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OprK and OprM Define Two Genetically Distinct Multidrug Efflux Systems in *Pseudomonas aeruginosa*

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Multidrug-resistant derivatives of *Pseudomonas aeruginosa* PAO1 were obtained after stepwise selection on tetracycline or erythromycin. Two phenotypes were generated. The tetracycline-resistant mutant (TETR) was phenotypically similar to OprM-overexpressing strains. This group displayed cross-resistance to quinolones, chloramphenicol, and all β-lactams tested except imipenem, with no changes in the erythromycin MICs for the strains. Sodium dodecyl sulfate-polyacrylamide gels showed the overproduction of an outer membrane protein in the range of 50 kDa and a 46-kDa inner membrane protein. The erythromycin-resistant mutant (ERYR) kept its susceptibility to all β-lactams tested with the exception of cefpirome, but it was resistant to chloramphenicol, quinolones, and tetracycline and was hypersusceptible to imipenem. This mutant also exhibited overexpression of a 50-kDa outer membrane protein that was different from OprM and of a 43-kDa inner membrane protein. The phenotype of ERYR was comparable to those of OprK- and OprJ-overexpressing strains. These strains were therefore classified as the OprK-like group. Transduction of the *oprK*::Ω-Hg mutation of strain K613 (K. Poole, K. Krebes, C. McNally, and S. Neshat, J. Bacteriol. 175:7363–7372, 1993) into the multidrug-resistant strains resulted in the loss of multidrug resistance and the acquisition of hypersusceptibility in the OprM group, while the phenotype of the OprK-like group was unaffected. These experiments demonstrated the existence of two genetically distinct efflux systems in *P. aeruginosa*. The identities of the operons encoding the two efflux systems and their physiological roles are discussed.

A number of bacteria possess active efflux pumps enabling them to avoid the deleterious effects of noxious agents (8, 9). In particular, active efflux proteins have been shown to contribute significantly to multidrug resistance (MDR) in *Pseudomonas aeruginosa* (16), a species characterized by intrinsic resistance to numerous antimicrobial agents. Poole et al. (17, 18) first described a siderophore-deficient mutant of *P. aeruginosa* overexpressing an outer membrane (OM) protein of 50 kDa (OprK). The OprK phenotype was associated with enhanced resistance to quinolones, tetracycline, chloramphenicol, and streptonigrin. The gene coding for OprK was recognized as being part of an operon composed of three genes encoding, respectively, OprK, which is homologous to OM export proteins; MexB, a 108-kDa cytoplasmic membrane protein acting as an active efflux pump; and MexA, a 40-kDa periplasmic component thought to link OprK and MexB. Recently, another three-protein efflux system was described in *P. aeruginosa* (10, 11). This system is composed of the 50-kDa OM protein OprM initially described by Masuda and Ohya (12) and MexC, a 46-kDa protein, and MexD, a 100-kDa protein, which are both found in the cytoplasmic membrane fraction. The MexC-MexD-OprM complex has been associated with resistance to tetracycline, chloramphenicol, and quinolones (10), as well as to some β-lactams such as carbenicillin (11). Masuda et al. (13) suggested the existence of three types of MDR mutants: (i) the *nalB* type (20, 21), showing cross-resistance to meropenem, cephems, and quinolones and overproducing OprM; (ii) the *nfxB* type (7), with cross-resistance to cefpirome, cefozopran, tetracycline, chloramphenicol, and quinolones and overproducing the 54-kDa OM protein OprJ; and (iii) the *nfxC* type (4), showing cross-resistance to quinolones and carbapenems and expressing decreased amounts of OprD and increased amounts of the 50-kDa OprN protein. OprM and OprN, which share almost the same apparent molecular size, were distinguishable by heat modifiability (13).

In the present study, we obtained MDR mutants of strain PAO1 by selecting for resistance to either tetracycline or erythromycin. We generated two phenotypic classes of mutants that we compared with various MDR strains described in the literature.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* strains used in the study are listed in Table 1.

