

## Use of an Efflux-Deficient *Streptococcus pneumoniae* Strain Panel To Identify ABC-Class Multidrug Transporters Involved in Intrinsic Resistance to Antimicrobial Agents

Gregory T. Robertson, Timothy B. Doyle, and A. Simon Lynch\*

*Cumbre, Inc., 1502 Viceroy Drive, Dallas, Texas 75235-2304*

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**Thirteen derivatives of the *Streptococcus pneumoniae* TIGR4 strain in which putative drug efflux pumps were genetically inactivated were constructed and characterized. The results indicate that two linked genes encoding the ABC-type transporters SP2073 and SP2075 function together to confer intrinsic resistance to a series of structurally unrelated compounds, including certain fluoroquinolones.**

Multidrug resistance efflux pumps are important components of innate and acquired bacterial resistance to antimicrobial agents (5). Herein we report the first systematic inactivation of genes encoding all nonessential candidate drug efflux systems in a gram-positive virulent pathogen, *Streptococcus pneumoniae* TIGR4 (12), and the characterization of the resulting mutants in terms of altered antimicrobial susceptibility.

Fourteen putative drug efflux transporters, identified by a combination of internal bioinformatics and by reference to the analysis of Paulsen and associates (<http://www.membranetransport.org/index.html>), were targeted for gene disruption (Table 1) using linear DNA fragments containing antibiotic resistance cassette-disrupted versions of the individual gene to be inactivated. Following the induction of genetic competence with competence-stimulatory peptide 2 (CSP-2), strains in which the targeted gene had been inactivated by allelic replacement were recovered by plating for the antibiotic resistance encoded by the marker used for gene disruption (as described previously in reference 8) (Table 1). Of the gene disruptions attempted, 13 yielded the anticipated insertion mutations, as verified by diagnostic PCR analysis (data not shown). In repeated attempts, mutants disrupted in open reading frame (ORF) SP1435 were not recovered, possibly indicating that either SP1435 is essential for growth or lethality resulted from the polar effect(s) on the expression of adjacent loci. Additional experiments would be necessary to resolve this issue.

Thirteen strains carrying disruptions in putative drug efflux pumps and a strain (CB318) lacking both SP2073 and SP2075 showed normal colony size and morphology relative to those of the parent strain TIGR4 on TSA II blood agar. These strains also exhibited similar growth kinetics in Todd-Hewitt broth supplemented with 5% (wt/vol) yeast extract and 0.1 mg of bovine catalase per ml. These 14 strains and the parent strain, TIGR4, were subsequently characterized in terms of altered susceptibility to 31 antimicrobial substances (7). Eleven strains, including a strain carrying a disruption in the SP0972 ORF encoding the previously described PmrA efflux pump (3), did not show appreciable changes in susceptibility to the com-

pounds tested (data not shown). The lack of effect of inactivation of SP0972 (*pmrA*) in this study indicates that PmrA is not intrinsically active in *S. pneumoniae* TIGR4, which is consistent with literature reports that indicate that PmrA is derepressed at the transcriptional level in clinical strains with PmrA-based efflux resistance (9, 10). The lack of effect following inactivation of the other tested pumps could indicate either that the appropriate substrates of these candidate efflux pumps were not tested or that these pumps are not intrinsically active in *S. pneumoniae* TIGR4. However, based on the studies completed to date, we cannot formally exclude the possibility that some or all of these putative efflux systems are under regulatory control mechanisms that might allow elevated efflux-based resistance if the cells were exposed to sub-MIC doses of the tested antibiotics prior to MIC challenge. In contrast, strains disrupted for genes encoding the putative ABC transporter SP2073 (strain CB069) or SP2075 (strain CB137) showed significantly increased (4- to 32-fold) susceptibility to 4 of the 31 agents tested (i.e., acriflavin, ethidium bromide, berberine, and norfloxacin) and marginal (2-fold) but highly reproducible changes in susceptibility to 4 additional agents (i.e., ciprofloxacin, oxolinic acid, novobiocin, and erythromycin) (Table 1). In all cases, the observed susceptibility differences were identical for strains lacking either SP2073 or SP2075, and combining the two mutations (strain CB318) was neither additive nor synergistic. Although the SP2073 and SP2075 genes are closely linked in the chromosome, they do not appear to constitute an operon since the middle gene (SP2074, encoding a degenerate transposase) in this three-gene cluster is predicted to be transcribed in the opposite direction of SP2075 and SP2073. Hence, the insertion of the chloramphenicol acetyltransferase (*cat*) resistance cassette in SP2075 (strain CB137) is unlikely to exert polar effects on the downstream SP2073 ORF. Further, SP2073 and SP2075 share significant amino acid homology to other ABC-class multidrug transporters of the MdB family, including the homodimeric drug efflux pump and lipid flipase MsbA from *Escherichia coli* (11). Thus, we conclude that ethidium bromide, berberine, novobiocin, acriflavin, erythromycin, and select quinolones are substrates for a single heterodimeric efflux pump consisting of both SP2073 and SP2075. This result is consistent with previous studies indicative of the existence of at least one drug efflux system in addition to PmrA

