ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2011, p. 190–196 Vol. 55, No. 1
0066-4804/11/$12.00 doi:10.1128/AAC.00672-10

Overexpression of *patA* and *patB*, Which Encode ABC Transporters, Is Associated with Fluoroquinolone Resistance in Clinical Isolates of *Streptococcus pneumoniae*▽

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Received 18 May 2010/Returned for modification 23 July 2010/Accepted 3 October 2010

Fifty-seven clinical isolates of *Streptococcus pneumoniae* were divided into four groups based on their susceptibilities to the fluoroquinolones ciprofloxacin and norfloxacin and the dyes ethidium bromide and acriflavine. Comparative reverse transcription-PCR was used to determine the level of expression of the genes *patA* and *patB*, which encode putative ABC transporters. Overexpression was observed in 14 of the 15 isolates that were resistant to both fluoroquinolones and dyes and in only 3 of 24 of those resistant to fluoroquinolones only. Isolates overexpressing *patA* and *patB* accumulated significantly less of the fluorescent dye Hoechst 33342 than wild-type isolates, suggesting that PatA and PatB are involved in efflux. Inactivation of *patA* and *patB* by *in vitro mariner* mutagenesis conferred hypersusceptibility to ethidium bromide and acriflavine in all isolates tested and lowered the MICs of ciprofloxacin in the *patAB*-overproducing and/or fluoroquinolone-resistant isolates. These data represent the first observation of overexpression of *patA* and *patB* in clinical isolates and show that PatA and PatB play a clinically relevant role in fluoroquinolone resistance.

Streptococcus pneumoniae is an important cause of community-acquired respiratory infections, including sinusitis, otitis media, and pneumonia, as well as serious invasive infections, such as septicemia and meningitis (9). Antibiotic resistance to β-lactams, macrolides, fluoroquinolones, and tetracycline is an increasing problem in many countries with *S. pneumoniae* infections (5, 13, 18, 29, 31). Fluoroquinolone resistance in particular is a cause for concern. Surveillance data supplied from the British Society for Antimicrobial Chemotherapy (BSAC) for 2007 to 2008 indicated that 95.6% of *S. pneumoniae* isolates from the United Kingdom show intermediate resistance to ciprofloxacin (0.12 μg/ml < MIC ≤ 2 μg/ml) and 4.4% of isolates are fully resistant to ciprofloxacin (MIC ≥ 4 μg/ml), while no isolates were susceptible according to the recommended breakpoint concentrations used. Fluoroquinolone resistance in *S. pneumoniae* is usually due to mutations in the genes encoding the target topoisomerase enzymes. Mutations frequently occur in *parC*, which encodes the A subunit of DNA topoisomerase IV, or *gyrA*, which encodes the A subunit of DNA gyrase (12). Less frequently, mutations can be found in the genes *parE* and *gyrB*, encoding the B subunits of these proteins (14, 23, 24). However, low-level resistance to fluoroquinolones can also be conferred by active efflux mediated by PmrA (6, 11) or PatA/PatB (10, 20, 21). Active efflux usually gives rise to smaller increases in the MIC of a fluoroquinolone than mutations affecting DNA topoisomerase IV and/or DNA gyrase. There have also been several studies whose results suggest that efflux pumps are a requirement for the selection of fluoroquinolone resistance in *S. pneumoniae* (6, 8, 16, 32). In addition, the overexpression of efflux pumps typically confers resistance to other antibacterial agents besides fluoroquinolones, such as some dyes (e.g., ethidium bromide), detergents (e.g., SDS), and disinfectants (e.g., cetrimide) (25). Genes encoding efflux pumps are typically chromosomally encoded, ensuring that mutations resulting in altered expression or function are retained in further generations in the absence of selective pressure or fitness cost.

Due to the lack of new antibacterial agents, there is considerable interest in potentiating the activity of current antibiotics, such as fluoroquinolones. Efflux pumps make attractive targets for drug discovery programs as inhibition confers multidrug susceptibility, reduces the frequency of selection of drug-resistant mutants, and potentiates the activity of existing drugs (19). *S. pneumoniae* contains a variety of different multidrug resistance (MDR) efflux pumps; in total, there are 25 transporters predicted to efflux antibiotics (http://www.membranetransport.org/index.html) (10, 11, 20, 21). Until recently, the only pneumococcal efflux pump implicated in fluoroquinolone efflux and resistance was PmrA (11). However, multidrug-resistant *S. pneumoniae* isolates carrying a nonfunctional *pmrA* gene have been described (7, 27). Marrer et al. (20, 21) identified overproduction of two putative ABC efflux transporters, PatA (SP2075) and PatB (SP2073), associated with fluoroquinolone resistance in a laboratory-selected ciprofloxacin-resistant mutant. In parallel, Robertson et al. (28) inactivated 13 genes encoding putative efflux pumps in *S. pneumoniae* strain R6 and found that the inactivation of *patA* and *patB* gave rise to hypersusceptibility to ciprofloxacin and norfloxacin, as well as to ethidium bromide and acriflavine. PatA

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▽ Published ahead of print on 11 October 2010.

