ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2002, p. 808–812 Vol. 46, No. 3
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.3.808–812.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Expression of Efflux Pump Gene *pmrA* in Fluoroquinolone-Resistant and -Susceptible Clinical Isolates of *Streptococcus pneumoniae*

Laura J. V. Piddock,* Maggie M. Johnson, S. Simjee†, and L. Pumbwe

Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom

Received 7 May 2001/Returned for modification 18 July 2001/Accepted 11 December 2001

Thirty-four ciprofloxacin-resistant (MIC ≥ 2 μg/ml) and 12 ciprofloxacin-susceptible clinical isolates of *Streptococcus pneumoniae* were divided into four groups based upon susceptibility to norfloxacin and the effect of reserpine (20 μg/ml). The quinolone-resistance-determining regions of *parC*, *parE*, *gyrA*, and *gyrB* of all ciprofloxacin-resistant clinical isolates were sequenced, and the activities of eight other fluoroquinolones, acriflavine, ethidium bromide, chloramphenicol, and tetracycline in the presence and absence of reserpine were determined. Despite a marked effect of reserpine upon the activity of norfloxacin, there were only a few isolates for which the activity of another fluoroquinolone was enhanced by reserpine. For most isolates the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine despite the lack of effect of this efflux pump inhibitor on fluoroquinolone activity. The strains that were most resistant to the fluoroquinolones were predominantly those with mutations in three genes. Expression of the gene encoding the efflux pump PmrA was examined by Northern blotting (quantified by quantitative competitive reverse transcriptase PCR) and compared with that of *S. pneumoniae* R6 and R6N. Within each group there were isolates that had high-, medium-, and low-level expression of this gene; however, increased expression was not exclusively associated with those isolates with a phenotype suggestive of an efflux mutant. These data suggest that there is another reserpine-sensitive efflux pump in *S. pneumoniae* that extrudes ethidium bromide and acriflavine but not fluoroquinolones.

Fluoroquinolone resistance in *Streptococcus 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 (for examples, see references 7 to 11). Mutations in the genes *parE* and *gyrB*, encoding the B subunits of these proteins, are reported less frequently (e.g., reference 8). Resistance to norfloxacin can also be due to active efflux (2, 6). This mechanism usually gives rise to smaller increases in the MIC of norfloxacin than in the MIC of this agent for strains containing mutations affecting DNA topoisomerase IV and/or DNA gyrase. The MICs of some fluoroquinolones are decreased in the presence of reserpine (an efflux pump inhibitor), leading to the conclusion that this suggests an active efflux system (3, 4, 12). Four studies have described mutant *S. pneumoniae* strains with phenotypes suggestive of an efflux mutant (2, 6, 13, 16), and in 1999 Gill et al. (6) described a putative efflux pump for fluoroquinolones encoded by the gene *pmrA*. This pump mediated low-level resistance to norfloxacin, ethidium bromide, and acriflavine.

There were several objectives of the present study: (i) to determine the level of expression of *S. pneumoniae pmrA* in wild-type and ciprofloxacin-resistant clinical isolates of *S. pneumoniae*; (ii) to determine the susceptibility of these isolates to newer fluoroquinolones in the presence and absence of reserpine; (iii) to determine the DNA sequence of the quinolone-resistance-determining regions (QRDRs) of *parC*, *parE*, *gyrA*, and *gyrB* for all resistant isolates; and (iv) to determine whether expression of *pmrA* is associated with higher MICs of fluoroquinolones with or without a mutation(s) in a topoisomerase gene.

* Corresponding author. Mailing address: Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 0121-414-6966. Fax: 0121-414-3454. E-mail: l.j.v.piddock@bham.ac.uk.

