Identification of an Efflux Pump Gene, *pmrA*, Associated with Fluoroquinolone Resistance in *Streptococcus pneumoniae*

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An open reading frame (ORF) homologous to *norA* was insertionally inactivated with *cat* in a fluoroquinolone-resistant pneumococcus with an efflux phenotype; this inactivation caused reversion to drug sensitivity. The ORF product has 24% amino acid sequence identity each to NorA and Bmr, which suggests that it is a major facilitator system pump of the 12-transmembrane-segment class.

Recent studies in our laboratories and elsewhere have described a putative efflux mechanism as a cause of low-level fluoroquinolone resistance in pneumococci (1–3, 13). As for other well-described multidrug efflux pumps, such as NorA and Bmr (9), concomitant reduced susceptibility to several diverse compounds is observed, and the pump is inhibited by reserpine. However, the genetic basis of this mechanism of resistance is not known. We therefore undertook this study to determine whether a homologue of *norA* or *bmr* is responsible for efflux-mediated resistance in *Streptococcus pneumoniae*.

Nucleotide sequence data from the Institute of Genome Research (Rockville, Md.; <http://www.tigr.org>) *S. pneumoniae* genome project were screened against the EMBL prokaryote library by using FASTA (10). One genomic sequence showed some homology with *norA*. An open reading frame (ORF) of 1,200 bp within this sequence showed 52% homology to a 430-nucleotide overlap with *norA*. By using PCR, DNA from 53 bp 5' of the start codon to 1,186 bp into this ORF was amplified from the chromosome of *S. pneumoniae* R6. The primer sequences were 5'-GGATCCGATATCAGAACCAGC TTCTTA3' (forward; *Bam*HI/*Eco*RV linker underlined) and 5'-GGTACCAAGCTTTTAATAATGTTTCGAAA3' (reverse; *Kpn*I/*Hind*III linker underlined). The conditions used were an initial extended denaturation cycle of 94°C for 5 min followed by 30 cycles of 1 min at 50°C, 1 min at 72°C, and 1 min at 94°C.

The ORF PCR product was cloned into pBluescript SK[–] (Stratagene, La Jolla, Calif.) and transformed into *Escherichia coli* XL1-Blue (Stratagene). *cat* excised from pR326 (4) was ligated into the cloned ORF. The resultant plasmid (pNBT5CAT), maintained in *E. coli* XL1-Blue, contained *cat* cloned into the *Acc*I site of the ORF, flanked by 478 and 764 bp of ORF sequence. pNBT5CAT was used to transform (6) *S. pneumoniae* R6N. Strain R6N is a strain with an efflux phenotype derived by transformation of strain R6 with DNA from strain 1N27; strain R6 is an easily transformed laboratory strain; strain 1N27 is spontaneous mutant (selected by norfloxacin) of *S. pneumoniae* ATCC 49619 (3) with an efflux phenotype and is not easily transformable. Transformants of R6N were selected on Columbia agar supplemented with 5% horse blood and containing chloramphenicol (8 µg/ml). The antibiotic susceptibility of transformants was tested by an agar dilution method (3) using Iso-Sensitest agar (Oxoid, Basing-

stoke, United Kingdom) supplemented with 5% horse blood and an inoculum of 10⁴ CFU per spot. Following incubation at 37°C in 4 to 6% CO₂ for 20 h, the MIC was taken as the lowest concentration completely inhibiting growth.

Table 1 shows the antibiotic susceptibilities of isogenic derivatives of R6. The efflux phenotype of R6N (increased MICs of norfloxacin, ciprofloxacin, acriflavine, and ethidium bromide [EtBr]; fourfold reduction of norfloxacin MIC in the presence of reserpine) (2, 3) was reversed to the fully susceptible phenotype in R6N-*cat*. Confirmation that these changes in susceptibility were due to altered efflux was made by demonstrating an increase in accumulation and a decrease in efflux of EtBr (1, 3) in R6N-*cat* in comparison to those of R6N. Briefly, for accumulation, bacteria were suspended in uptake buffer (NaCl, 110 mM; KCl, 7 mM; NH₄Cl, 50 mM; Na₂HPO₄, 0.4 mM; glucose, 0.2%; Tris base, 52 mM adjusted to pH 7.5 with HCl) and then exposed to EtBr (2 µg/ml). The increase in fluorescence as EtBr entered the cells was recorded fluorometrically (excitation λ = 530 nm; emission λ = 600 nm) at 30°C. EtBr accumulation at 20 min was significantly greater (Student's *t* test, *P* < 0.02) in R6N-*cat* than in R6N (Fig. 1A). For determining EtBr loss, bacteria were first exposed to EtBr (2 µg/ml) in the presence of reserpine (1 µg/ml) for 20 min at 37°C. The cells were then pelleted by centrifugation and resuspended in fresh uptake buffer. The loss of EtBr from the cells was measured as a decrease in fluorescence. EtBr efflux was significantly lower in R6N-*cat* than in R6N (Fig. 1B). There was no significant difference in the accumulation of efflux of EtBr between R6 and R6N-*cat*.