TABLE 1. Wild-type and control *S. pneumoniae* strains used in the present study

S. pneumoniae strain	Comment	Reference
R6	Unencapsulated	3
M4	Wild-type (type 2) NCTC 7465	
M3	Wild-type (type 1) NCTC 7466	
M168	Spontaneous reserpine-resistant mutant of M4	10
M169	Spontaneous reserpine-resistant mutant of R6	10
M22	Spontaneous ciprofloxacin-resistant mutant of M4	26
M240	R6::*patB magellan2*	10
M246	R6::*patA magellan2*	10
M260	M22::*patB magellan2*	This study
M276	M22::*patA magellan2*	This study

and PatB also play a role in efflux inhibitor resistance in laboratory-selected reserpine-resistant mutants of *S. pneumoniae* (10). However, although several studies suggest that PatA and PatB play a role in fluoroquinolone resistance in laboratory strains of *S. pneumoniae*, there is little direct evidence to show whether PatA and PatB are overproduced in clinical isolates. In the present study, it is shown that *patA* and *patB* are overexpressed in a significant proportion of clinical isolates of *S. pneumoniae*. These isolates also accumulated less Hoechst 33342, a fluorescent dye which is a substrate of efflux pumps (30), and most were additionally resistant to the dyes ethidium bromide and acriflavine. This is the first report showing direct evidence of the involvement of PatA and PatB in fluoroquinolone resistance in clinical isolates of *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains, storage, and growth. *S. pneumoniae* M4 (NCTC 7465 type 1), *S. pneumoniae* M3 (NCTC 7466 type 2), *S. pneumoniae* R6 (3), and the well-characterized laboratory strains M169, M168, M22, M240, M246, M260, and M276 (10) were used throughout as control strains (Table 1). Fifty-seven clinical isolates were obtained from a variety of sources (27): 15 isolates were from MRL Pharmaceutical Services (14), 26 isolates were from the Lung Investigation Unit, University Hospital, Birmingham, United Kingdom (26), 14 clinical isolates were from the Centers for Disease Control and Prevention, Atlanta, GA (15), and 2 laboratory-selected mutants were from George Drusano, Albany, NY (16). All strains were grown in brain heart infusion broth (BHI; Oxoid, Basingstoke, United Kingdom) for 24 h at 37°C in 5% CO_2. The identity of each species was confirmed by Gram stain, optochin sensitivity, and the presence of capsule, which was determined by using a Slidex Pneumo-Kit (BioMerieux, France).

Antibiotics and susceptibility determination. All antibiotics, dyes, and efflux pump inhibitors were made up and used according to the manufacturer's instructions; they included ciprofloxacin, norfloxacin, levofloxacin, acriflavine, ethidium bromide, sodium orthovanadate, and reserpine (Sigma-Aldrich Company Ltd., Dorset, United Kingdom). The MIC of each antibiotic, dye, and efflux pump inhibitor for each strain was determined by using the standardized agar doubling dilution method according to the guidelines of BSAC (2). The effects of the efflux pump inhibitors reserpine (20 μg/ml) and sodium orthovanadate (50 μM) on the MICs of these agents were also measured. Synergy between reserpine or orthovanadate and a fluoroquinolone or a dye was defined as a reduction in the MIC of the agent of ≥2 dilutions in the presence of the inhibitor.

Growth kinetics. The growth kinetics of all the *S. pneumoniae* strains used in this study were determined by monitoring optical density (read at 600 nm) every 10 min using a FLUOstar Optima (BMG Labtech, Aylesbury, United Kingdom). Samples were prepared by adding a 5% inoculum of *S. pneumoniae* culture pregrown to logarithmic phase (optical density at 600 nm [OD_{600}] of 0.4, equivalent to 10^5 CFU/ml) to prewarmed BHI broth. Approximately 100 μl of sample for each strain was added to 3 separate wells on a microtiter tray and grown statically at 37°C for 24 h in aerobic conditions, with 5 s of shaking (300 rpm) before each reading. Samples from BHI broths were also removed and visualized microscopically in order to detect any gross changes to cell morphology. Generation times were calculated from the logarithmic phase of growth using Microsoft Excel. Differences in average generation time between groups were assessed for statistical significance using the nonpaired Student's *t* test.

Measurement of the expression of *patA* and *patB*. To measure the levels of expression of *patA* and *patB* in parallel from a single mRNA preparation, comparative reverse transcription-PCR (c-RT-PCR) was combined with the rapid and high-throughput technique of denaturing high-pressure liquid chromatography (DHPLC) analysis of amplimers, as previously described by Garvey and Piddock (10). *S. pneumoniae* strains were grown on three separate occasions, and RNA was isolated from each of these three samples as described previously (10). To determine whether there was a significant difference in the expression of *patA* and *patB* between strains R6, M3, M4, and the 57 clinical isolates, mean peak areas were compared using the two-tailed Student's *t* test.

