Characterization of TolC Efflux Pump Proteins from *Pasteurella multocida*

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Two TolC homologs, PM0527 and PM1980, were identified for *Pasteurella multocida*. A pm0527 mutant displayed increased susceptibility to a range of chemicals, including rifampin (512-fold) and acridine orange (128-fold). A pm1980 mutant showed increased susceptibility to rifampin, cefazidime, and vancomycin.

*Pasteurella multocida* is a gram-negative bacterial pathogen that can cause disease in a wide range of animals (10, 40). Like other pathogens, *P. multocida* must survive within diverse host niches during the infection process. To this end, pathogens have evolved different systems for the import and export of certain molecules to help them survive and disseminate within the host. Efflux pumps actively export substances from the bacterial cell. These systems can be specific for one substrate or can transport a range of structurally dissimilar compounds. TolC family export systems typically export several unrelated substances, including molecules produced by the host, such as bile (26, 35), indicating that these systems might have a role in facilitating bacterial survival in particular niches. Efflux systems that transport several compounds can also be associated with multidrug resistance (32). While these systems have been found in many species, there are no data for *P. multocida*.

Efflux systems consist of multiple protein components. Some multidrug resistance efflux systems comprise three proteins, viz., a transporter, accessory protein, and outer membrane protein channel (32). These tripartite systems are often encoded as a single operon. However, some efflux systems, such as AcrAB from *Escherichia coli* (27), have the outer membrane component encoded elsewhere on the chromosome (25).

Envelope proteins of the TolC family are key components of both the type I secretion system and efflux pumps. The crystal structure of *E. coli* TolC revealed a channel-tunnel that spans the bacterial outer membrane and periplasm, providing a large exit duct for protein export and multidrug efflux when recruited by the substrate-engaged inner membrane complexes (5, 6).

There is accumulating evidence that efflux pumps that confer clinically relevant antibiotic resistance are important for bacterial pathogenesis. The reported properties associated with pump expression include adherence to, and invasion of, host cells by *Salmonella enterica* and colonization and persistence in chickens both by *S. enterica* (9) and by *Campylobacter jejuni* (26).

In this study we have characterized two outer membrane proteins, encoded by the genes pm0527 and pm1980, predicted to be TolC homologues in *P. multocida*.

PM0527 was recently identified experimentally as an outer membrane protein (8) with a predicted molecular mass of 50 kDa. PM0527 showed similarity to a number of bacterial TolC proteins, including those from *Haemophilus influenzae* (HI1462; 65% identity), *C. jejuni* (CmeC; 22% identity), and *E. coli* (TolC; 22% identity). PM1980, a predicted 52-kDa protein, showed similarity to *Mannheimia succiniciproducens* TolC (41% identity), *E. coli* CusC (26% identity), and *H. influenzae* HI1462 (21% identity). Each of the candidate genes encoding these proteins was inactivated in a tetracycline-resistant derivative of a *P. multocida* VP161 strain (AL435) (for strains, see Table S1 in the supplemental material) as described previously (16, 17, 18, 29) using single-crossover insertional mutagenesis (primers are listed in Table S2 in the supplemental material). Each mutation was confirmed by PCR (95°C, 5 min; 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 2 min; and finally 72°C for 5 min). Each mutant strain was complemented in *trans* with the intact gene generated using flanking oligonucleotides (for primers, see Table S2 in the supplemental material). The amplified DNA fragments were ligated into the Sall- and BamHI-digested expression vector pAL99 (for plasmids, see Table S1 in the supplemental material) such that transcription of the gene was driven by the constitutive *P. multocida* tipA promoter. As a control, the vector pAL99 was transformed separately into each mutant (for strains, see Table S1 in the supplemental material).

As a secondary confirmation of the mutants, we used Western blotting with chicken antiserum raised against recombinant PM0527 and recombinant PM1980 (4). For immunoblotting, approximately 10⁶ whole cells were loaded in each lane, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride...
P. multocida PM1980 failed to detect a protein of the predicted size in the mutant expresses no PM0527. The antiserum produced against are consistent with the PCR data showing that the pm0527 strain is due to the multicopy gene dosage effect. These data PM0527. The high level of PM0527 in the complemented lane 4), confirming the identity of the 50-kDa protein as not in the mutant strain transformed with vector only (Fig. 1, of 50 kDa in the wild-type strain (Fig. 1, lane 1) which was membrane. For PM0527, the antiserum recognized a protein a small number of antimicrobials (Table 1). The MICs of rifampin, ceftazidime, trimethoprim, and vancomycin decreased 64-fold, 8-fold, 4-fold, and 4-fold, respectively. MICs of novobiocin, erythromycin, taurocholic acid, cyclophosphamide, dithiothreitol, ethidium bromide, SDS, Triton X-100, and fusaric acid were decreased slightly (two-fold).

