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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, ceftazidime, 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 SalI- 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 tpiA* 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 10^8 whole cells were loaded in each lane, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride

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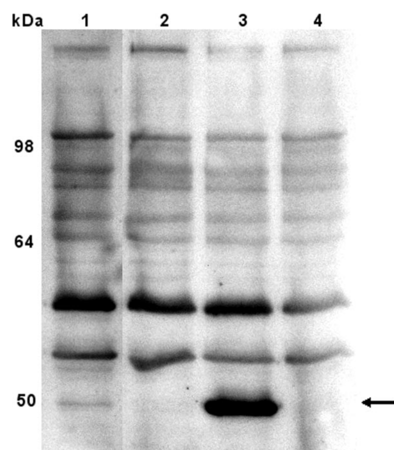

FIG. 1. Immunoblot analysis of PM0527 TolC expression in *P. multocida* whole-cell lysate probed with chicken antiserum against recombinant PM0527. Lanes: 1, AL435 parent strain; 2, pm0527 mutant; 3, complemented mutant; 4, mutant complemented with empty vector. The positions of standard molecular mass markers are shown on the left. The 50-kDa PM0527 is indicated with an arrow. Prebleed serum showed no reactivity.

membrane. For PM0527, the antiserum recognized a protein of 50 kDa in the wild-type strain (Fig. 1, lane 1) which was absent in the mutant (Fig. 1, lane 2). Importantly, this protein was restored in the complemented strain (Fig. 1, lane 3) but not in the mutant strain transformed with vector only (Fig. 1, lane 4), confirming the identity of the 50-kDa protein as PM0527. The high level of PM0527 in the complemented strain is due to the multicopy gene dosage effect. These data are consistent with the PCR data showing that the pm0527 mutant expresses no PM0527. The antiserum produced against PM1980 failed to detect a protein of the predicted size in the *P. multocida* wild-type strain grown in vitro.

To determine whether the proteins PM0527 and PM1980 were involved in drug efflux, we tested the susceptibilities of parent (AL435), mutant (pm0527 and pm1980), and complemented strains to various chemicals (Table 1). The MICs were defined in twofold dilution steps in triplicate using the standard microtiter broth dilution method (20) in brain heart infusion broth. Microtiter plates were incubated for 24 h under constant aeration at 37°C. The antibiotics and other compounds used in this study were purchased from Sigma Chemical Co., Merck, or BDH Chemicals. Compared to the parent, AL435, the pm0527 mutant showed significantly increased susceptibilities to many compounds (Table 1). For the pm0527 mutant, the MICs of aminoglycoside decreased fourfold for gentamicin; the MICs of two bacteristatic antibiotics, rifampin and trimethoprim, decreased 512-fold and 32-fold, respectively, while the MICs of ceftazidime, novobiocin, vancomycin, and lincomycin decreased 16-fold, 16-fold, 8-fold, and 4-fold, respectively. Furthermore, the MICs of two macrolides decreased eightfold (erythromycin) and twofold (spiramycin) and the MICs of sulfathiazole, cycloheximide, and polymyxin B decreased fourfold, twofold, and fourfold, respectively. MICs of the bile salts taurocholic acid, sodium deoxycholate, chloramphenicol, dithiothreitol, $HgCl_2$, fusaric acid, and mucin were decreased slightly (two-fold) but reproducibly for the pm0527 mutant. Furthermore, the MICs of the dyes acridine orange, crystal violet, and ethidium bromide and the detergent SDS decreased 128-fold, 2-fold, 4-fold, and 4-fold, respectively. The pm0527 mutant demonstrated levels of resistance similar to those of wild-type *P. multocida* for neomycin sulfate, nalidixic acid, deoxycholic acid, cyclophosphamide, the heavy metals $CoCl_2$, $CuCl_2$, and $ZnCl_2$, and the surfactants Triton X-100 and Tween 20, suggesting that these compounds are not substrates of the PM0527 TolC efflux system. Complementation with the full-length pm0527 gene restored resistance to the majority of the compounds to approximately wild-type levels, indicating that the observed phenotype was not due to polar effects (Table 1).

We tested the second TolC homologue, PM1980, for susceptibility to the same set of compounds. Compared with the parent, AL435, the pm1980 mutant showed increased susceptibility to 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 (twofold).

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* Vcec (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 insertional 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-

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 pm1980 strain		Fold difference[a]
		AL435 (parent)	pm0527 strain					
			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
Sulfinamide	Sulfathiazole	2,500	625	1,250	4	2,500	2,500	1
Other antibiotics	Cycloheximide	500	250	500	2	500	500	1
	Polymyxin B	12.5	3.125	6.25	4	12.5	6.25	1
Bile salts	Deoxycholic 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	Acridine 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	$CoCl_2$	1,250	1,250	1,250	1	1,250	1,250	1
	$CuCl_2$	312.5	312.5	312.5	1	312.5	312.5	1
	$HgCl_2$	2.5	1.25	2.5	2	2.5	5	1
	$ZnCl_2$	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	20,000	40,000	2
	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

[a] *n*-fold difference between MICs for mutant strain and strain AL435.