Accumulation of Hoechst 33342 with or without reserpine by *S. pneumoniae*. The efflux activities of the clinical isolates, grown to mid-log phase in BHI broth, were compared with those of the laboratory strains R6, M3, and M4 by monitoring the uptake of Hoechst 33342 added to a final concentration of 2.5 μM, with measurements taken at excitation and emission wavelengths of 350 and 460 nm, respectively, over 30 min using a FLUOstar Optima (BMG Labtech). Differences in accumulation between strains were analyzed for statistical significance using Student's *t* test. In parallel, the accumulation of Hoechst 33342 with or without reserpine (20 μg/ml) was measured.

PCR and sequencing of *patA* and *patB*. One set each of primers specific for *patA* and *patB* were designed to amplify these genes for sequencing (Table 2). The primer sets encompassed the entire coding sequence for *patA* and *patB*, as well as ~200 bp upstream of each gene, covering the putative promoter regions. PCR was performed under the same conditions as described previously (10). The

TABLE 2. Details of the DNA primers used in this investigation

Gene amplified or primer set (purpose or description)	Forward primer	Reverse primer	Amplimer size (bp)
16S rRNA	5′-GAGAAGAACGAGTGTGAGAG-3′	5′-CTAACACCTAGCACTCATCG-3′	391
patA (sequencing)	5′-TCTTGCTCAGTCCATCATCGAATAT-3′	5′-CCGCTGTGGATTAGTTCATTTCC-3′	2,963
patB (sequencing)	5′-AGAATCCAGTCCAGCGAAAGCT-3′	5′-GAAAGAACGACCAGATGTTCCAAT-3′	2,959
patA (expression)	5′-ATGTTGTCCTCGCAGCCTAT-3′	5′-ACGAACCGATGAACAAGAGG-3′	212
patB (expression)	5′-TTGCTGGTTCGGCTGTACTT-3′	5′-AACTGCTGTCATCTGGCCTT-3′	330
hexA (expression)	5′-GAGAATGCTCGCTCAGGTAA-3′	5′-TCACATGAGGAGCTTCAGGA-3′	421
1 (*patA* forward primer + transposon primer)	5′-TCTTGCTCAGTCCATCATCGAATAT-3′	5′-CCGGGGACTTATCAGCCAACC-3′	1,445
2 (*patA* reverse primer + transposon primer)	5′-CCGCTGTGGATTAGTTCATTTCC-3′	5′-CCGGGGACTTATCAGCCAACC-3′	1,566
3 (*patB* forward primer + transposon primer)	5′-AGAATCCAGTCCAGCGAAAGCT-3′	5′-CCGGGGACTTATCAGCCAACC-3′	1,487
4 (*patB* reverse primer + transposon primer)	5′-GAAAGAACGACCAGATGTTCCAAT-3′	5′-CCGGGGACTTATCAGCCAACC-3′	1,520

TABLE 3. Susceptibility of all strains and isolates to fluoroquinolones and dyes

Agent and efflux inhibitor[a]	MIC (μg/ml) for[b]:										
	Wild-type strain			FQDR isolates		FQ-R isolates		DR isolates		S isolates	
	M3	M4	R6	Range	MIC_{90}	Range	MIC_{90}	Range	MIC_{90}	Range	MIC_{90}
Cip	**1**	**1**	**0.5**	**2–64**	**16**	**1–64**	**8**	**0.25–0.25**	**0.25**	**0.25–0.5**	**0.25**
Cip + res	0.5	0.5	0.5	0.5–32	4	0.5–64	4	0.5–0.5	0.5	0.25–1	0.5
Cip + NaO	1	1	0.1	0.12–64	8	0.12–32	2	0.5–0.5	0.5	0.12–1	0.5
Nor	**4**	**8**	**4**	**16–64**	**32**	**16–32**	**32**	**4–4**	**4**	**2–4**	**4**
Nor + res	2	4	2	1–16	8	2–16	8	1–1	1	1–2	1
EtBr	**2**	**2**	**1**	**4–16**	**8**	**1–8**	**2**	**16–16**	**16**	**1–4**	**2**
EtBr + res	0.5	0.3	0.3	0.12–0.5	0.5	0.12–4	0.5	0.5–0.5	0.5	0.06–0.5	0.25
EtBr + NaO	0.3	0.3	0.1	0.12–2	1	0.06–4	0.5	1–1	1	0.06–1	0.25
Acr	**4**	**4**	**2**	**8–16**	**8**	**2–4**	**4**	**16–16**	**16**	**2–4**	**4**
Acr + res	1	1	1	0.25–2	1	0.25–4	0.5	2–2	2	0.25–2	1
Acr + NaO	0.5	0.5	0.3	0.25–2	1	0.25–2	0.5	2–2	2	0.25–1	0.5