PCR of chromosomal DNA using the above primers and *Cla*I restriction digest analysis of the resultant amplicons showed that the putative efflux pump gene was present in R6, R6N, and R6N-*cat*. In addition, PCR product digestion with *Cla*I showed that the *cat* orientation in the pump gene in R6N-*cat* was such that its transcription was in the opposite direction to that predicted for the pump gene. Southern blotting was performed on *Pst*I- and *Hind*III-digested chromosomal DNA (from R6, R6N, and R6N-*cat*) (13). The Southern blot was probed with an efflux gene probe (made by using the above PCR product) or a *cat* probe (derived by *Sma*I and *Hind*III digestion from p326). Probes were labelled with the ECL random prime labelling system (Amersham Life Science Ltd., Little Chalfont, United Kingdom). Hybridizations were carried out for 18 h at 60°C; the membrane was washed three times for 15 min at 60°C in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate and then washed three times for 15 min at 60°C in 0.1× SSC

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TABLE 1. Characteristics and antibiotic susceptibilities of *S. pneumoniae* strains

Strain	Characteristic	MIC (μ g/ml) ^a						
		Nor	Nor + R	Cip	Moxi	Spar	EtBr	Acri
R6	Transformation recipient	2	2	0.5	0.12	0.25	2	4
R6N	R6 transformed with 1N27 DNA (3)	16	4	2.0	0.12	0.25	16	16
R6N-cat	R6N transformed with pNBT5CAT	2	2	0.5	0.12	0.25	2	4
								Cm
								32

^a Nor, norfloxacin; R, reserpine; Cip, ciprofloxacin; Moxi, moxifloxacin (Bay 12-8039); Spar, sparfloxacin; Acri, acriflavine; Cm, chloramphenicol.

containing 0.1% sodium dodecyl sulfate. Probe signal generation was detected on chemiluminescence film in accordance with the manufacturer's instructions (Amersham Life Science Ltd.). Probing with the efflux gene demonstrated that there was

only one copy of the efflux pump gene on the chromosome of R6 and its isogenic derivatives. The efflux pump gene probe and the *cat* probe both hybridized to the same 1.8-kb *Pst*I fragment of R6N-cat chromosomal DNA. Finally, DNA sequencing of PCR products generated from chromosomal DNA of R6N and R6N-cat also confirmed that *cat* had inserted into the *Acc*I site of the putative efflux gene in the latter strain.

Alignment of the putative pneumococcal efflux pump sequence with the amino acid sequences of Bmr and NorA is shown in Fig. 2. Overall, it has 24% sequence identity each with Bmr and NorA. Transmembrane segment (TMS) predictions using SOSUI (7) indicate that the pneumococcal efflux pump has 12 TMSs. The TMSs are in positions comparable to those in other 12-TMS proton-dependent efflux pumps (9). Further evidence that it is an efflux pump comes from the finding that it contains sequences similar to those of motifs A, B, C, D2, and G (Fig. 2) that have been described in other 12-TMS proton-dependent efflux pumps (8). These motifs are thought to be essential for protein structure and function but are unlikely to be involved in pump substrate specificity (9). Analysis of sequence found at the 5' end of the ORF revealed the presence of promoter, transcription start, and Shine-Dal-

FIG. 1. EtBr accumulation (A) and efflux (B) in isogenic pneumococci. Fluorescence is directly proportion to EtBr concentration. For accumulation (A), fluorescence is in arbitrary units; for efflux (B), fluorescence is expressed as a percentage of that at the first time point (15 s) for each strain. Each point represents the mean of three separate determinations; error bars show standard deviations. Symbols: ■, R6, susceptible strain; ▲, R6N, efflux strain; ●, R6N-cat, strain with putative efflux pump gene insertionally inactivated by *cat*.

