Functionally Cloned *pdrM* from *Streptococcus pneumoniae* Encodes a Na⁺ Coupled Multidrug Efflux Pump

Kohei Hashimoto, Wakano Ogawa*, Toshihiro Nishioka, Tomofusa Tsuchiya*, Teruo Kuroda

Department of Molecular Microbiology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Tsushima, Okayama, Japan

**Abstract**

Multidrug efflux pumps play an important role as a self-defense system in bacteria. Bacterial multidrug efflux pumps are classified into five families based on structure and coupling energy: resistance—nodulation—cell division (RND), small multidrug resistance (SMR), major facilitator (MF), ATP binding cassette (ABC), and multidrug and toxic compounds extrusion (MATE). We cloned a gene encoding a MATE-type multidrug efflux pump from *Streptococcus pneumoniae* R6, and designated it *pdrM*. PdrM showed sequence similarity with NorM from *Vibrio parahaemolyticus*, YdhE from *Escherichia coli*, and other bacterial MATE-type multidrug efflux pumps. Heterologous expression of PdrM let to elevated resistance to several antibacterial agents, norfloxacin, acriflavine, and 4',6-diamidino-2-phenylindole (DAPI) in *E. coli* KAM32 cells. PdrM effluxes acriflavine and DAPI in a Na⁺- or Li⁺-dependent manner. Moreover, Na⁺ efflux via PdrM was observed when acriflavine was added to Na⁺-loaded cells expressing *pdrM*. Therefore, we conclude that PdrM is a Na⁺/drug antiporter in *S. pneumoniae*. In addition to *pdrM*, we found another two genes, spr1756 and spr1877, that met the criteria of MATE-type by searching the *S. pneumoniae* genome database. However, cloned spr1756 and spr1877 did not elevate the MIC of any of the investigated drugs. mRNA expression of spr1756, spr1877, and *pdrM* was detected in *S. pneumoniae* R6 under laboratory growth conditions. Therefore, spr1756 and spr1877 are supposed to play physiological roles in this growth condition, but they may be unrelated to drug resistance.

**Introduction**

*Streptococcus pneumoniae* is the most common pathogenic bacterium in community-acquired pneumonia [1,2,3,4,5]. In Japan, it has been reported that *S. pneumoniae* is detected in 10–36% of investigated episodes of community-acquired pneumonia [6,7,8,9]. This bacterium colonizes the upper respiratory tract and sometimes causes pneumonia, but it is also responsible for acute otitis media, bacteremia, and bacterial meningitis [10]. β-lactams and macrolides are usually used to treat *S. pneumoniae* infections, but the recent increase in the prevalence of antibiotic-resistant *S. pneumoniae* is a serious problem in clinical sites worldwide [8,11,12,13,14,15,16].

The frequency of isolating penicillin-resistant *S. pneumoniae* (PRSP), including penicillin-intermediate resistant *S. pneumoniae* (PISP), is as high as 25–50% in Japan [17,18]. This, in addition to emerging multidrug-resistant *S. pneumoniae* (MDRSP), makes therapy difficult.

Several mechanisms contribute to bacterial resistance to drugs: 1) inactivation of drugs by degradation or modification, 2) modification of drug targets, 3) emergence of a bypass mechanism that is not inhibited by drugs, 4) changes in membrane permeability for drugs, and 5) drug efflux from cells. Among these five mechanisms, drug efflux, especially multidrug efflux, is known to play an important role in multidrug resistance in bacteria [19,20,21,22].

Multidrug efflux pumps actively expel various chemical compounds, including antibiotics, dyes, and detergents. So far, three drug efflux pumps, PmrA [23], MefE [24], and PatAB [25] have been reported in *S. pneumoniae*. PmrA and MefE are classified as MF-type pumps and PatAB is an ABC-type efflux pump [26,27]. However, more drug efflux pump genes are predicted to exist in the *S. pneumoniae* genome and remain uncharacterized (http://www.strept pneumoniae.com/).

We found a new gene for multidrug resistance in *S. pneumoniae* by shot-gun cloning. The gene was predicted to encode a MATE-type efflux pump because of the similarity of its primary structure with known MATE-type efflux pumps [28].