† Present address: U.S. Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD 20708.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. pneumoniae* M4 (NCTC 7465 type 1), *S. pneumoniae* M3 (NCTC 7466 type 2), and *S. pneumoniae* R6 and R6N (6) were used throughout as control strains. M3 and M4 produce a capsule whereas R6 and R6N do not. Strain R6N is a strain with an efflux phenotype derived from strain R6 transformed by Gill et al. (6) with DNA from 1N27, a spontaneous norfloxacin-resistant laboratory mutant of ATCC 49619. Clinical isolates were obtained from a variety of sources: 16 isolates were from MRL Pharmaceutical Services (8); 15 isolates were from the Lung Investigation Unit, University Hospital, Birmingham, United Kingdom (12); and 13 clinical isolates were from the Centers for Disease Control and Prevention, Atlanta, Ga. (9). All strains were maintained at −80°C on Protect beads (Protect Bacterial Preservers, TSC Ltd., Heywood, United Kingdom) without antibiotic and grown overnight in brain heart infusion broth (Unipath, Basingstoke, United Kingdom) incubated at 37°C in 5% CO_2. The solid medium was Isosensitest agar supplemented with 5% defibrinated horse blood. The identification of each species was confirmed by Gram stain and optochin sensitivity, and the presence of capsule was determined with the Slidex Pneumo-Kit (bioMeriéux SA). Thirty of the clinical isolates were shown to be capsule producers.

Antibiotics and susceptibility determination. The MIC of each antibiotic for each strain was determined by a standard agar doubling dilution method (1). All of the following antibiotics were gifts and were made up and used according to the manufacturers' instructions: ciprofloxacin and moxifloxacin (Bayer AG, Leverkusen, Germany); sparfloxacin (Rhone DPC Europe, Paris, France); grepafloxacin (Glaxo Wellcome, London, United Kingdom); gatifloxacin (Grunenthal GmbH, Stolberg, Germany); clinafloxacin (Parke-Davis Warner Lambert, Ann Arbor, Mich.); levofloxacin (Aventis, Strasbourg, France); sitafloxacin (Daiichi, Tokyo, Japan); norfloxacin, tetracycline, chloramphenicol, and acriflavine (Sigma); and ethidium bromide (BDH). Reserpine (Sigma) was added to a final concentration of 20 μg/ml.

PCR and DNA sequencing. The QRDRs of *gyrA* (nucleotides [nt] 137 to 408), *gyrB* (nt 1096 to 1553), *parC* (nt 104 to 465), and *parE* (nt 981 to 1334) of each

strain were amplified by PCR from a whole-cell lysate. The primers were designed with Primer computer software from the DNA sequences of each gene available in the EMBL database (GenBank accession numbers: *parC* and *parE*, X95717; *gyrA*, X95718; *gyrB*, Z67740). The DNA sequences of all amplimers were determined by MWG Biotech.

Expression of *pmrA*. Northern blotting was performed with RNA (20 μg) extracted with Trizol (Gibco BRL) and as described in the Amersham Gene Images kits. To remove any contaminating DNA, the samples were treated with DNase I (Roche Diagnostics; catalog no. 776785), and complete removal was verified by direct PCR with the RNA as a template. Sample-to-sample RNA uniformity was determined by examining 16S rRNA expression in parallel. The PCR was used to generate a 558-bp fragment of the structural gene for PmrA (GenBank accession no. AJ007367; nt 431 to 988), which was used as the probe. The intensities of each band on the Northern blot were determined by computer scanning and image analysis, and scores to the nearest full integer were assigned. Growing cells to different growth stages showed that expression of *pmrA* was not growth dependent (data not shown); despite this finding cells were grown to early logarithmic phase for all RNA preparations, as this is the phase which produces cells that give the most reliable accumulation data (L. J. V. Piddock, unpublished data). RNA was prepared for all isolates on at least three separate occasions, and Northern blotting was performed on at least three separate occasions. For quantitative competitive (QC) reverse transcriptase PCR (RT-PCR), total cellular RNA (5 μg) was reverse transcribed into cDNA as previously described (5, 14). An internal competitor DNA standard for *pmrA* was generated by PCR amplification of genomic DNA using primer PmrA/R (GCATTGGCACAGAG GAGATA) and the 40-mer forward primer Pmr40mer (TGTTCCTAATGCAA CGGCACTGCAGGTACTCTAACTGGT). RT-PCR was performed on the RNA template to generate cDNA of *pmrA*. Competitor DNA was added at concentrations from 0.01 to 2 pg to replicate tubes containing identical aliquots of cDNA. The PCR was performed on the competitor DNA-cDNA mixture using primers PmrA/F (TGTTCCTAATGCAACGGCAC) and PmrA/R. The two products were separated by polyacrylamide gel electrophoresis, the gel was silver stained, and the amplimers were quantified by densitometry. The concentration of competitor DNA at which the two amplimers were of equal density was taken to be the concentration of the target cDNA. The banding intensities were determined using IPLab Spectrum software, which gave band intensities as peak values, or Adobe PhotoShop version 4.0 software, which gave band intensities as pixel area means ± standard deviations.