Chemicals. Imipenem and norfloxacin (Merck Sharp & Dohme-Chibret, Zürich, Switzerland), meropenem (Imperial Chemical Industries PLC, Macclesfield, Great Britain), ceftazidime (Glaxo, Bern, Switzerland), cefpirome and ofloxacin (Roussel Uclaf, Romainville, France), cefoperazone (Pfizer AG, Zürich, Switzerland), carbenicillin (SmithKline Beecham Pharmaceuticals, Worthing, Great Britain), aztreonam (Squibb & Sons Inc., Princeton, N.J.), ciprofloxacin (Bayer AG, Wuppertal, Germany), gentamicin (Biochrom KG, Berlin, Germany), erythromycin (Abbott Laboratories, North Chicago, Ill.), and chloramphenicol and tetracycline (Sigma, St. Louis, Mo.) were provided in the form of sterile powders of known potencies and were dissolved extemporaneously according to the recommendations of the manufacturers. Other chemicals were of analytical grade.

Media and selection of MDR mutants. Luria-Bertani (LB) medium was used for bacterial growth. Selection of resistant strains was performed with a single agent on LB agar-antibiotic gradient plates (2). Gradients were prepared in square petri dishes (9 by 9 cm), and the antibiotic concentrations provided ranged linearly from zero to the chosen maximum (14). After incubation for 24 h at 37°C, bacterial growth was examined. Typical growth aspects on the gradients included confluent growth at lower antibiotic concentrations and nonconfluent colonies at higher concentrations. In order to provide an inoculum of 10^9 CFU per gradient plate, 1.5 ml of an overnight bacterial broth culture was centrifuged in an Eppendorf tube for 1 min. The supernatant was discarded and the pellet was well homogenized in 0.15 ml of LB medium. A total of 0.1 ml of this suspension, corresponding to approximately 10^9 CFU, was uniformly plated

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TABLE 1. Bacterial strains used in the study

P. aeruginosa strain	Relevant characteristics	Reference
PAO1	Wild type	Laboratory collection
PAO1ERYR	MDR mutant selected on erythromycin	This study
PAO1TETR	MDR mutant selected on tetracycline	This study
PAO4098	FP^- *met-9020 pro-9024 blaP9208*	10
PAO4098E	MDR mutant overproducing OprM, selected on ciprofloxacin and cefsulodin	10
PAO1OCR03	MDR mutant overproducing OprM, selected on ofloxacin and cefsulodin	13
PAO1OR01	MDR mutant overproducing OprJ, selected on ofloxacin	13
K372	Derivative of PAO6609 deficient in production of pyoverdine and the ferripyochelin receptor	19
K385	Dipyridyl-resistant derivative of K372, MDR mutant overproducing OprK	18
K613	K372 ORFC::Ω-Hg	18

onto either erythromycin or tetracycline gradient plates, and the plates were incubated at 37°C for 24 h. Colonies growing at the highest antibiotic concentrations were sampled, streaked onto antibiotic-free LB agar for a further incubation (24 h at 37°C), checked for purity, and replated onto new antibiotic gradient plates when necessary.

Definition of resistance. The acquisition of resistance was defined as a fourfold increase in the MIC. MDR designated the simultaneous acquisition of resistance as defined above for various structurally unrelated antibiotics.

Mutational frequency. Portions (0.1 ml) of an overnight broth culture were uniformly spread onto antibiotic-free LB agar (after proper dilution) and onto antibiotic-containing agar plates (without dilution). Antibiotic concentrations corresponded to four times the MICs. Colonies were counted after 48 h of incubation at 37°C and were compared with the original inoculum size. Mutants appeared at frequencies of 10^{-7} to 10^{-8}.

Susceptibility testing. Susceptibility to antimicrobial agents was assayed by a microdilution method in Mueller-Hinton broth (23). The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth after 18 h at 37°C.

OM and IM membrane preparations. Each strain was grown in prewarmed flasks containing 300 ml of LB medium, inoculated with 3 ml of an overnight preculture, and incubated at 37°C under rotatory shaking (350 rpm). The cells were harvested at the midexponential phase of growth (optical density at 650 nm = 0.6 to 0.7) by centrifugation at 5,000 × *g* for 20 min at 4°C, washed once with 30 mM morpholinepropanesulfonic acid (MOPS)–200 mM NaCl buffer (pH 8.0), and resuspended (100 mg/ml [wet weight]) in the same buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 10 mM 2-mercaptoethanol. The cells were then passed twice through a French press cell (SLM Instruments, Inc., Urbana, Ill.) at 15,000 lb/in^2. Unbroken cells were eliminated by centrifugation at 5,000 × *g* for 20 min at 4°C. The membranes were pelleted by centrifugation at 110,000 × *g* for 50 min and were washed twice with 15 mM MOPS (pH 8.0). Membrane suspensions were then incubated with 2% (wt/vol) Sarkosyl NL-97 detergent at room temperature for 20 min. The insoluble OM fraction was pelleted by centrifugation at 40,000 × *g* at 10°C and was resuspended in distilled water at a protein concentration of about 10 mg/ml. The supernatant contained the soluble inner membrane (IM) fractions. The OM and IM fractions were stored at −20°C. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was used as a standard.