The crystal structure of the MATE-type efflux pump, NorM from *Vibrio cholerae* was solved in 2010 and results showed that this protein possesses twelve transmembrane helices, composed of two bundles of six transmembrane helices (TM 1–6 and TM 7–12) [29]. Coupling with Na⁺ and substrate is an interesting characteristic of MATE-type efflux pumps, and this phenomenon has been reported in most MATE-type efflux...
erythromycin and 10 μg/mL of the respective antibiotic, and NorM from *N. gonorrhoeae* [35]. Here we report the characteristics of a novel MATE-type efflux pump from *S. pneumoniae*, including its Na⁺ coupling ability.

**Methods**

**Bacterial Strains and Growth**

*S. pneumoniae* R6 (ATCC number, BAA-255), which was purchased from the American Type Culture Collection, was used as a source of chromosomal DNA. *S. pneumoniae* was grown in brain heart infusion (BHI, Difco) at 37°C for 16 hr ~ 24 hr without shaking. *E. coli* KAM32 (ΔaacB, ΔupdeF) [32], a drug-hypersusceptible strain, was used as a cloning host. *E. coli* was grown aerobically in LB broth at 37°C.

**Cloning of the pdrM Gene**

Chromosomal DNA was prepared from *S. pneumoniae* R6 by the method of Berns and Thomas [36]. Chromosomal DNA was partially digested with *Sma*I, and fragments ranging in size from 3 to 10 kb were separated by sucrose density gradient centrifugation. Plasmid pHY300PLK (Takara Co.) was digested with *Bam*HI, dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics K.K.), and then ligated to chromosomal DNA fragments with a Ligation-Convenience Kit (Nippon Gene Co.). Competent *E. coli* KAM32 cells were transformed with recombinant plasmids and incubated at 37°C for 48 h on LB plates containing an antimicrobial agent. We also used 10 μg/ml erythromycin and 10 μg/ml tetraphenylphosphonium chloride to clone drug efflux pump genes, but could not isolate any candidates. We isolated eight candidates from a plate containing 5 μg/ml acriflavine. Single colonies were isolated on the same medium as the selection medium, and plasmids were extracted from cells. *E. coli* KAM32 was re-transformed with the plasmids to confirm that it was responsible for the observed resistance to the antimicrobial agent used for the selection. Subsequently, restriction maps of candidate plasmids were determined.

**PCR Cloning of Putative MATE Efflux Pump Genes**

The *pdrM* gene was also cloned by PCR to compare its activity with the products of spr1756 and spr1876 under the same promoter. A DNA fragment containing the *pdrM*, spr1756, or spr1876 gene was amplified by PCR using *S. pneumoniae* R6 chromosomal DNA as a template. Primers used for gene cloning are listed in Table S1. The amplified DNA fragment containing each gene was ligated into *Bam*HI sites of pSTV29 (Takara Co.) or pUC19.

**Drug Susceptibility Test**

The MICs of various antimicrobial agents were determined by the microdilution method according to the recommendations of the Japanese Society of Chemotherapy [39]. Briefly, MICs were determined in Mueller-Hinton broth (Difco) containing each compound in a twofold serial dilution series. About 10⁶ cells were inoculated in each well of a microtiter plate, incubated in the test medium at 37°C for 24 h, and growth was examined visually. The MIC of each compound was defined as the lowest concentration that prevented visible growth.

**Energy Starvation and Loading Fluorescent Substance**

*E. coli* KAM32 harboring the recombinant plasmid was grown in LB medium at 37°C until OD₆₀₀=0.8. Cells were harvested and washed twice with modified Tanaka buffer (pH 7.0) containing 2 mM MgSO₄ [37]. Cells were resuspended in this buffer containing acriflavine (final concentration: 4.2 μM) or 4', 6-diamidino-2-phenylindole (DAPI, final concentration: 5 μM). Then, in order to load fluorescent dye into the cells, cells were incubated at 37°C for 2.5 hr in the presence of 100 μM carboxyfluorescein-dimalonylglucosamine (CCCP). Control cells and pdrM–possessing cells were normalized according to cell density and fluorescent intensity. By initially adjusting the cell density of control and sample cells, the fluorescent intensity was roughly adjusted. Then the fluorescent intensity was slightly adjusted last before the assay.