RESULTS

Susceptibility of strains to fluoroquinolones and other agents. Four control stains were used throughout this study: M3 and M4 were NCTC type strains that produced a capsule and were susceptible to norfloxacin; addition of reserpine had a minimal effect upon susceptibility to fluoroquinolones or other agents. Neither strain R6N (derived from R6) nor strain R6 produced a capsule. Strain R6N had the published phenotype of low-level resistance to norfloxacin with corresponding decreased susceptibility to ethidium bromide and acriflavine.

The clinical isolates were divided into four groups based upon their susceptibility to norfloxacin and the effect of reserpine. Group 1 consisted of 14 isolates which required ≥16 μg of norfloxacin/ml for inhibition. In the presence of 20 μg of reserpine/ml the MIC of norfloxacin was reduced by fourfold or more. Group 2 consisted of four isolates which required 4 μg of norfloxacin/ml for inhibition and for which the MIC was also reduced with reserpine (by ≥4-fold). Group 3 consisted of 20 isolates which required 16 μg of norfloxacin/ml for inhibition but for which reserpine did not lower the MIC by more than 1 dilution. Group 4 consisted of eight isolates which were susceptible to norfloxacin (MIC ≤ 4 μg/ml) and for which reserpine did not lower the MIC by more than 1 dilution.

There were some highly resistant isolates in group 1 requiring 64 μg of ciprofloxacin/ml for inhibition (Table 1). While some of the isolates of this group remained susceptible to some of the other fluoroquinolones, only clinafloxacin and sitafloxacin remained active against all the isolates. Despite a marked effect upon the activity of norfloxacin by reserpine, there were only a few isolates for which the activity of another fluoroquinolone was enhanced by reserpine. Only for two isolates in this group were the MICs of ciprofloxacin lowered by fourfold. For the majority of isolates the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine. The MIC of chloramphenicol was unaffected by the presence of reserpine, and the MIC of tetracycline was lowered by only 1 dilution, if at all. Of interest, the isolates in group 1 were more susceptible to all fluoroquinolones than were those in group 3 (as shown by the MIC at which 50% of the isolates tested were inhibited [MIC_{50}]).

The four isolates of group 2 were susceptible to ciprofloxacin; however, the MIC of this agent was lowered by no more than 1 dilution in the presence of reserpine. In the presence of reserpine the MICs of all other fluoroquinolones were lowered by no more than 1 dilution. For three isolates the MIC of ethidium bromide was lowered by reserpine, and for one isolate the MIC of tetracycline was also reduced.

All isolates in group 3 required 16 μg or more of norfloxacin/ml for inhibition. Reserpine had a minimal effect upon these MICs. Four isolates were much more resistant to ciprofloxacin (MICs of 32 to 128 μg/ml) than were any of the other isolates and also much less susceptible, if not resistant, to all other fluoroquinolones in this study. Reserpine had no effect on the activity of any of the other fluoroquinolones for the isolates in this group. However, the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine despite the lack of effect of this efflux pump inhibitor on fluoroquinolone activity. The isolates that were most resistant to the fluoroquinolones were predominantly those with mutations in three genes.