\* Corresponding author. Mailing address: Cumbre, Inc., 1502 Viceroy Dr., Dallas, TX 75235-2304. Phone: (214) 631-4700. Fax: (214) 631-4710. E-mail: [simon.lynch@cumbre.biz](mailto:simon.lynch@cumbre.biz).

TABLE 1. Susceptibilities of *S. pneumoniae* TIGR4 derivatives to antibiotics, antibacterials, and biocides<sup>a</sup>

| Compound               | MIC (μg/ml) for <sup>b</sup> : |   |   |   |   |
|------------------------|--------------------------------|---|---|---|---|
|                        | CB067<br>(WT parent)           | CB069<br>(ABC-SP2073::Sp <sup>r</sup> ) | CB137<br>(ABC-SP2075::Cm <sup>r</sup> ) | CB318 (ABC-SP2073::Sp <sup>r</sup> ;<br>ABC-SP2075::Cm <sup>r</sup> ) | CB065<br>(MFS-SP0972::Sp <sup>r</sup> ) |
| Acridine               | 128                            | 128                                     | 128                                     | 128   | 128                                     |
| Acriflavin             | 16                             | <b>4</b>                                | <b>4</b>                                | <b>4</b>  | 16                                      |
| Berberine              | 256                            | <b>16</b>                               | <b>16</b>                               | <b>16</b>   | 256                                     |
| EtBr (+R) <sup>c</sup> | 16 (1)                         | <b>0.5 (0.5)</b>                        | <b>0.5 (0.5)</b>                        | <b>0.5 (0.5)</b>  | 16 (1)                                  |
| Etoposide              | 16                             | 8                                       | 8                                       | 8   | 8                                       |
| Triclosan              | 8                              | 8                                       | 8                                       | 8   | 8                                       |
| Rifampin               | 0.015                          | 0.015                                   | 0.015                                   | 0.015   | 0.015                                   |
| Ciprofloxacin          | 2                              | <b>1</b>                                | <b>1</b>                                | <b>1</b>  | 2                                       |
| Gatifloxacin           | 0.5                            | 0.5                                     | 0.5                                     | 0.5   | 0.5                                     |
| Levofloxacin           | 1                              | 1                                       | 1                                       | 1   | 1                                       |
| Moxifloxacin           | 0.24                           | 0.24                                    | 0.24                                    | 0.24  | 0.24                                    |
| Nalidixic acid         | 256                            | 256                                     | 256                                     | 256   | 256                                     |
| Norfloxacin            | 16                             | <b>4</b>                                | <b>4</b>                                | <b>4</b>  | 16                                      |
| Oxolinic acid          | >32                            | <b>32</b>                               | <b>32</b>                               | <b>32</b>   | >32                                     |
| Sparfloxacin           | 0.5                            | 0.5                                     | 0.5                                     | 0.5   | 0.5                                     |
| Novobiocin             | 1                              | <b>0.5</b>                              | <b>0.5</b>                              | <b>0.5</b>  | 1                                       |
| Trimethoprim           | 4                              | 8                                       | 8                                       | 8   | 4                                       |
| Chloramphenicol        | 2                              | 4                                       | <b>32</b>                               | <b>32</b>   | 4                                       |
| Clindamycin            | ≤0.03                          | ≤0.03                                   | ≤0.03                                   | ≤0.03   | ≤0.03                                   |
| Erythromycin           | 0.03                           | <b>≤0.015</b>                           | <b>≤0.015</b>                           | <b>≤0.015</b>   | 0.03                                    |
| Fusidic acid           | >256                           | >256                                    | >256                                    | >256  | >256                                    |
| Kanamycin              | 64                             | 32                                      | 64                                      | 64  | 32                                      |
| Linezolid              | 1                              | 1                                       | 1                                       | 1   | 1                                       |
| Minocycline            | 0.12                           | 0.12                                    | 0.12                                    | 0.12  | 0.12                                    |
| Purromycin             | 8                              | 8                                       | 8                                       | 8   | 8                                       |
| Spectinomycin          | 16                             | <b>&gt;256</b>                          | 16                                      | <b>&gt;256</b>  | <b>&gt;256</b>                          |
| Streptomycin           | 64                             | 32                                      | 32                                      | 32  | 32                                      |
| Tetracycline           | 0.5                            | 0.5                                     | 0.5                                     | 0.5   | 0.5                                     |
| Ampicillin             | 0.12                           | 0.12                                    | 0.24                                    | 0.12  | 0.12                                    |
| Polymixin B            | >256                           | >256                                    | >256                                    | >256  | >256                                    |
| Vancomycin             | 1                              | 1                                       | 1                                       | 1   | 1                                       |