A phylogenetic analysis was performed to investigate the evolutionary relationships of PM0527 and PM1980 to 21 functionally characterized TolC family members (Fig. 2; Table 2). Multiple sequence alignment of the TolC sequences was produced using the ClustalX software program (eBiotools software) (36). Manual curation of the multiple sequence alignment was performed using the Seaview software program (14). All sequences in the alignment were cropped to the common topology core equivalent to residues 24 to 430 of the E. coli TolC protein (sequence identifier, P02930). Domain boundaries and multiple sequence alignment validation used a structural alignment of structures of E. coli TolC (PDB identifier, 1EK9), Vibrio cholerae Vcc (PDB identifier, 1YC9), and Pseudomonas aeruginosa OprM (PDB identifier, 1WP1) (aligned using the MUSTANG software program [21]). The multiple sequence alignment was used to produce a bootstrapped neighbor-joining tree, using 1,000 bootstrap trials (36). The neighbor-joining tree was drawn using the NJplot software program (31). The tree shows clustering of TolC members into clades with conserved efflux function: multidrug, cation, or protein efflux. PM0527 and PM1980 are phylogenetically most related to the TolC proteins with multidrug efflux function. Sequence alignment of the 21 TolC family members is shown in Table S3 in the supplemental material.

These results clearly demonstrated that PM0527 and PM1980 are TolC homologues which contribute to the intrinsic resistance of P. multocida to diverse antimicrobial agents. This conclusion is based on several lines of evidence. First, PM0527 shares significant sequence and predicted structural similarity with many known tripartite efflux systems in gram-negative bacterial pathogens. Second, inactivation of the pm0527 and pm1980 proteins by insertion mutagenesis substantially increased the susceptibility of P. multocida to structurally diverse antimicrobial agents (Table 1). Furthermore, complementation of the mutants with the intact genes restored the resistance to numerous compounds. Phylogenetic analysis showed that both the PM0527 and PM1980 proteins align with other TolC homologues with specific drug efflux function. To de-

**FIG. 1.** Immunoblot analysis of PM0527 TolC expression in P. multocida whole-cell lysate probed with chicken antiserum against recombinant PM0527. Lanes: 1, AL435 parent strain; 2, pm0527 mutant; 3, complemented mutant; 4, mutant complemented with empty vector. The positions of standard molecular mass markers are shown on the left. The 50-kDa PM0527 is indicated with an arrow. Prebleed serum showed no reactivity.
termine if either efflux pump might also be involved in the export of proteins, supernatants from 30 ml of overnight brain heart infusion cultures were concentrated 300-fold by ultrafiltration (10-kDa cutoff) and examined by SDS-polyacrylamide gel electrophoresis. We observed no difference in the profiles of secreted proteins between the wild type and mutants. Together, these findings define the active role of PM0527 and PM1980 in the export of chemical compounds and antimicrobial agents.

The genomic location of pm0527 in P. multocida resembles that of acrAB in E. coli and tolC in H. influenzae, where the gene encoding the channel-tunnel is unlinked to those encoding the proteins of the inner-membrane complex (27, 37). Of particular interest is the high functional and sequence identity of PM0527 with the H. influenzae TolC homologue, HI1462. PM0527 and HI1462 share 65% sequence identity (from PM0527 residues 1 to 452), and the HI1462 mutant showed a susceptibility profile similar to that of the pm0527 mutant against 9 out of 12 compounds tested (37). The model of HI1462 (37) predicts that a pair of oppositely charged residues (R396 and E397) forms a circular network of salt bridges at the periplasmic tunnel entrance. In addition, R397 in HI1462 was reported to be responsible for the anion selectivity (33). This pair of oppositely charged residues is also found in the PM0527 sequence (residues R414 and D415) (Table 3), suggesting a common efflux mechanism. Interestingly, although PM1980 shares only 20% sequence identity with HI1462, it also contains a conserved pair of oppositely charged residues (D363 and R364), albeit in reverse orientation (Table 3).