The eight isolates in group 4 were all susceptible to norfloxacin and ciprofloxacin, and the MICs were lowered at most by only 1 dilution in the presence of reserpine. Reserpine had little or no effect upon the activities of any of the fluoroquinolones. However, as for the other groups the MICs of acriflavine and ethidium bromide were lowered by ≥4-fold in the presence of reserpine.

Mutations in the QRDRs of topoisomerase genes. The QRDRs of *parC*, *parE*, *gyrA*, and *gyrB* were determined for all control strains and those that were resistant to norfloxacin. The four control strains had wild-type DNA sequences for all four genes. All resistant strains harbored one or more mutations in the QRDR of one or more genes.

Ten of the isolates in group 1 contained a mutation in *parC* (most substituting phenylalanine for serine 79). Four isolates had a mutation in *parE* (three of which substituted valine for isoleucine 460). Eight isolates had a mutation in *gyrA* (all substituting phenylanaline for serine 81), and none had a mutation in *gyrB*. Four isolates contained a mutation(s) in only a single gene (two had a mutation in *parC*, and two had a mutation in *gyrA*). Six isolates had a mutation in two genes (one had a mutation in *parC* and *parE*, three had a mutation in *parC* and *gyrA*, and one had a mutation in *parE* and *gyrA*). Two isolates had a mutation in three genes (*parC*, *parE*, and *gyrA*).

Seventeen of the isolates in group 3 contained a mutation in *parC* (11 substituting phenylalanine for serine 79). Eight isolates had a mutation in *parE* (two substituting asparagine for

TABLE 1. Summary of MIC data for the four groups of clinical strains compared with control strains[a]

Drug	MIC for control strain:				Group 1			Group 2 (range)	Group 3			Group 4 (range)
	M3	M4	R6	R6N	Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}	
NOR	2	4	2	4	16–32	32	32	4	16–32	32	32	2.0–4.0
NOR + R	1	2	1	1	2–8	4	8	1–2	8.0–16	16	16	1.0–2.0
CIP	1	1	0.5	1	2–64	4	64	0.5–1	2.0–128	16	64	0.5–2.0
CIP + R	0.5	1	0.5	0.5	0.5–64	2	64	0.25–2	2.0–128	8	64	0.25–1.0
CLIN	0.06	0.06	0.03	0.06	0.03–0.5	0.25	0.5	0.06	0.06–2.0	0.5	0.5	0.03–0.25
CLIN + R	0.03	0.03	0.03	0.06	0.03–0.25	0.12	0.25	0.03–0.06	0.06–1.0	0.25	0.5	0.03–0.25
GATI	0.5	0.5	0.12	0.25	0.25–8	0.5	8	0.12	0.5–16	4	8	0.25–1.0
GATI + R	0.25	0.25	0.12	0.12	0.12–8	0.5	8	0.12–0.25	0.5–8	2	8	0.12–25
GRP	0.25	0.25	0.12	0.12	0.25–16	2	16	0.12–0.25	0.5–16	2	16	0.06–0.25
GRP + R	0.12	0.25	0.06	0.06	0.12–16	1	16	0.12	0.25–16	2	16	0.03–0.25
LEV	1	1	0.25	0.25	1–64	4	16	1	2.0–32	8	32	0.5–1.0
LEV + R	0.5	1	0.25	0.25	1–16	4	8	0.5–1	2.0–32	8	16	0.5–1.0
MXF	0.25	0.25	0.06	0.06	0.25–4	1	4	0.06–0.25	0.25–8	1	4	0.12–0.25
MXF + R	0.12	0.25	0.06	0.06	0.12–4	0.25	2	0.06–0.12	0.12–4.0	0.5	4	0.06–0.25
SITA	0.03	0.03	0.03	0.06	0.03–0.5	0.25	0.25	0.03–0.06	0.03–1.0	0.25	0.5	0.03–0.25
SITA + R	0.03	0.03	0.03	0.03	0.03–0.25	0.06	0.25	0.03–0.06	0.03–0.5	0.25	0.25	0.03–0.06
SPAR	0.25	0.25	0.06	0.06	0.25–16	1	16	0.06–0.5	0.5–16	2	16	0.06–1.0
SPAR + R	0.12	0.06	0.06	0.06	0.25–16	0.5	16	0.12–0.25	0.25–16	2	8	0.06–0.5
ACR	4	4	2	8	2–16	4	16	1–4	2.0–16	4	8	4.0–8.0
ACR + R	1	1	0.5	0.5	0.5–4	1	2	0.5–1	1.0–2.0	1	2	1.0–2.0
CHL	2	2	0.06	0.06	2–16	2	16	1–32	2.0–16	2	16	2.0–16
CHL + R	2	2	0.06	0.06	2–16	2	16	1–32	2.0–16	2	16	2.0–16
ETBR	1	2	8	32	1–32	4	32	1–2	2.0–16	4	16	1.0–16
ETBR + R	0.5	0.5	1	2	0.12–4	1	4	0.25–1	0.5–2.0	1	2	0.25–2.0
TET	1	2	1	1	0.06–32	0.5	16	0.12–8	0.12–16	2	16	0.06–16
TET + R	0.5	0.5	1	1	0.06–16	0.25	8	0.12–4	0.06–16	2	16	0.06–16