Gel electrophoresis. OM and IM proteins were analyzed by electrophoresis with a Protean II Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.). Protein fractions were solubilized in sample buffer, heated at 95°C for 5 min, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 10% (wt/vol) acrylamide and 0.1% (wt/vol) piperazine diacrylyl in the running gel at a constant current of 10 mA per gel. The gels were stained with Coomassie blue solution.

Transduction. The general transducing phage E79*tv-2* (15) was used for transduction. Transduction was performed as described by Haas et al. (6). The donor strain was K613 (K372 ORFC::Ω-Hg). Recipient strains were MDR mutants and the corresponding wild-type strains listed in Table 1.

DNA hybridization. Chromosomal DNA was prepared from exponentially growing cultures of *P. aeruginosa* as described previously (1) and was digested with either *Eco*RI or *Sal*I. After electrophoretic separation, Southern hybridization (22) was performed by using a digoxigenin (DIG)-labelled 4.8-kb *Bam*HI DNA fragment from plasmid pHP45Ω-Hg as a probe (3). Hybridization at 42°C in the presence of 50% formamide and subsequent development of the blot were carried out according to the instructions of the manufacturer (1a).

RESULTS

Isolation of erythromycin- and tetracycline-resistant mutants of PAO1. Spontaneous resistant mutants were isolated by stepwise selection on LB agar gradient plates. Resistant colonies were selected after one (erythromycin) or three (tetracycline) steps. One resistant mutant, called ERYR and TETR, respectively, was chosen from each plate and was tested for its susceptibility to antibiotics. Two phenotypes appeared (Table 2). TETR was characterized by cross-resistance to quinolones, chloramphenicol, and all β-lactams tested except imipenem. In addition, it was slightly more susceptible to gentamicin than its parent, whereas its susceptibility to erythromycin was not affected. ERYR, by contrast, kept its susceptibility to all β-lactams tested, with the notable exception of cefpirome, but it was resistant to chloramphenicol, quinolones, and tetracycline and was hypersusceptible to imipenem.

OM and IM proteins. SDS-polyacrylamide gels of OM proteins (Fig. 1A) displayed the overproduction of a protein band of about 50 kDa in both mutants. The mobility of the band from the ERYR strain (lane 4) was slightly slower than that of the TETR strain (lane 3), but it was identical to that of the OprK protein band overexpressed in strain K385 (lane 2). By using different acrylamide concentrations for examining lower-molecular-mass components of the OM, no other significant changes were observed (data not shown). IM preparations (Fig. 1B) demonstrated the overproduction of a protein of about 46 kDa in the TETR strain (lane 3) and one of about 43 kDa in strains ERYR (lane 2) and K385 (lane 4) as well as in the OprJ-overproducing strain OR01 (data not shown). The protein band with a higher molecular mass, of about 100 kDa,

TABLE 2. MICs for *P. aeruginosa* PAO1 and MDR derivatives ERYR and TETR

Strain	MICs (μg/ml)[a]													
	AZM	CRB	CAZ	CPA	CPM	MER	IMI	CAM	TET	ERY	CIP	NOR	OFL	GEN
PAO1	2	64	1	4	2	0.5	1	64	16	128	0.03	0.25	1	0.5
TETR	32	512	8	64	8	2	0.5	512	64	128	0.5	2	8	0.125
ERYR	1	16	1	4	16	0.5	0.125	256	64	512	1	4	8	0.25

[a] Abbreviations: AZM, aztreonam; CRB, carbenicillin; CAZ, ceftazidime; CPA, cefoperazone; CPM, cefpirome; MER, meropenem; IMI, imipenem; CAM, chloramphenicol; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; OFL, ofloxacin; GEN, gentamicin.