**Efflux of Fluorescent Substance**

Efflux of acriflavine or DAPI was carried out as previously described [32,38]. Energy starved and fluorescent dye–loaded cells were washed three times with 0.1 M 3-morpholinopropanesulfonic acid (MOPS)–tetramethylammonium hydroxide (TMAH) (pH 7.0) containing 2 mM MgSO₄ and the appropriate concentration of fluorescent dye (4.2 μM acriflavine or 5 μM DAPI), and were resuspended in the same buffer. Fluorometric measurements were performed at 37°C with a Hitachi F-2000 fluorescence spectrometer. Excitation and emission wavelengths used for the acriflavine efflux assay were 468 nm and 499 nm, respectively, whilst 332 nm and 462 nm were used for the DAPI efflux assay. After preincubation at 37°C for 4 min, lactate-TMAH (pH 7.0) (final concentration 20 mM) was added to energize the cells, and then NaCl, LiCl, or KCl (final concentration 10 mM) was added to the assay mixture.

**Acriflavine Efflux from Cells Induced by an Artificial Inward Na⁺ Gradient**

Based on our previous report, accelerated acriflavine efflux was measured by generating a transient Na⁺ gradient [30]. Energy-starved and fluorescent dye–loaded cells were washed three times with 0.1 M MOPS–TMAH (pH 7.0) containing 2 mM MgSO₄ and 4.2 μM acriflavine, and were resuspended in the same buffer. Buffer containing NaCl or KCl (final concentration 100 mM) was incubated at 37°C for 4 min to draw a base line. Then, prepared cells were added to the incubating buffer to make cells impose an inward Na⁺ gradient and acriflavine fluorescence at 499 nm was monitored with excitation at 468 nm.

**Efflux of Na⁺ Induced by Addition of Acriflavine**

Na⁺ efflux by the addition of acriflavine was observed as previously described [30]. Briefly, *E. coli* KAM32 harboring recombinant plasmids were grown in modified Tanaka medium [37] supplemented with 1% tryptone, 5 mM MgSO₄, and 10 mM melibiose at 30°C [39]. Cells were harvested at the late-exponential phase of growth, and washed three times with 0.1 M MOPS–TMAH (pH 7.0) containing 5 mM MgSO₄, and were resuspended in the same buffer. A portion of this suspension was diluted to approximately 9 mg protein/ml in the same buffer containing 250 μM NaCl.

Cells were incubated at 25°C in a plastic vessel with rapid stirring, and N₂ gas was blown continuously to maintain anaerobic conditions. A Na⁺-electrode (Radiometer, Copenhagen, Denmark) and reference electrode were put into the vessel [40]. Methyl-β-D-galactopyranoside (MβGal), a substrate of the melibiose transport-er, which is coupled to Na⁺ efflux [31], was added [final
Drug performed according to the method by Ochi et al [43]. Primers were designed as follows: (NH₄)₂SO₄, 0.1% Na citrate 2 H₂O, 0.5% glucose, 0.02% MgSO₄, 1.5% agar) was used to select a strain using the reverse-transcriptase reaction was performed to confirm no detectable DNA contamination. RT-PCR products were analyzed by 3% agarose gel electrophoresis.

**Results**

**Functional Cloning of pdrM**

We cloned genes relating to drug resistance from *S. pneumoniae* R6 [44] using a shot-gun method, and isolated eight hybrid plasmids that conferred acriflavine resistance to the heterologous host, *E. coli* KAM32. *E. coli* cells possessing hybrid plasmids showed resistance not only to acriflavine but also to norfloxacin, suggesting that the cloned gene is related to multidrug resistance. All hybrid plasmids possessed a common region of approximately 3 kb, and we chose one of the eight plasmids, pHAP5, for the following analysis (Figure S1).

**Sequence Analysis of pHAP5**

We identified the DNA region cloned in pHAP5 by comparing the partially determined sequence of pHAP5 with the *S. pneumoniae* R6 genome sequence [9]. The cloned region on pHAP5 contained one intact ORF (spr1052) and parts of two additional ORFs (spr1051 and spr1053).