[a] The groups of clinical strains were as follows: group 1, norfloxacin resistant (MIC ≥ 16 μg/ml), MIC lowered by reserpine, n = 14; group 2, norfloxacin sensitive (MIC ≤ 4 μg/ml), MIC lowered by reserpine, n = 4; group 3, norfloxacin resistant (MIC ≥ 16 μg/ml), MIC not lowered by reserpine, n = 20; group 4, norfloxacin sensitive (MIC ≤ 4 μg/ml), MIC not lowered by reserpine, n = 8. Abbreviations: NOR, norfloxacin; R, reserpine; CIP, ciprofloxacin; CLIN, clinafloxacin; GATI, gatifloxacin; GRP, grepafloxacin; LEV, levofloxacin; MXF, moxifloxacin; SITA, sitafloxacin; SPAR, sparfloxacin; ACR, acriflavine; CHL, chloramphenicol; ETBR, ethidium bromide; TET, tetracycline. All values are in micrograms per milliliter.

aspartate 435, five substituting valine for isoleucine 460, and one isolate having both substitutions). Fourteen isolates had a mutation in *gyrA* (10 substituting phenylalanine for serine 81 and 4 substituting tyrosine for serine 81), and none had a mutation in *gyrB*. Three isolates contained a mutation(s) in only a single gene (two had a mutation in *parC*, and one had a mutation in *gyrA*). Six isolates had a mutation(s) in two genes (four had a mutation in *parC* and *gyrA*, and two had a mutation in *parE* and *gyrA*). Seven isolates had a mutation in three genes (six had a mutation in *parC*, *parE*, and *gyrA*, and one had a mutation in *parC*, *gyrA*, and *gyrB*). Some isolates had two mutations in one gene.

In general the highest MICs of fluoroquinolones were seen for those isolates with multiple mutations in the topoisomerase genes. The most resistant isolate had mutations in three genes. For such isolates the activities of sitafloxacin and clinafloxacin were reduced to 0.5 μg/ml.