[a] Cip, ciprofloxacin; Nor, norfloxacin; EtBr, ethidium bromide; Acr, acriflavine; res, reserpine; NaO, sodium orthovanadate.
[b] FQDR, resistant to both fluoroquinolones and dyes ($n = 15$); FQ-R, resistant to fluoroquinolones alone ($n = 24$); DR, dye resistant ($n = 2$); S, susceptible to all agents ($n = 16$). MICs of each agent in the absence of an efflux inhibitor are highlighted in boldface.

resulting amplimers were cleaned using a QIAquick PCR purification kit (Qiagen), and the products were eluted in 30 μl of *Ultra*Pure distilled water (Gibco), separated by 1% agarose gel electrophoresis (100 V for 35 min), and then quantified using GeneTools software (Syngene) and Hyperladder 1 (Bioline) as a quantification standard. The PCR sequencing reaction was performed using a BigDye terminator version 3.1 cycle sequencing kit (Applied Biosytems Ltd., United Kingdom) following the protocol outlined by the Functional Genomics Laboratory (School of Biosciences, University of Birmingham, Birmingham, United Kingdom). The sequences were read on an ABI Prism 3700 DNA analyzer, and the data analyzed using Chromas (chromatogram evaluation; Technelysium Pty. Ltd.) and GeneDoc tools (22) (DNA-protein translation).

***In vitro mariner* transposon mutagenesis of *patA* and *patB*.** Insertional inactivation of *patA* and *patB* was performed essentially as described by Garvey and Piddock (10). In brief, *patA* and *patB* PCR amplimers from R6 were used as targets for transposition and were inactivated by transfer of the *magellan2* minitransposon catalyzed by the *Himar1* transposase, as described previously (17). The transposition products were repaired (1) and transformed into target isolates as described below. Two test PCRs were used to confirm the insertion of the *magellan2* transposon, as described previously (10).

Insertional inactivation of *patA* and *patB* in clinical isolates of *S. pneumoniae*. Isolates were incubated at 37°C in 5% CO_2 until an OD_{550} of 0.4 was reached (mid-logarithmic phase growth). The cultures were then diluted 1:20 in competence medium (Todd-Hewitt broth [THB; Oxoid, Basingstoke, United Kingdom], 1 mM calcium chloride [BDH, Poole, United Kingdom], 0.2% bovine serum albumin [Sigma-Aldrich Company Ltd., Poole, United Kingdom] and 100 ng/ml competence-stimulating peptide [CSP] 1 or 2 [Perbio Science United Kingdom Limited, Chester, United Kingdom]). Immediately after the addition of CSP 1 or 2, 1 μg of either the M246 PCR amplimer *patA*::*magellan2* or the M240 PCR amplimer *patB*::*magellan2* was added to 500 μl of the pneumococcal suspension and the reaction mixture incubated statically at 37°C in air for 150 min. In parallel, a duplicate mixture in which the DNA was replaced with an equivalent volume of sterile water or chromosomal DNA from a wild-type control strain, such as R6 (to provide a spontaneous mutation control) was incubated. After incubation, 100 μl of the transformation reaction mixture was inoculated onto Columbia blood agar (Oxoid, Basingstoke, United Kingdom) containing spectinomycin at 100 mg/liter (the antibiotic resistance cassette in *magellan2* codes for spectinomycin resistance) and incubated overnight at 37°C in 5% CO_2. Transformants growing on spectinomycin-containing agar were then subcultured onto fresh Columbia blood agar medium and incubated at 37°C in 5% CO_2 overnight. Spontaneous mutations giving rise to spectinomycin resistance were detected by inoculating the transformation reaction mixture containing no DNA. Any colonies growing on the selective medium in the absence of donor DNA invalidated the transformation experiment.

RESULTS

Fifteen of 57 clinical isolates are resistant to both fluoroquinolones and dyes. Three wild-type control strains were used throughout this study (10). M3 and M4 are NCTC type strains that produce a capsule and are susceptible to norfloxacin; the addition of reserpine had minimal effect upon susceptibility to fluoroquinolones or other agents. Strain R6 does not produce a capsule and is susceptible to norfloxacin. The wild-type strains of *S. pneumoniae* R6, M3, and M4 showed the typical susceptibility of this species to all agents tested (Table 3).