PM0527 appears to be the predominant TolC protein in P. multocida. Compared with PM0527, the level of resistance

### Table 1. Susceptibilities of P. multocida AL435, its tolC mutants (pm0527 and pm1980 strains), and their complemented strains to different compounds

| Group          | Compounds       | MIC (µg/ml) of compound for: | Fold difference* a | MIC (µg/ml) of compound for: | Fold difference* a |
|----------------|-----------------|-------------------------------|--------------------|-------------------------------|--------------------|
|                | AL435 (parent)  | pm0527 strain                | Complemented       | Mutant Complemented           | Mutant Complemented |
| Antibiotics    |                 |                               |                    |                               |                    |
| Aminoglycoside | Gentamicin      | 20                            | 5                  | 20                            | 4                  | 40                  | 40                 | 0.5                |
|                | Neomycin sulfate| 125                           | 125                | 125                           | 1                 | 1,000               | 500                | 0.125              |
| Bacteriostatic | Chloramphenicol | 1                             | 0.5                | 1                             | 2                 | 1                   | 1                  | 1                  |
|                | Trimethoprim    | 8                             | 0.25               | 0.5                           | 32                | 2                   | 1                  | 4                  |
|                | Rifampin        | 50                            | 0.1                | 0.2                           | 512               | 0.78                | 0.78               | 64                 |
| Beta-lactam    | Ceftazidime     | 250                           | 15.6               | 7.8                           | 16                | 31.2                | 31.2               | 8                  |
| Coumarin       | Novobiocin      | 40                            | 2.5                | 40                            | 16                | 20                  | 40                 | 2                  |
| Fluoroquinolone| Nalidixic acid  | 25                            | 25                 | 25                            | 1                 | 25                  | 12.5               | 1                  |
| Glycopeptide   | Vancomycin      | 1,000                         | 125                | 250                           | 8                 | 250                 | 250                | 4                  |
| Lincosamide    | Lincomycin      | 100                           | 25                 | 25                            | 4                 | 100                 | 50                 | 1                  |
| Macrolides     | Erythromycin    | 3.8                           | 0.47               | 1.88                          | 8                 | 1.88                | 1.88               | 2                  |
|                | Spiramycin      | 25                            | 12.5               | 25                            | 2                 | 25                  | 25                 | 1                  |
| Sulfonamide    | Sulfathiazole   | 2,500                         | 625                | 1,250                         | 4                 | 2,500               | 2,500              | 1                  |
| Other antibiotics | Cyclodexamide | 500                           | 250                | 500                           | 2                 | 500                 | 500                | 1                  |
|                | Polymyxin B     | 12.5                          | 3.125              | 6.25                          | 4                 | 12.5                | 6.25               | 1                  |
| Bile salts     | Decoxycholic acid | 625                    | 625                 | 312.5                         | 1                 | 625                 | 625                | 1                  |
|                | Sodium deoxycholate | 625                  | 312.5              | 312.5                         | 2                 | 625                 | 625                | 1                  |
|                | Taurocholic acid | 3,125                         | 1,562.5            | 781.25                        | 2                 | 1,562.5             | 1,562.5            | 2                  |
| Chemicals      |                 |                               |                    |                               |                    |                    |
| Alkylating agent | Cyclophosphamide | 4,000                      | 4,000              | 2,000                         | 1                 | 2,000               | 2,000              | 2                  |
| Redox agent    | Dithiothreitol  | 4,000                         | 2,000              | 2,000                         | 2                 | 2,000               | 4,000              | 2                  |
| Dyes           | Acidine orange  | 30                            | 0.234              | 15                            | 128               | 30                  | 30                 | 1                  |
|                | Crystal violet  | 12.5                          | 3.125              | 6.25                          | 4                 | 12.5                | 12.5               | 1                  |
|                | Ethidium bromide | 20                           | 5                  | 10                            | 4                 | 10                  | 10                 | 2                  |
| Metals         | CoCl₂           | 1,250                         | 1,250              | 1,250                         | 1                 | 1,250               | 1,250              | 1                  |
|                | CuCl₂           | 312.5                         | 312.5              | 312.5                         | 1                 | 312.5               | 312.5              | 1                  |
|                | HgCl₂           | 2.5                           | 1.25               | 2.5                           | 2                 | 2.5                 | 5                  | 1                  |
|                | ZnCl₂           | 62.5                          | 62.5               | 62.5                          | 1                 | 62.5                | 125                | 1                  |
| Surfactants    | SDS             | 50                            | 12.5               | 25                            | 4                 | 25                  | 25                 | 2                  |
|                | Triton X-100    | 40,000                        | 40,000             | 40,000                        | 1                 | 40,000              | 40,000             | 1                  |
|                | Tween 20        | 40,000                        | 40,000             | 40,000                        | 1                 | 40,000              | 40,000             | 1                  |
| Toxin          | Fusaric acid    | 100                           | 50                 | 50                            | 2                 | 50                  | 100                | 2                  |
| Mucosal protein| Mucin           | 8,000                         | 4,000              | 8,000                         | 2                 | 8,000               | 8,000              | 1                  |

* n-fold difference between MICs for mutant strain and strain AL435.
conferred by PM1980 was relatively moderate, and it may be
masked by the function of PM0527 in wild-type P. multocida.