Expression of *pmrA* in clinical isolates. Northern blotting was used to assess the expression of *pmrA* in all control strains and clinical isolates. A band with heavy density was deemed to have high-level expression and was scored as 4; low-level expression was scored as 1, and medium-level expression was scored as 2 to 3 (Fig. 1). The lack of detectable expression was designated by 0. Strain R6N was shown to have high-level expression of *pmrA*, confirming that R6N overexpresses this gene; wild-type strain R6 had low-level expression. Control strain M3 had medium-level expression, while strain M4 did not express *pmrA*. It has since been shown elsewhere that strain M4 has a large deletion within this gene (L. Weigel, personal communication). Three clinical isolates also did not express *pmrA*. Eight isolates expressed high levels of *pmrA* mRNA. QC RT-PCR was used to quantify the expression of *pmrA* by 18 isolates. Two strains which gave no detectable signal with Northern blotting expressed no *pmrA* mRNA and 0.037 pg of *pmrA* mRNA/μl, respectively. The isolates that gave a low signal (=1) on Northern blotting were divided into three groups by QC RT-PCR: R6 and three isolates expressed 0.011 pg of *pmrA* mRNA/μl, six isolates expressed 0.037 pg of *pmrA* mRNA/μl, and one isolate expressed 0.11 pg of *pmrA*

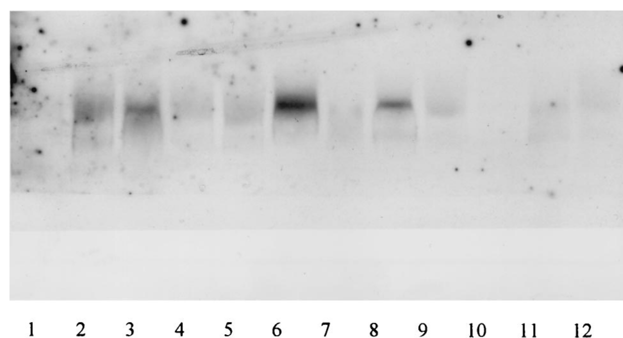

FIG. 1. Northern blot of *pmrA* of clinical isolates of *S. pneumoniae*. Lane 1, R6; lane 2, R6N; lanes 3 to 12, selected clinical isolates showing range of expression of *pmrA*.