<sup>a</sup> Determined in Todd-Hewitt broth supplemented with 5% (wt/vol) yeast extract and 0.1 mg of bovine catalase per ml. Values in bold differ from the TIGR4 parent (CB067) control values; those changes likely resulting from the inserted resistance cassette are underlined.

<sup>b</sup> Drug efflux pump classes were as follows: ABC (ATP-binding cassette), MFS (major facilitator superfamily), and MATE (multidrug and toxic compound extrusion family). The specific gene(s) that was disrupted is denoted by the TIGR SP number designation. Resistance encoded by the antibiotic cassette used in gene inactivation are indicated as follows: Sp<sup>r</sup>, resistance to spectinomycin; Kn<sup>r</sup>, resistance to kanamycin; Cm<sup>r</sup>, resistance to chloramphenicol; and Er<sup>r</sup>, resistance to erythromycin. Not shown are MICs for CB068 (MFS-SP1116::Sp<sup>r</sup>), CB330 (MFS-SP0101::Er<sup>r</sup>), CB328 (MFS-SP1600::Er<sup>r</sup>), CB329 (MFS-SP0168::Er<sup>r</sup>), CB071 (ABC-SP1342::Kn<sup>r</sup>), CB074 (ABC-SP1062::Cm<sup>r</sup>), CB327 (ABC-SP1426::Er<sup>r</sup>), CB347 (MATE-SP1166::Kn<sup>r</sup>), CB354 (ABC-SP1526::Er<sup>r</sup>), and CB356 (ABC-SP1358::Er<sup>r</sup>). WT, wild type.

<sup>c</sup> EtBr, ethidium bromide; (+R), the MIC was determined in the presence of reserpine at a final concentration of 20 μg per ml.

in *S. pneumoniae* (9, 10) and with the observation that the SP2073 and SP2075 ORFs share significant amino acid similarity with genes (*efrB* and *efrA*, respectively) encoding a two-component ABC-class multidrug resistance efflux transporter in *Enterococcus faecalis* (4). Also consistent with previous studies (1, 2, 9, 10), we found that ethidium bromide efflux was sensitive to reserpine; however, reserpine did not further decrease the sensitivity of strains that were mutated for SP2073 and/or SP2075. This pharmacological result, in combination with the lack of observed phenotypes for mutants rendered defective in the other candidate efflux systems, suggests that SP2073/SP2075 is the principal, intrinsically active system for drug efflux in *S. pneumoniae* TIGR4. Clearly, additional studies aimed at determining the in vivo significance of the SP2073/SP2075 candidate efflux pump (or the other putative efflux pumps tested here) toward the potential failure of experimental chemotherapies and/or the acquisition and development of specific antibiotic resistance are warranted. Such studies may be particularly informative, especially given the expanded use of the fluoroquinolone class of antibiotics for the treatment of certain pneumococcal infections in humans (6).

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