**Table 1. Similarity of putative MATE transporters in *S. pneumoniae* R6 with characterized MATE-type transporters.**

| Cluster | spr1052 (PdrM) | spr1756 | spr1877 |
|---------|----------------|---------|---------|
| Cluster 1* | spr1756 (Sp) | 22      | 59      |
|          | spr1777 (Sp) | 63      | 59      | –        |
|          | AbeM (Ab)    | 72      | 60      | 20       |
|          | NorM (Vp)    | 71      | 60      | 49       |
|          | HmrM (Hi)    | 69      | 18      | 63       |
|          | VcmA (Vc)    | 69      | 35      | 31       |
| Cluster 2* | ALF5 (Af)    | 62      | 58      | 10       |
|          | ERC1 (Sc)    | 55      | 48      | 23       |
|          | hMATE1 (Hs)  | 20      | 49      | 17       |
|          | TT12 (Af)    | 24      | 41      | 22       |
| Cluster 3* | DinF (Ec)    | 32      | 59      | 29       |
|          | VmrA (Vp)    | 28      | 63      | 41       |
|          | MepA (Sa)    | 53      | 43      | 62       |

*The classification was referred to the result of analysis with ClustalW and reference [47].

**Drug Susceptibility Testing**

The MIC of various antibiotics was measured in *E. coli* KAM32 transformed with the pdrM-expressing plasmid (Table 2). The MIC of acriflavine in KAM32/pHAP5 was four times higher than that of KAM32/pHY300PLK. The MICs of norfloxacin and DAPI in KAM32/pHY300PLK were increased four and sixteen times, respectively, demonstrating that pdrM is indeed a gene involved in multidrug resistance. We suspected that PdrM may be more active in Gram positive bacteria than in *E. coli* because *S. pneumoniae* is a Gram positive bacterium. *B. subtilis* is one of the legally recognized hosts of genetic transformation in Japan, and we planned to investigate the activity of PdrM in *B. subtilis*. However, we knew that the basic drug resistant levels of wild type strains often prevented detection of the activity of a drug efflux pump, and...
we constructed *B. subtilis* TN26 from *B. subtilis* 168 before the investigations. Six genes (*bmr* [48], *ebrAB* [49], *blt* [50], *bmrA* [51], *yerP* [52], and *bmr3* [53]) for the drug efflux pump were disrupted in *B. subtilis* TN26. The MIC of tetraphenylphosphonium chloride in *B. subtilis* TN26 was sixteen times lower than that of the parental strain 168 and the MICs of norfloxacin, acriflavine, and ethidium bromide were also four times lower than that of the parental strain (Table 2). We then measured the drug resistant level in *B. subtilis* TN26 transformed with *pdrM*. However, *pdrM* was unable to elevate the MIC of the chemicals we investigated although the host was the same Gram positive bacterium *B. subtilis* as *S. pneumoniae*. We considered that the slightly decreased MIC of ciprofloxacin were in a range for error. We confirmed the mRNA expression of *pdrM* in *B. subtilis* TN26/pHAP5 using RT-PCR (Figure S3), and there may be some difficulty with translation or transition to the membrane in *B. subtilis*. As another reason, it may be possible that the basic resistant levels for quinolones and DAPI are still too high in *B. subtilis* TN26 to detect MIC changes.

**Drug Efflux Assay**

PdhM recognized DAPI and acriflavine as its good substrates. Therefore, we investigated the efflux activity of DAPI and acriflavine in *E. coli* KAM32/pHAP5 (Figure 1). No active efflux activity was observed when only lactate was added to the cell suspension. Consequently, NaCl was added to the assay mixture, and significant DAPI efflux was observed. The addition of LiCl also activated DAPI efflux as strongly as NaCl, although the efflux of DAPI was not observed when KCl was added to the assay mixture. Thus, Na⁺ and Li⁺ cations, but not Cl⁻, facilitate the efflux activity of PdrM. A similar result was observed when acriflavine was used as the substrate for the efflux assay (data not shown).

Next, we investigated the dependency of DAPI efflux on NaCl concentrations (Figure 2(A)). DAPI efflux in *E. coli* KAM32/pHAP5 was accelerated with increasing NaCl concentrations, and reached a plateau at 100 mM NaCl. DAPI efflux was also stimulated in a concentration-dependent manner by LiCl (data not shown). DAPI efflux curves almost coincided when the same concentration of LiCl was added (data not shown). Thus, NaCl and LiCl are compatible with one another in DAPI efflux.