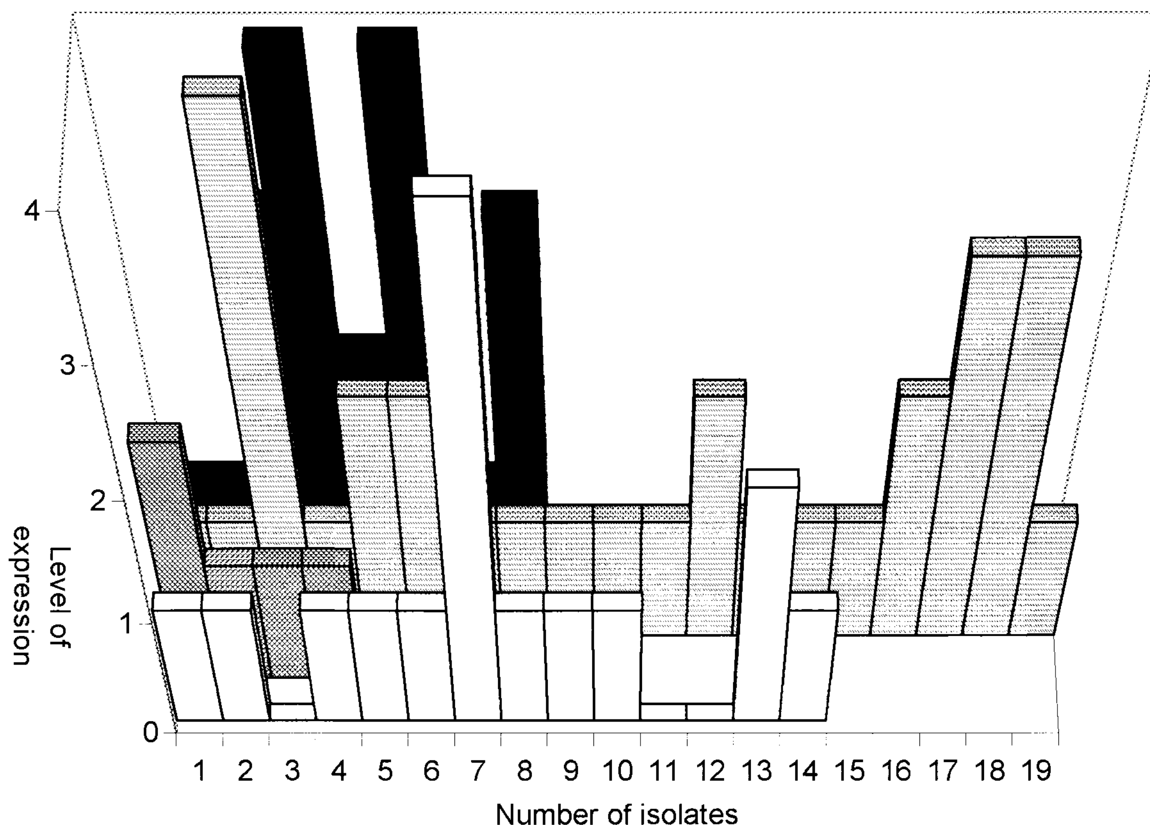

FIG. 2. Expression of *pmrA* by each group of clinical isolates. Open bars, group 1; darkly shaded bars, group 2; lightly shaded bars, group 3; solid bars, group 4. Levels of expression: 0, no detectable expression; 1, low-level expression; 2 to 3, medium-level expression; 4, high-level expression.

mRNA/μl. Three strains (R6N and two isolates) with a high signal (=4) on Northern blotting expressed 1, 0.37, and 0.33 pg of *pmrA* mRNA/μl, respectively. Four clinical isolates which were susceptible to norfloxacin and for which reserpine did not lower the MIC expressed high levels of *pmrA* mRNA. Increased *pmrA* expression was also not associated with those isolates that required the highest concentration of fluoroquinolones for inhibition (Fig. 2). In summary, within each group there were isolates that had high-, medium-, and low-level expression of this gene, and increased expression was not exclusively associated with those isolates with a phenotype suggestive of an efflux mutant.

DISCUSSION

Analyses of the DNA sequences of the QRDRs of *parC*, *parE*, *gyrA*, and *gyrB* revealed no novel mutations. Some isolates contained multiple mutations in one, two, or three genes; while the origins of these isolates are not fully known, it is clear that each bacterium must have been exposed to a fluoroquinolone on multiple occasions for these mutations to have accumulated, as it is extremely 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.

Despite the grouping of the isolates based upon the effect of reserpine on norfloxacin activity, there was no clear association between the groups and *pmrA* expression, as within each group there were isolates that had high-, medium-, and low-level expression of this gene. QC RT-PCR was found to be more accurate than was Northern blotting and, based upon expression of *pmrA* mRNA, further divided the isolates into smaller groups. It had been anticipated that, as strain R6N was selected on the basis of low-level resistance to norfloxacin and the MIC for it was decreased in the presence of reserpine, isolates with a similar phenotype would also overexpress *pmrA*. However, the isolates in this study have shown that this is not the case. In addition, the enhancing effect between norfloxacin and reserpine was seen even for isolates that expressed little or no *pmrA* mRNA. For other bacterial species several efflux pumps have now been described, and so it is suggested that *S. pneumoniae* also possesses more than one efflux pump and that reserpine inhibits another pump in addition to PmrA; this inhibition could give rise to the lower MICs of norfloxacin in the presence of this inhibitor. Reserpine may also interact with multiple efflux pumps with overlapping substrate profiles. It was also interesting that the majority of the clinical isolates were cross resistant to ethidium bromide and acriflavine and that the MICs of these agents were also lowered by fourfold or more by reserpine irrespective of norfloxacin susceptibility. These data suggest that *S. pneumoniae*, irrespective of antibiotic susceptibility, possesses an efflux pump that is inhibited by reserpine, is constitutively expressed, and pumps out ethidium bromide and acriflavine. It is interesting that few of the MICs

of the newer fluoroquinolones were reduced by reserpine. The data from the present study suggest that the normal efflux pump(s) in wild-type *S. pneumoniae* and/or overexpression of *pmrA* does not contribute to resistance to fluoroquinolones other than norfloxacin. As the pneumococcal genome is now available (15) and it is clear that several putative efflux pump genes are present, identification of those involved in antibiotic transport can proceed.

