enabled a more efficient selection of *parC* mutants.

In summary, our data support the hypothesis that DinF is directly involved in quinolone and, especially, moxifloxacin efflux. Since quinolones are synthetic compounds, they clearly do not represent the natural substrates of DinF. However, our results may help in identifying the natural substrate or substrate class transported by DinF, thus leading to a deeper understanding of the role of efflux pumps in pneumococcal metabolism and drug resistance.

Conclusions. Our data confirm previous work which assigns to PatAB, the heterodimeric ABC half-transporter SP2073-5, a role as an MDR efflux pump in pneumococci (10). The fold change in MIC conferred by PatAB deletion classifies this transporter as the only transporter responsible for resistance to a given compound (10, 39). Moderate changes in susceptibility to various compounds, which do not allow for an unambiguous association of a specific substrate to each transporter, were observed for the other transporter mutants. A novel observation enabled us to link the MATE DinF transporter SP1939 to quinolone susceptibility and, in particular, moxifloxacin susceptibility.

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