It was also observed that acriflavine was effluxed in a Na⁺ concentration-dependent manner (Figure 2(B)). Incongruously, however, DAPI efflux was maximal at ~100 mM Na⁺ whereas maximal acriflavine efflux occurred at ~10 mM Na⁺. Various reasons were conceived to explain this discrepancy. However, the most possible reason we could think of was that the lowest concentrations of DAPI and acriflavine detectable by our luminometric method may be different. Alternatively, stoichiometry with Na⁺ may be different between DAPI and acriflavine.

| Antimicrobial agents | MIC (µg/ml) *E. coli* KAM32 pHY300PLK (control) pHAP5 (pdrM) | MIC (µg/ml) *B. subtilis* TN26 pHY300PLK (control) pHAP5 (pdrM) | MIC (µg/ml) *B. subtilis* 168 pHY300PLK (control) pHAP5 (pdrM) |
|----------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Norfloxacin          | 0.015                                           | 0.06                                           | 0.25                                            |
| Ciprofloxacin        | 0.004                                           | 0.008                                          | 0.25                                            |
| Enoxacin             | 0.06                                            | 0.06                                           | 0.5                                             |
| Ofloxacin            | 0.015                                           | 0.015                                          | ND                                              |
| Chloramphenicol      | 0.25                                            | 0.5                                            | ND                                              |
| Erythromycin         | 2                                               | 4                                              | ND                                              |
| Acriflavine          | 2                                               | 8                                              | 0.25                                            |
| DAPI                 | 0.125                                           | 2                                              | 0.5                                             |
| EtBr                 | 4                                               | 4                                              | 0.5                                             |
| Benzalkonium Cl      | 4                                               | 4                                              | ND                                              |
| Rhodamine 6G         | 8                                               | 8                                              | ND                                              |
| TPPCl                | 4                                               | 4                                              | ND                                              |

Table 2. Drug susceptibility in cells transformed with *pdrM*.

[DAPI: 4’,6-diamidino-2-phenylindole, EtBr: ethidium bromide, TPPCl: tetraphenylphosphonium chloride, ND: not determined.

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Acriflavine should be expelled in the presence of an artificial Na⁺ concentration gradient if PdrM antiports Na⁺ and acriflavine. To investigate this, acriflavine efflux via PdrM was measured in cells with a reduced intracellular Na⁺ concentration. Energystarved and acriflavine-loaded cells, in which Na⁺ concentrations are low, were prepared. Then, the cells were rapidly added to the assay mixture containing NaCl (final concentration 100 mM) to form an inward Na⁺ gradient. As a result, acriflavine was effluxed from pdrM–possessing cells in response to the Na⁺ gradient (Figure 4).

In contrast, this phenomenon was not observed with KCl instead of NaCl. This result also shows that acriflavine efflux is dependent on Na⁺ and is not due to the effect of osmotic pressure or Cl⁻.

We also investigated the antiport activity of norfloxacin and H⁺ via PdrM using a quinacrine fluorescence method [54], but the movement of H⁺ was not induced by the addition of norfloxacin or DAPI (Figure 5).

Putative MATE Efflux Pumps in S. pneumoniae R6

We found three genes – spr1756, spr1877, and pdrM – that met the criteria of the MATE family by searching the S. pneumoniae genome database (Figure S2). Of these three genes, spr1756 was reported to encode a protein whose function is similar to DinF [55]. Recently, Tocci et al reported three ORFs (SP1166, SP1939, and SP2065) belonging to MATE in S. pneumoniae DP1004 [56]. From the putative amino acid sequence, SP1166, SP1939, and SP2065 in S. pneumoniae DP1004 are thought to encode the orthologs of pdrM, spr1756, and spr1877 in S. pneumoniae R6, respectively. We cloned spr1756, spr1877, and pdrM by PCR, and measured the MIC of several antibiotics in E. coli KAM32 expressing the cloned gene (Table 3). Expression of pdrM cloned by PCR also led to elevated resistance against acriflavine and DAPI to the same extent as the pdrM cloned functionally (described above), spr1756 and spr1877 did not elevate the MIC of any of the drugs. mRNA of all three genes, however, were detected in S. pneumoniae R6 under laboratory growth conditions (Figure 6). Tocci et al constructed a spr1756-disrupted mutant from S. pneumoniae R6 and revealed that the MICs of moxifloxacin and levofloxacin were slightly lower in the mutant. Spr1756 may be able to efflux moxifloxacin; however, we speculate that the main role of spr1756 may be different from expelling antimicrobial