ACKNOWLEDGMENTS

We are grateful to Grunenthal GmbH for supporting this study.
We are grateful also to Mark Webber and Vito Ricci for technical support.

REFERENCES

1. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(Suppl. 1)**:**5–16.
2. **Baranova, N. N., and A. A. Neyfakh.** 1997. Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **41:**1396–1398.
3. **Brenwald, N. P., M. J. Gill, and R. Wise.** 1997. The effect of reserpine, an inhibitor of multi-drug efflux pumps, on the in vitro susceptibilities of fluoroquinolone-resistant strains of *Streptococcus pneumoniae* to norfloxacin. J. Antimicrob. Chemother. **40:**458–460.
4. **Brenwald, N. P., M. J. Gill, and R. Wise.** 1998. Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **42:**2032–2035.
5. **Celi, F. S., M. E. Zenilman, and A. R. Shuldiner.** 1993. A rapid and versatile method to synthesise internal standards for competitive PCR. Nucleic Acids Res. **21:**1047.
6. **Gill, M. J., N. P. Brenwald, and R. Wise.** 1999. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **43:**187–189.
7. **Janoir, C., V. Zeller, M. D. Kitzis, N. J. Moreau, and L. Gutmann.** 1996. High-level fluoroquinolone resistance in *Streptococcus pneumoniae* requires mutations in *parC* and *gyrA*. Antimicrob. Agents Chemother. **40:**2760–2764.
8. **Jones, M. E., D. F. Sahm, N. Martin, S. Scheuring, P. Heisig, C. Thornsberry, K. Kohrer, and F.-J. Schmitz.** 2000. Prevalence of *gyrA*, *gyrB*, *parC*, and *parE* mutations in clinical isolates of *Streptocococcus pneumoniae* with decreased susceptibilities to different fluoroquinolones and originating from worldwide surveillance studies during the 1997–1998 respiratory season. Antimicrob. Agents Chemother. **44:**462–466.
9. **Jorgensen, J. H., L. M. Weigel, M. J. Ferraro, J. M. Swenson, and F. C. Tenover.** 1999. Activities of newer fluoroquinolones against *Streptococcus pneumoniae* clinical isolates including those with mutations in the *gyrA*, *parC*, and *parE* loci. Antimicrob. Agents Chemother. **43:**329–334.
10. **Pan, X. S., and L. M. Fisher.** 1996. Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: role in fluoroquinolone resistance. J. Bacteriol. **178:**4060–4069.
11. **Pan, X. S., J. Ambler, S. Mehtar, and L. M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **40:**2321–2326.
12. **Piddock, L. J. V., M. Johnson, V. Ricci, and S. L. Hill.** 1998. Activities of new fluoroquinolones against fluoroquinolone-resistant pathogens of the lower respiratory tract. Antimicrob. Agents Chemother. **42:**2956–2960.
13. **Piddock, L. J. V., Y. F. Jin, and M. J. Everett.** 1997. Non-*gyrA*-mediated ciprofloxacin resistance in laboratory mutants of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **39:**609–615.
14. **Pumbwe, L., and L. J. V. Piddock.** 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**2861–2864.
15. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498–506.
16. **Zeller, V., C. Janoir, M. D. Kitzis, L. Gutmann, and N. J. Moreau.** 1997. Active efflux as a mechanism of resistance to ciprofloxacin in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **41:**1973–1978.