The MICs of antibiotics for 46 of the clinical isolates have been described previously, detailing their phenotype and the occurrence of mutations in the quinolone resistance-determining regions (QRDRs) of the topoisomerase genes (27). In the present study, the susceptibility of all isolates to two fluoroquinolone antibiotics, ciprofloxacin and norfloxacin, and two dyes, ethidium bromide and acriflavine, was determined in parallel (Table 3). Isolates were defined as fluoroquinolone resistant (FQ-R) if they were resistant to either ciprofloxacin (MIC ≥ 2 μg/ml) or norfloxacin (MIC ≥ 16 μg/ml) according to the BSAC guidelines. BSAC breakpoint concentrations are not available for ethidium bromide and acriflavine, so isolates were defined as dye resistant (DR) if the MICs of both dyes were two or more dilutions higher than for the control strain R6 (MIC of ≥4 μg/ml for ethidium bromide and ≥8 μg/ml for acriflavine). Of the 57 clinical isolates in the study, 15 were resistant to both fluoroquinolones and dyes (FQDR), 24 were FQ-R only, two were DR only, and the remaining 16 were sensitive to all agents tested (S).

The MICs of these agents for these isolates were also measured in the presence of 20 μg/ml of the efflux pump inhibitor reserpine. The MICs of the dyes for all 15 FQDR isolates were reduced by reserpine, and the fluoroquinolone MICs were reduced by reserpine for 10 isolates. Of the 24 isolates that were FQ-R only, the MICs of dyes were reduced for 21 isolates, and fluoroquinolone MICs were reduced for 13. Reserpine reduced the MICs for the two isolates that were resistant to dyes only. Finally, the MICs of fluoroquinolones were reduced by reserpine in 8 of the 16 sensitive strains, and the MICs of dyes were reduced in 15. Similar reductions in MICs were observed with the addition of 50 μM sodium orthovanadate.

Of the 15 FQDR isolates, only one isolate did not possess mutations in the QRDRs of the topoisomerase genes *parC*,

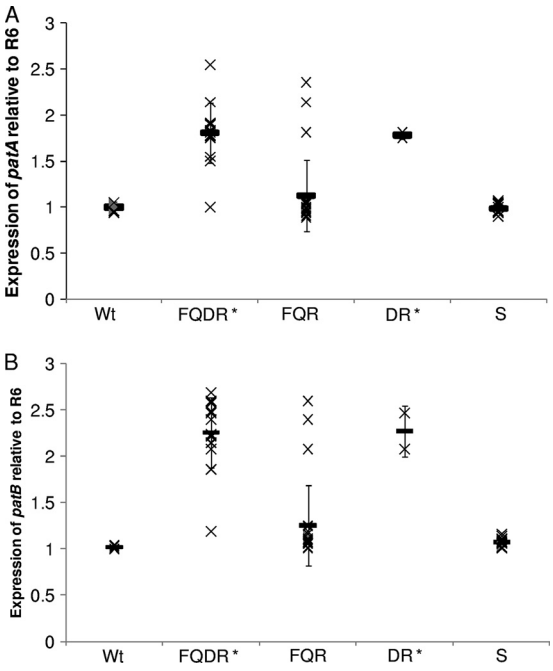

FIG. 1. Fold expression of *patA* (A) and *patB* (B) relative to that of R6, measured by c-RT-PCR. Crosses represent results for individual isolates, and solid lines represent the mean expression level for each group. Error bars show standard deviations. Wt, wild type; FQDR, isolates resistant to both fluoroquinolones and dyes ($n = 15$); FQ-R, isolates resistant to fluoroquinolones only ($n = 24$); DR, isolates resistant to dyes only ($n = 2$); S, isolates sensitive to all agents tested ($n = 16$). Asterisks denote groups of isolates for which mean levels of expression are significantly different from the results for the wild type ($P < 0.01$).

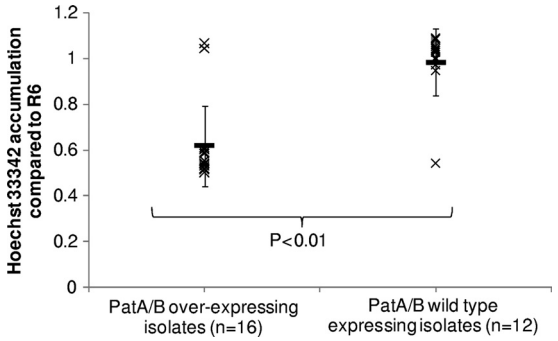

FIG. 2. Accumulation of Hoechst 33352 by representative isolates of each group compared to that by R6. Crosses represent results for individual isolates, and solid lines represent the mean accumulation of Hoechst by each group. Error bars show standard deviations.

parE, *gyrA*, and *gyrB*. The remaining 14 isolates possessed mutations in one ($n = 4$), two ($n = 4$), or three ($n = 6$) of these genes. All 24 of the FQ-R isolates possessed mutations in one ($n = 8$), two ($n = 11$), or three ($n = 5$) QRDRs. Neither of the DR isolates contained mutations in these genes. One of the fluoroquinolone- and dye-susceptible isolates had a lysine-to-asparagine mutation in the *parC* gene. This mutation was also found in combination with other mutations in two FQ-R isolates and two FQDR isolates and in isolation in one FQDR isolate.