Figure 2. Salt concentration-dependent fluorescent dye efflux via PdrM. DAPI efflux activity (A). The concentration of NaCl or LiCl is (a) 0 mM, (b) 1 mM, (c) 10 mM, (d) 30 mM, (e) 50 mM, (f) 80 mM, and (g) 100 mM. Acriflavine efflux activity (B). The concentration of NaCl or LiCl is (a) 0 μM, (b) 10 μM, (c) 100 μM, (d) 1 mM, (e) 10 mM, and (f) 20 mM. Acriflavine fluorescence was quenched in cells because of its concentration. When an energy source was added, acriflavine was expelled and acriflavine fluorescence increased. Different from DAPI, the fluorescence of acriflavine does not change by DNA binding.

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Na⁺ Efflux Elicited by Acriflavine Influx

Several reported MATE-type multidrug efflux pumps are known to utilize Na⁺ to extrude their substrates [30,31,32,33,34,35]. Therefore, we investigated whether Na⁺ movement accompanied substrate extrusion via PdrM (Figure 3). Indeed, Na⁺ was extruded when acriflavine was added to a cell suspension of E. coli expressing pdrM. This result demonstrates that PdrM acts as an antiporter to exchange Na⁺ and acriflavine, and that Na⁺ is utilized as a coupling cation to transport drugs.

Figure 3. Na⁺ efflux from Na⁺-loaded cells elicited by an inwardly directed acriflavine gradient. Cells of E. coli KAM32/pHAP5 or E. coli KAM32/pHY300PLK (control) were diluted with 0.1 M MOPS-TMAH (pH7.0) until approximately 9 mg protein/ml. Na⁺ was loaded to cells via MelB by the addition of Mjgal (the first arrow), and acriflavine (final 200 μM) was added to the assay mixture at the time point indicated by the second arrow. Upward deflection of the curve indicates Na⁺ influx into cells and downward deflection indicates Na⁺ efflux from cells.

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Figure 4. Acriflavine efflux from cells elicited by an inwardly directed artificial Na⁺ gradient. An inwardly directed chemical gradient of Na⁺ was imposed by the addition of the cell suspension into the assay medium containing salt at the time point indicated by the arrow. (A) E. coli KAM32/pHAP5, (B) E. coli KAM32/pHY300PLK. The final concentration of the salt was 100 mM NaCl (curve a) or 100 mM KCl (curve b). The assay was performed at 37°C.

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was used for the assay. The final concentrations of DAPI and NFLX were lactate, potassium salt was 5 mM. Potassium lactate adjusted at pH 7.0 added at the time point shown by the arrow. The final concentration of (solid line) and KAM32/pHY300PLK (dashed line). Each reagent was tested with inverted membrane vesicles from P. aeruginosa and YdhE from V. parahaemolyticus. MATE-type pumps are phylogenetically classified into three clusters. PdrM is categorized into cluster 1 [57], along with typical bacterial MATE-type efflux pumps such as NorM from V. parahaemolyticus and YdhE from E. coli (Figure S2). In contrast, spr1756 and spr1877 are classified into cluster 3 [57]. Bioinformatic analysis predicted that many bacterial membrane proteins belong to cluster 3, but only a few of them, (e.g. MepA from S. aureus [58], VmrA from V. parahaemolyticus [32], and DinF from Ralstonia solanacearum [59]) were actually shown to possess the drug efflux activity. Indeed, DinF from E. coli is a well-known protein that belongs to cluster 3 in the MATE family but has not been reported to contribute to drug resistance. Proteins in cluster 3 may be rarely involved in drug resistance in bacteria.