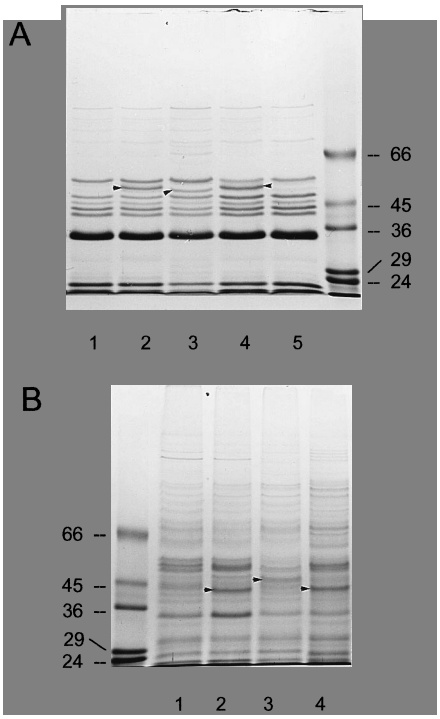

FIG. 1. SDS-PAGE of OM (A) and IM (B) proteins of *P. aeruginosa* ERYR and TETR strains compared with those of the OprK-overproducing strain K385. The migrations of standard proteins are indicated (in kilodaltons). (A) Lanes: 1, K613; 2, K385; 3, TETR; 4, ERYR; 5, PAO1. (B) Lanes: 1, PAO1; 2, ERYR; 3, TETR; 4, K385. Arrowheads indicate overproduced protein bands.

reported in the literature (18) was not detectable in our preparations.

Transduction of the ORFC(*oprK*)::Ω-Hg mutation into MDR strains. In order to further characterize ERYR and TETR and to prove the existence of two separate efflux systems in *P. aeruginosa*, we transduced the Ω-Hg cassette inactivating ORFC, which is supposed to code for the OM protein OprK, from strain K613 into the six MDR strains and into their corresponding wild-type strains (Table 1). Ten Hg-resistant transductants from each strain were tested for their antibiotic susceptibilities on pefloxacin gradient plates and compared with those of the corresponding parental MDR and wild-type strains. With a few exceptions, all transductants obtained with each strain had the same antibiotic resistance profile (data not shown). We therefore selected one representative transductant of each strain for further analysis.

In order to verify the relevant genotype of the selected transductants, chromosomal DNAs from strain K613 and the transductants were digested with either *Eco*RI or *Sal*I and were analyzed by Southern hybridization by using as a probe the DIG-labelled Ω-Hg cassette of plasmid pHP45Ω-Hg. The observed banding pattern was the same in all strains and was identical to that of K613, demonstrating that the ORFC::Ω-Hg mutation was correctly transferred.

Antibiotic susceptibility patterns of MDR strains and transductants. Analysis of antibiotic susceptibility patterns allowed us to distinguish two groups of MDR strains (Table 3). One group, called the OprM-like group (strains TETR, OCR03, and 4098E), was characterized by a complete loss of the MDR phenotype and the acquisition of hypersusceptibility after the transduction, whereas the antibiotic susceptibility profiles of the strains of the so-called OprK-like group (strains ERYR, K385, and OR01) were unaffected by the transduction (with

TABLE 3. MIC ratios for *P. aeruginosa* wild-type, MDR, and corresponding transduced strains