Nineteen of 57 clinical isolates overexpress *patA* and *patB*. To determine whether overexpression of *patA* and/or *patB* was associated with a particular phenotype, the expression of *patA* and *patB* in all strains was measured by c-RT-PCR (Fig. 1). In total, 19 isolates expressed both *patA* and *patB* at levels significantly higher than those of the control strain R6. In all cases, *patA* and *patB* were overexpressed together, suggesting coregulation. Fourteen of the 15 FQDR isolates and both of the DR-only isolates overexpressed *patA* and *patB*, while only 3 of the 24 isolates that were FQ-R only did so. Overexpression of these two genes was not found in any of the fluoroquinolone- and dye-susceptible isolates.

Isolates overexpressing *patA* and *patB* accumulate significantly less Hoechst 33342. Overexpression of *patA* and *patB* has been associated with increased efflux (10). To confirm that the same was true for the clinical isolates in this study, the accumulation of the dye Hoechst 33342 was measured in 28 randomly chosen clinical isolates, including isolates from all groups. Out of 17 isolates overexpressing *patA* and *patB*, 15 showed significantly reduced accumulation of Hoechst 33342 in comparison to its accumulation in R6 (Fig. 2). In all isolates accumulating lower levels of Hoechst 33342, accumulation was increased in the presence of 20 μg/ml reserpine. Of the 11 isolates tested that did not overexpress *patA* and *patB*, all accumulated wild-type levels of Hoechst 33342 (Fig. 2).

Inactivation of *patA* and *patB* in the overexpressing clinical isolates confers loss of resistance. To confirm whether increased expression of *patA* and/or *patB* conferred the FQDR phenotype, single mutants in which *patA* or *patB* was inactivated were constructed in 16 representative isolates that overexpressed *patA* and *patB* (consisting of 8 FQDR isolates, 2 DR isolates, and 1 FQ-R isolate) and 4 FQ-R isolates and one sensitive isolate that did not. When *patA* was inactivated, the *magellan2* minitransposon had inserted between nucleotides 833 and 834 of *patA*, while when *patB* was inactivated, the minitransposon had inserted between nucleotides 1082 and 1083 of *patB*. The susceptibilities of the inactivated strains to ciprofloxacin, ethidium bromide, and acriflavine were determined and compared to those of the parental isolates (Table 4). Inactivation of either *patA* or *patB* conferred hypersusceptibility to ethidium bromide in 13 of the 15 drug-resistant isolates, while for one of the FQDR isolates and one of the FQ-R isolates, the hypersusceptibility phenotype was only observed when *patA* was inactivated. Ciprofloxacin resistance was reduced by 2 to 4 dilutions (4- to 8-fold) by inactivation of *patA* and *patB* in five of the eight FQDR isolates and four of the five FQ-R isolates, although in no case was the fluoroquinolone susceptibility returned to wild-type levels. For all but two of the inactivated strains, reserpine and sodium orthovanadate did not change the MIC of any agent by more than one dilution.

Susceptibility to levofloxacin is not affected by lack of or overexpression of the PatAB transporter in these isolates. For a representative subset of isolates (consisting of five FQDR isolates, three FQR isolates, one DR isolate, and three S isolates) and their corresponding mutants in which *patA* or *patB* have been inactivated, the MICs of levofloxacin were determined as described above in the presence or absence of 20 μg/ml reserpine or 50 μM sodium orthovanadate. According

TABLE 4. Susceptibility of isolates in which *patA* or *patB* has been inactivated to ciprofloxacin and ethidium bromide[a]