What are the roles of spr1756 and spr1877? They are expressed under laboratory conditions, and we suggested that they have some as of yet undetermined role under these culture conditions. Muñoz-Elías’s group isolated a mutant possessing a transposon in spr1756 (dinF), corresponding to sp1939 in S. pneumoniae TIGR4 [60], and the transposon mutant in sp1939 formed poorer biofilms than the parent, S. pneumoniae TIGR4. Their results may support our hypothesis that spr1756 and spr1877 gene products play some role unrelated to drug resistance in S. pneumoniae, although a specific physiological role of spr1756 is still unknown.

Discussion

We cloned a MATE-type multidrug efflux pump gene, pdrM from S. pneumoniae R6. PdrM effluxes acriflavine and DAPI in a Na⁺ dependent manner. We could not detect the antiport activity of norfloxacin or DAPI and H⁺ via PdrM though we previously found AheM from A. baumannii, which is a H⁺-coupled MATE-type efflux pump [61]. Therefore, we considered H⁺ was unlikely to be moved via PdrM.

Sodium circulation in S. pneumoniae has not been investigated extensively. Kakimoto et al reported that Na⁺-ATPase classified into V-type ATPase in Enterococcus hirae was closely related to Streptococcus [62] and the Na⁺/proton antipporter in Enterococcus faecalis [63]. In Streptococcus bovis, sodium-dependent transport of neutral amino acids was reported in both whole cells and membrane vesicles [64]. Because these closely related bacteria possess systems coupling with Na⁺, we assumed that the existence of systems to utilize Na⁺ should also be expected in S. pneumoniae.

Table 3. Drug susceptibility in cells transformed with genes predicted from genome information.

| Antimicrobial agents | pSTV29 (control) | pdrM (spr1052) | spr1756 | spr1877 |
|---------------------|-----------------|----------------|---------|---------|
| Streptomycin        | 0.03            | 0.03           | 0.03    | 0.03    |
| Erythromycin        | 0.03            | 0.03           | 0.03    | 0.03    |
| Oxacillin           | 0.03            | 0.03           | 0.03    | 0.03    |
| Cefotaxime          | 0.03            | 0.03           | 0.03    | 0.03    |
| Methyl viologen     | 4.07            | 4.07           | 4.07    | 4.07    |
| Trimethoprim        | 0.03            | 0.03           | 0.03    | 0.03    |
| Safranin O          | 0.50            | 0.50           | 0.50    | 0.50    |
| Methyl viologen     | 4.07            | 4.07           | 4.07    | 4.07    |
| Trimethoprim        | 0.03            | 0.03           | 0.03    | 0.03    |
| Safranin O          | 0.50            | 0.50           | 0.50    | 0.50    |

Figure 5. H⁺ efflux assay in the inverted vesicle. H⁺ efflux activity was tested with inverted membrane vesicles from E. coli KAM32/pHAPS (solid line) and KAM32/pHY300PLK (dashed line). Each reagent was added at the time point shown by the arrow. The final concentration of lactate, potassium salt was 5 mM. Potassium lactate adjusted at pH 7.0 added at the time point shown by the arrow. The final concentration of (solid line) and KAM32/pHY300PLK (dashed line). Each reagent was tested with inverted membrane vesicles from P. aeruginosa and YdhE from V. parahaemolyticus. MATE-type pumps are phylogenetically classified into three clusters. PdrM is categorized into cluster 1 [57], along with typical bacterial MATE-type efflux pumps such as NorM from V. parahaemolyticus and YdhE from E. coli (Figure S2). In contrast, spr1756 and spr1877 are classified into cluster 3 [57]. Bioinformatic analysis predicted that many bacterial membrane proteins belong to cluster 3, but only a few of them, (e.g. MepA from S. aureus [58], VmrA from V. parahaemolyticus [32], and DinF from Ralstonia solanacearum [59]) were actually shown to possess the drug efflux activity. Indeed, DinF from E. coli is a well-known protein that belongs to cluster 3 in the MATE family but has not been reported to contribute to drug resistance. Proteins in cluster 3 may be rarely involved in drug resistance in bacteria.