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

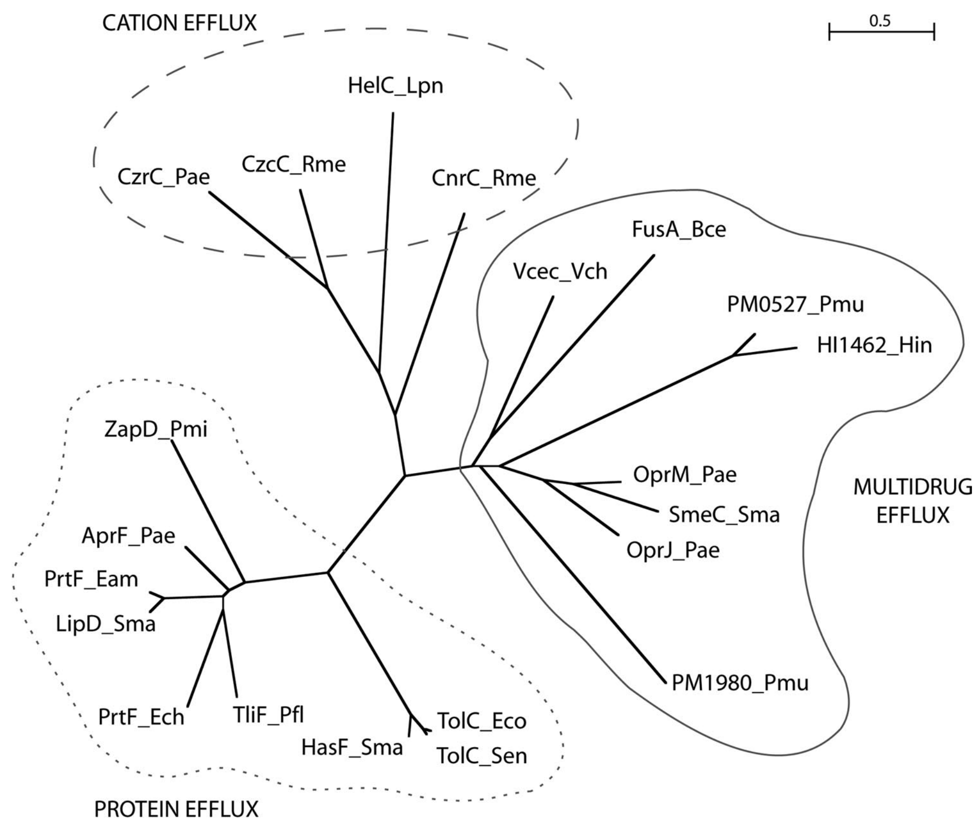

FIG. 2. Location of PM0527 and PM1980 proteins in the TolC family phylogenetic tree. The TolC homologues fall into three major clades, which reflect the efflux function of the TolC proteins: multidrug, cation, or protein efflux. The distance bar indicates 50 substitutions per 100 residues. Proteins are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

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

TABLE 2. Known TolC homologues for which functional data are published[a]

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
PrtF_Eam	*Erwinia amylovora*	CAB42876	Protein efflux		41
LipD_Sma	*Serratia marcescens*	BAA25796	Protein efflux		3
PrtF_Ech	*Erwinia chrysanthemi*	CAA37344	Protein efflux		23
TliF_Pfl	*Pseudomonas fluorescens*	AAD09855	Protein efflux		1
HasF_Sma	*Serratia marcescens*	CAA67136	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		This study
OprJ_Pae	*Pseudomonas aeruginosa*	AAB41958	Multidrug efflux		34
SmeC_Sma	*Stenotrophomonas maltophilia*	AAD51346	Multidrug efflux		24
OprM_Pae	*Pseudomonas aeruginosa*	Q51487	Multidrug efflux	1WP1	2
HI1462_Hin	*Haemophilus influenzae*	P45217	Multidrug efflux		
PM0527_Pmu	*Pasteurella multocida*	NP_245464	Multidrug efflux		This study
FusA_Bce	*Burkholderia cepacia*	P24126	Multidrug efflux		38
Vcec_Vch	*Vibrio cholerae*	ZP_01680658	Multidrug efflux	1YC9	13
CzrC_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

[a] Database annotation, NCBI database (http://www.ncbi.nlm.nih.gov) accession number, function, and reference are also given.

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

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

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

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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