Isolate	Group	Gene inactivated	MIC (μg/ml) of:					
			Cip	Cip + res	Cip + NaO	EtBr	EtBr + res	EtBr + NaO
R6	Control		0.5	0.5	0.25	1	0.25	0.12
M42	FQDR		**64**	**32**	**16**	**8**	**0.5**	**1**
M298		*patA*	8	8	8	0.5	0.5	0.5
M299		*patB*	8	8	8	0.5	0.5	0.5
M45	FQDR		**4**	**1**	**1**	**4**	**0.5**	**1**
M45A		*patA*	1	1	1	0.5	0.5	0.5
M45B		*patB*	1	1	1	0.5	0.5	0.5
M50	FQDR		**64**	**32**	**32**	**4**	**0.5**	**1**
M50A		*patA*	4	4	4	0.5	0.5	0.5
M50B		*patB*	4	4	4	0.5	0.5	0.5
M74	FQDR		**64**	**32**	**64**	**4**	**0.5**	**1**
M301		*patA*	8	8	8	0.5	0.5	0.25
M305		*patB*	8	8	8	0.5	0.5	0.25
M86	FQDR		**2**	**0.5**	**0.5**	**4**	**0.5**	**0.5**
M86A		*patA*	1	0.5	0.5	0.5	0.5	0.5
M86B		*patB*	1	0.5	0.5	0.5	0.5	0.5
M87	FQDR		**32**	**8**	**16**	**4**	**0.5**	**1**
M87A		*patA*	8	8	8	0.5	0.5	0.5
M87B		*patB*	8	8	8	0.5	0.5	0.5
M296	FQDR		**2**	**0.5**	**8**	**8**	**0.5**	**2**
M304		*patA*	4	1	2	0.5	0.5	0.25
M310		*patB*	4	1	2	16	0.5	8
M297	FQDR		**8**	**2**	**8**	**8**	**0.5**	**1**
M311		*patA*	8	4	4	0.5	0.5	8
M311B		*patB*	8	4	4	0.5	0.5	8
M79	FQ-R		**32**	**32**	**16**	**2**	**0.5**	**0.5**
M316		*patA*	8	8	8	0.25	0.5	0.5
M317		*patB*	8	8	8	0.5	0.5	0.5
M83	FQ-R		**64**	**64**	**1**	**8**	**4**	**4**
M318		*patA*	16	16	16	0.5	0.5	0.5
M319		*patB*	16	16	16	0.5	0.5	0.5
M97	FQ-R		**8**	**4**	**8**	**4**	**0.5**	**1**
M302		*patA*	4	4	4	0.5	0.5	0.5
M306		*patB*	4	4	4	0.25	0.5	0.5
M98	FQ-R		**32**	**2**	**32**	**4**	**0.12**	**1**
M314		*patA*	8	8	8	0.5	0.5	0.5
M307		*patB*	8	8	8	2	0.5	2
M100	FQ-R		**8**	**2**	**1**	**4**	**0.25**	**1**
M315		*patA*	2	1	0.12	0.25	0.12	0.25
M308		*patB*	2	1	0.12	0.25	0.25	0.5
M55	DR		**0.25**	**0.5**	**0.5**	**16**	**0.5**	**1**
M300		*patA*	2	2	2	0.5	0.5	0.5
M55B		*patB*	2	2	2	0.5	0.5	0.5
M46	DR		**0.25**	**0.5**	**0.5**	**16**	**0.5**	**1**
M46A		*patA*	0.12	0.12	0.12	0.5	0.5	0.5
M46B		*patB*	0.12	0.12	0.12	0.5	0.5	0.5
M295	S		**1**	**0.5**	**0.25**	**2**	**0.25**	**0.5**
M303		*patA*	2	1	0.12	1	0.5	1
M309		*patB*	4	1	2	16	0.5	8

[a] Abbreviations are as defined for Table 3. MICs of each agent for the parent strains in which *patA* and *patB* are active are highlighted in boldface.

to the BSAC guidelines, all of the FQDR and FQR isolates were resistant to levofloxacin (MIC $>$ 2 μg/ml), whereas all DR and S isolates were susceptible (MIC $\leq$ 2 μg/ml). These MICs were not reduced by more than one dilution by either of the efflux inhibitors or by the inactivation of *patA* or *patB* (data not shown).

No mutations were found in *patA* or *patB* or the upstream region of the MDR clinical isolates. The protein-coding regions of *patA* and *patB* from 21 randomly selected clinical isolates were sequenced to rule out the possibility that any increases in PatA and PatB activity could be due to a functional change in the transporter protein itself. In all isolates tested, the nucleotide sequences of *patA* and *patB* were identical to those of the wild-type strains R6, M3, and M4.

It was hypothesized that the overexpression of *patA* and *patB* could be due to a nucleotide change in the *patAB* promoter region that increases the activity of the promoter. To check this, a 200-bp region upstream of the start site of *patA* was sequenced from the same set of 21 isolates. Again, no differences in sequence compared to that of the wild type were found in any of the isolates tested.

No differences in growth rate were observed between the clinical isolates and control strains. To determine whether the overexpression of *patA* and *patB* confers a fitness cost on

the clinical isolates that also carry topoisomerase mutations, the growth kinetics of a randomly chosen selection of isolates (consisting of six isolates that overexpressed *patA* and *patB* and eight that did not) were determined by measuring the optical density of cultures over time. The wild-type strain R6 was used as a control. The average generation time of R6 under the conditions used was 55 min. Generation times ranged from 29 min to 74 min for the isolates that did not overexpress *patA* and *patB* and from 35 to 100 min for the isolates that did (data not shown). There was no statistically significant difference in growth rates between the two groups, indicating that overproducing PatA and PatB confers no gross fitness costs on the clinical isolates under the conditions tested.