		TMS1	TMS2	
Bmr	MEKKN--ITLTILLTNFLIAFLGIGLVPTPTIMNELHLSGTAV---GYMVAFAITQ	54		
NorA	---MN--KQIFVLYFNIFLIFLIGLVIVLVFVYKDLGLTGSDL---GLLVAAFAALSQ	51		
Pmr	MTEINWKNLRIAWFGNFLTGSISLVVPMPIFVENLVGVSGQQVAFYAGLAISVSAISA	60		
		lgxxxxxPxvP		
		Motif D2		
		TMS 2	TMS 3	TMS 4
Bmr	LIVSPIAGRWVDRFGKIMIVIGLLFFSVSEFLFGIGKTVEMLFISRLGGISAPFIMP	114		
NorA	MIISPFGGTLADKLGKLIICIGLILFSVSEFMAVGHNVSVLMLSRVLGMSAGMMPG	111		
Pmr	ALFSPWIGLADKYGRKPMIRAGLAMITMGGLAFVFNINYLIFLRLNLGVFPAGEV-PN	119		
		GxLaDrxgrkxxl		lxxxRxxqGxgaa
		Motif A		Motif B
		TMS 4	TMS 5	TMS 6
Bmr	VTAFIADITIKTRPKALGYMSAISTGPIIGPGIGGFLAEVHS-RLPFFFAAFALLAA	173		
NorA	VTGLIADISPSHQKAKNFQYMSALINSGFILPGIGGFMAEVSH-RMPFYTAGALGILAF	170		
Pmr	ATALIASQVKEKSGSALGTSLTGVAAGTLTGPFGIGFIAELFGIRTVFLVLGVSLFLAA	179		
		gxxxGpxxgxl		
		Motif C		
		TMS 6	TMS 7	
Bmr	ILSILTLREFERNPENQEIKQKTKFKRIFAPMYFIAFLII-LISSFGLASFESFLALFV	232		
NorA	IMSIVLIHDPKSTSTSGFQKLEPQLTKINWKVETIPVILT-LVLSFGLSAFETLYSLYT	229		
Pmr	ILTICFIKEDFQPAKEKAIPTELFTSVKYPYLLNLFLTSFVIQPSAQSIGPILALYV	239		
		TMS 8	TMS 9	
Bmr	DHKFGF-TASDIAIMITGGAIVGALTQVLFDRFTRWFGIEHLIRYSLILSTSLVFLTT	291		
NorA	ADKVN-SPKDISAITGGGIFGALFQTYFFDKFMKYSELTFIAWSLLYSVVVLILLVF	288		
Pmr	RDLGQTENLFLVSGLIVSSMGFSMSAGVMGLGDKVGNHRLVVAQFYSVITLYLCA	299		
		TMS 10	TMS 11	
Bmr	VHSYVAILLVTVTVFVGFDLMRFVAVTTLYSKIAGNEQ-GFAGGNMSMFTSIGNVFGPIIG	350		
NorA	ANGYWSIMLSFVVFVGFDIMRPAITNYFSNIAGERQ-GFAGGINSTFTSMGNFIQPLIA	347		
Pmr	ASSPLQLGLYRFLFLGTGALIFGVNALLSKMTPKAGISRVFAFNQVFFYLGGVGVGPMAG	359		
		GxxxGPL		
		Motif G		
		TMS 12		
Bmr	GMLFDIDVNPYFAT-VTLAIGALTIAWKAFAHLKAST-	389		
NorA	GALFDVHIEAPIYMAIGVSLAGVIVLVIEKQHRAKLKEQNM	388		
Pmr	SAVAGQFGYHAFYATSLCAVAFSCLFNLII-QFRLLKVKKEI	399		

FIG. 2. Amino acid sequence alignment of *B. subtilis* Bmr (EMBL accession no. M33768), *S. aureus* NorA (EMBL accession no. D90119), and the pneumococcal efflux pump (Pmr). Alignments were performed by using Multalin (5). Amino acids in bold are those common to the three proteins. Motifs A, B, C, D2, and G are those described by Paulsen and Skurray (8). TMS predictions are from SOSUI predictions (7) of the pneumococcal sequence.

garno sequences consistent with those found in pneumococci (11). In common with other efflux pumps, the pneumococcal pump gene was found in all strains examined by PCR. It is therefore likely that the associated drug resistance phenotype results from increased pump expression.

In summary, we have insertionally inactivated an ORF that had sequence homology with *norA* in a pneumococcus with a drug efflux phenotype. This inactivation resulted in the loss of the efflux phenotype. The corresponding amino acid sequence of this ORF is consistent with it encoding a 12-TMS proton-dependent multidrug efflux pump of the major facilitator family. Since there may be other multidrug efflux pumps in pneumococci, we propose calling this pump PmrA (pneumococcal multidrug resistance protein).

Nucleotide sequence accession number. The efflux gene sequence is deposited in the EMBL sequence database (accession no. AJ007367; 16 June 1998).

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