In conclusion, data from the present study demonstrated
that PM0527 and PM1980 are TolC proteins of P. multocida,
since their corresponding mutants show susceptibility to a
range of substances. Based on our functional analyses and
amino acid sequence similarity, both PM0527 and PM1980 can
be classified as components of efflux pump systems of the

![TolC Phylogenetic Tree](image)

**FIG. 2.** Location of PM0527 and PM1980 proteins in the TolC family phylogenetic tree. The TolC homologues fall into three major clades,
which reflect the efflux function of the TolC proteins: multidrug, cation, or protein efflux. The distance bar indicates 50 substitutions per 100
residues. Proteins are labeled as “protein name_species name” according to details in Table 2.

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**TABLE 2.** Known TolC homologues for which functional data are published

| Name            | Organism                     | NCBI accession no. | Function               | Protein Data Bank structure code | Reference |
|-----------------|------------------------------|--------------------|------------------------|---------------------------------|-----------|
| ZapD_Pmi        | Proteus mirabilis            | AAC33452           | Protein efflux         |                                 | 39        |
| AprF_Pae        | Pseudomonas aeruginosa       | CAA45857           | Protein efflux         |                                 | 12        |
| PrlF_Eam        | Erwinia amylovora            | CAB42876           | Protein efflux         |                                 | 41        |
| LipD_Sma        | Serratia marcescens          | BAA25796           | Protein efflux         |                                 | 3         |
| PrlF_Ech        | Erwinia chrysanthemi         | CAA37344           | Protein efflux         |                                 | 23        |
| TliF_Pfl        | Pseudomonas fluorescens      | AAD09855           | Protein efflux         |                                 | 1         |
| HasF_Sma        | Serratia marcescens          | CA67136            | Protein efflux         |                                 | 7         |
| TolC_Eco        | Escherichia coli             | P02930             | Protein efflux         | 1EK9/1TQ                       | 22        |
| TolC_Sen        | Salmonella enterica          | AAL22060           | Protein efflux         |                                 | 28        |
| PM1980_Pmu      | Pasteurella multocida        | NP_246919          | Multidrug efflux       |                                 |           |
| OprI_Pae        | Pseudomonas aeruginosa       | AAB41985           | Multidrug efflux       |                                 | 34        |
| SmeC_Sma        | Stenotrophomonas maltophilia| AAD51346           | Multidrug efflux       |                                 | 24        |
| OprM_Pae        | Pseudomonas aeruginosa       | Q51487             | Multidrug efflux       | 1WP1                            | 2         |
| H11462_Hin      | Haemophilus influenzae       | P45217             | Multidrug efflux       |                                 |           |
| PM0527_Pmu      | Pasteurella multocida        | NP_245464          | Multidrug efflux       |                                 |           |
| FusA_Bce        | Burkholderia cepacia         | P24126             | Multidrug efflux       |                                 |           |
| VceC_Vch        | Vibrio cholerae              | ZP_01680658        | Multidrug efflux       | 1YC9                           | 13        |
| CzcC_Pae        | Pseudomonas aeruginosa       | CAB56469           | Cation efflux          |                                 | 19        |
| CzcC_Rme        | Ralstonia metallidurans      |CAA67082            | Cation efflux          |                                 | 30        |
| CnrC_Cme        | Ralstonia metallidurans      | CAB82451           | Cation efflux          |                                 | 15        |
| HelC_Lpn        | Legionella pneumophila       | CAH15280           | Cation efflux          |                                 | 11        |

* Database annotation, NCBI database (http://www.ncbi.nlm.nih.gov) accession number, function, and reference are also given.
resistance nodulation family. The characterized proteins are likely components of a tripartite efflux system, but the precise nature of protein interactions within the different TolC complexes must await future studies.

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TABLE 3. Sequence region that is predicted to form a circular network of salt bridges at the periplasmic tunnel entrance

| Name          | Organism            | NCBI accession no. | Salt bridges | Position |
|---------------|---------------------|---------------------|--------------|----------|
| HI1462_Hin    | Haemophilus influenzae | P45217              | G V S E L R E W L V A A | 391–402 |
| PM0527_Pmu    | Pasteurella multocida | NP_245464           | G V S P L R D W L S A A | 409–420 |
| PM1980_Pmu    | Pasteurella multocida | NP_246919           | G D Y T F D R V L Q A R | 358–369 |

* Database annotation, NCBI database (http://www.ncbi.nlm.nih.gov) accession numbers are given. Underlining indicates acidic residues, and boldface indicates basic residues.
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