DISCUSSION

Overexpression of PatA and PatB has been previously shown to be responsible for multidrug resistance phenotypes of laboratory-selected ciprofloxacin- and efflux pump inhibitor-resistant mutants (10, 21). However, to date, this resistance mechanism has not been shown to be relevant in a clinical setting. Here, we report that in a set of 57 clinical isolates, 14 out of 15 isolates that were resistant to both fluoroquinolones and dyes overexpressed *patA* and *patB*. In addition, 3 of 24 isolates that were resistant to fluoroquinolones only and 2 isolates that were resistant to dyes only also overexpressed *patA* and *patB*. This suggests that overexpression of *patA* and *patB* occurs in a clinical setting and, in most cases, appears to confer a phenotype similar to that observed in the laboratory mutants. In all cases, *patA* and *patB* were overexpressed together, suggesting that they are likely to be coregulated.

As observed in laboratory mutants, overexpression of *patA* and *patB* in these clinical isolates confers an efflux phenotype, demonstrated by the decreased accumulation of Hoechst 33342 observed in isolates with increased expression of *patA* and *patB*. The majority of isolates overexpressing *patA* and *patB* showed reduced susceptibility to both fluoroquinolones and dyes, which was partially (fluoroquinolones) or fully (dyes) reversible by the addition of an efflux inhibitor. This is consistent with an increase in efflux conferred by a multidrug efflux pump.

The inactivation of *patA* conferred hypersusceptibility to ethidium bromide and acriflavine in all strains tested, regardless of whether *patA* and *patB* were overexpressed. This suggests that the PatA and PatB transporters are required for low-level, intrinsic resistance to these agents, even when expressed at wild-type levels. The MICs of ciprofloxacin in 9 of 13 isolates resistant to fluoroquinolones were reduced by 2 or more dilutions when *patA* was inactivated. The MICs were not reduced to the wild-type levels observed for R6, presumably due to the coexisting presence of mutations in the QRDRs of the fluoroquinolone-resistant isolates. The fact that this decrease in the ciprofloxacin MIC was not observed in all fluoroquinolone-resistant isolates may indicate that the relative contribution of QRDR mutations and efflux to ciprofloxacin resistance varies between different genetic backgrounds. For some isolates, the inactivation of *patB* alone did not result in increased sensitivity to dyes. This could suggest that PatA is capable of providing some efflux activity when expressed alone. In a previous study on reserpine-selected mutants overexpressing *patA* and *patB* (10), the inactivation of *patB* reversed the multidrug-resistant phenotype but was unable to restore reserpine sensitivity. To resolve this, more work is needed to precisely define the roles of PatA and PatB in the formation of the efflux pump.

The inactivation of *patA* or *patB* or the addition of efflux inhibitors did not increase susceptibility to levofloxacin in any isolate tested. This result is consistent with those of previous reports suggesting that levofloxacin, as a hydrophobic fluoroquinolone, is poorly transported by the PatA and PatB transporters compared to their transport of the hydrophilic fluoroquinolones ciprofloxacin and norfloxacin (4).

Analysis of the DNA sequences of the QRDRs of *parC*, *parE*, *gyrA*, and *gyrB* of 37 of the 39 fluoroquinolone-resistant isolates in this study revealed that 36 isolates contain mutations in one ($n = 11$), two ($n = 14$), or three ($n = 11$) genes. While the origins of these isolates are not fully known, it is clear that many must have been exposed to a fluoroquinolone for a prolonged period or repeatedly for these mutations to have accumulated, as it is unlikely that such isolates could have emerged after a single exposure. To obtain a similar mutant in the laboratory would require at least three exposures to a fluoroquinolone. However, one isolate that overexpressed *patA* and *patB* possesses no QRDR mutations. Another isolate has only one (lysine-to-asparagine) mutation in *parC*, which is also found in one of the susceptible isolates, suggesting that it is not involved in conferring fluoroquinolone resistance. These isolates are resistant to fluoroquinolones, which suggests that, although overexpression of *patA* and *patB* is observed in conjunction with QRDR mutations, it is also able to confer resistance to fluoroquinolones independently in clinical isolates.

Previous work showed that laboratory-selected strains that overexpress *patA* and *patB* grow at a similar rate to their parental strains. Similarly, in this study, no significant differences in growth rate were observed between isolates overexpressing and not overexpressing *patA* and *patB*. This suggests that this overexpression does not confer a fitness cost on the organism under the conditions used.

This study provides evidence to show that overexpression of *patA* and *patB* is found in clinical isolates and, as in laboratory mutants, leads to reduced susceptibility to the hydrophilic fluoroquinolones ciprofloxacin and norfloxacin, as well as other agents. Overexpression of the transporter did not affect growth under laboratory conditions, suggesting that this resistance mechanism may carry a low fitness cost and so may be easily selected in a clinical setting.

ACKNOWLEDGMENTS

This work was supported by a Bristol Myers Squibb Unrestricted Grant in Infectious Diseases to L.J.V.P. A.J.B. is supported by an MRC DTG to the University of Birmingham.

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