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Discussion

We cloned a MATE-type multidrug efflux pump gene, pdrM from S. pneumoniae R6. PdrM effluxes acriflavine and DAPI in a Na⁺ dependent manner. We could not detect the antiport activity of norfloxacin or DAPI and H⁺ via PdrM though we previously found AheM from A. baumannii, which is a H⁺-coupled MATE-type efflux pump [61]. Therefore, we considered H⁺ was unlikely to be moved via PdrM.

Sodium circulation in S. pneumoniae has not been investigated extensively. Kakimoto et al reported that Na⁺-ATPase classified into V-type ATPase in Enterococcus hirae was closely related to Streptococcus [62] and the Na⁺/proton antipporter in Enterococcus faecalis [63]. In Streptococcus bovis, sodium-dependent transport of neutral amino acids was reported in both whole cells and membrane vesicles [64]. Because these closely related bacteria possess systems coupling with Na⁺, we assumed that the existence of systems to utilize Na⁺ should also be expected in S. pneumoniae.
We searched the genome database of *S. pneumoniae* R6 to determine whether the genes encoding similar proteins to the Na+/proton antiporters (e.g., NhaP from *Pseudomonas aeruginosa* [65], NhaH from *E. coli* [66], MnaA from *S. aureus* [67], and human Na+/H+ antiporter, NHE-2 [68]) are present or not. However, no such genes were found in the genome of *S. pneumoniae* R6.

We did find spr0573, described to be a sodium/hydrogen exchanger family protein Nha2, in the genome database ([http://www.ncbi.nlm.nih.gov/nuccore/NC_003098](http://www.ncbi.nlm.nih.gov/nuccore/NC_003098), but spr0573 is barely similar to NhaP from *P. aeruginosa* (less than 40% similarity). spr0573 also showed little similarity with NhaB from *E. coli*, MnaA from *S. aureus*, human NHE-2, and other known Na+/H+ antiporters. Therefore, it is doubtful whether Nha2 (spr0573) is actually a Na+/H+ antiporter.

We found at least three proteins possibly using Na+ as a coupling ion based on the genome information of *S. pneumoniae* R6. They are spr1598, spr0369, and spr0648. Among them, we could not find any reports relating to spr0468. spr1598 is presumed to encode a serine transporter because of its high similarity (90%) to Spr4969 from *M. tuberculosis*. spr0369 has been renamed dagA and it has been proposed to be a protein in the sodium/alanine symporter family. More than half of N-terminal of DagA from *S. pneumoniae* R6 is highly similar to DagA from the marine bacterium, *Alteromonas haloplanktis* [70].

The presence of these genes and *pdhM* suggests that *S. pneumoniae* also utilizes secondary transporters coupled with Na+. Therefore, we think that *S. pneumoniae* possesses machineries to form a Na+ motive force although we cannot predict any possible candidate genes now.

Na+ and Li+ significantly promoted expulsion of the fluorescent dyes, acrillavine and DAPI via PdrM, whilst K+ did not. Minimal efflux of the fluorescent dyes was observed in both KAM32/ pHAP5 and control cells when KCl was added to the assay mixture, and this phenomenon was observed in *N. meningitidis* from *V. parahaemolyticus* [30]. This result suggested that *E. coli* KAM32 possesses a weak acrillavine efflux system that is promoted in the presence of KCl, and is independent of PdrM.

On the other hand, KAM32/pHAP5 seemed to show higher acrillavine efflux activity in Figure 4(A) than in Figure 4(B). This may have been caused by PdrM though acrillavine efflux was not observed in Figure 1 and 2. Contaminated Na+ in high concentration KCl solution may stimulate the activity of PdrM or K+ at high concentrations may play a role similar to Na+

In summary, *pdhM* from *S. pneumoniae* has been revealed to be a MATE-type multidrug efflux pump. We would emphasize that PdrM is the first multidrug efflux pump that shows Na+ -coupling availability in Gram-positive bacteria.

### Supporting Information

**Figure S1** Restriction map of plasmid pHAP5, Horizontal bar indicates DNA regions derived from chromosomal DNA of *S. pneumoniae* R6. Arrows indicate ORFs of *pdhM* (spr1052), spr1051, and spr1053. The vector plasmids for pHAP5 and pUAP19 are pH300PLK and pUC19, respectively.

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