Strains[a]	MIC ratio[b]													
	AZM	CRB	CAZ	CPA	CPM	MER	IMI	CAM	TET	ERY	CIP	NOR	OFL	GEN
OprM-like														
TETR	16	8	8	16	4	4	0.5	8	4	1	16	8	8	0.25
OCR03	8	4	8	8	2	8	2	4	4	1	8	8	4	1
4098E	4	4	4	4	2	4	0.5	4	4	2	8	8	4	0.5
TETRT	0.5	0.01	0.5	0.12	0.5	0.12	1	0.12	0.03	0.06	<0.1	0.12	0.06	0.25
OCR03T	<0.06	<0.01	1	0.06	0.06	0.25	1	0.06	0.01	0.12	<0.1	<0.06	0.01	0.12
4098ET	<0.06	<0.01	0.5	0.06	0.12	0.06	1	0.06	<0.01	0.12	<0.06	<0.12	0.03	0.25
OprK-like														
ERYR	0.5	0.25	1	1	8	0.5	0.12	4	4	4	32	16	8	0.5
OR01	1	0.5	1	1	8	1	1	4	4	4	32	16	8	1
K385	0.5	0.5	1	1	2	0.5	1	4	4	4	16	16	8	0.5
ERYRT	0.5	0.25	1	1	8	1	1	4	4	4	32	32	8	0.5
OR01T	0.5	0.06	0.5	1	4	1	0.5	4	2	4	32	16	8	0.12
K385T	0.25	<0.01	1	1	2	0.5	1	4	2	4	8	16	4	0.25
Wild type														
PAO1T	0.125	0.01	1	0.25	0.12	1	1	0.06	0.01	0.12	<0.1	0.12	0.12	0.5
4098T	0.06	0.01	1	0.12	0.25	0.06	0.5	0.06	0.01	0.25	<0.06	0.12	0.03	0.12
ORFC::Ω-Hg														
K613	0.25	<0.01	1	0.12	0.06	0.5	1	0.06	0.03	0.06	<0.06	0.12	0.06	0.5

[a] Transductants are designated by a T following the name of the corresponding parental strain.
[b] Ratio indicates the MICs for the MDR and transduced strains divided by the MIC for the corresponding wild-type strain. See footnote of Table 1 for definitions of abbreviations.

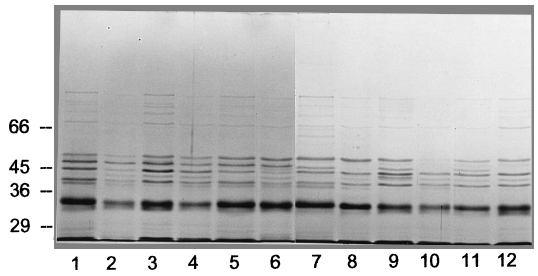

FIG. 2. OM proteins of MDR *P. aeruginosa* and corresponding transduced strains of the OprK-like (lanes 1 to 6) and the OprM-like (lanes 7 to 12) groups. Odd-numbered lanes contain the MDR strains (lanes: 1, ERYR; 3, OR01; 5, K385; 7, TETR; 9, OCR03; 11, 4098E), and the adjacent even-numbered lanes contain the corresponding transductants. The positions of the molecular mass standards are indicated on the left (in kilodaltons).

the exception of an increase in susceptibility to carbenicillin in strains OR01T and K385T). This observation was surprising since we had anticipated the opposite result, supposing that OprK was the gene product of ORFC (see Discussion). The susceptibility profiles of the transductants of the wild-type strains PAO1 and 4098 were similar to that of the hypersusceptible strain K613, for which MIC ratios were calculated relative to those for the corresponding "wild-type" strain K372 (Table 3).

Membrane profiles of transductants. Comparison of the OM preparations of the transduced and parental strains clearly demonstrated that the overproduced 50-kDa protein of the resistant OprM-like group had disappeared from the susceptible transductants (Fig. 2), whereas the OM protein profiles of the transductants from the OprK-like group were unchanged.

DISCUSSION

Several MDR strains of *P. aeruginosa* have been described in the literature. Although different selection methods have been used for their isolation, all of them are characterized by the overexpression of OM proteins in the range of 50 kDa. Poole et al. (18) isolated an MDR strain by looking for mutants resistant to the iron chelator 2,2-dipyridyl; Li et al. (10) and Masuda et al. (13) isolated an MDR strain by selecting for simultaneous resistance to a β-lactam and a quinolone. The MDR strains described in the present study were obtained by selection for resistance to either tetracycline or erythromycin, two antibiotics with unrelated structures and modes of action. Despite the different selection methods, comparison of the six different MDR strains has shown that all of the strains can be classified into two groups with respect to their antibiotic susceptibilities and OM profiles. This relatively clear-cut classification seems to be in contrast to the broad substrate specificities of the two efflux systems which are highlighted by the differences in the sizes, chemical structures, and physical properties of the substrate molecules.

An intriguing result of the present study was obtained by transducing into the MDR strains the DNA region containing the *mexA-mexB*-ORFC operon from strain K613 in which ORFC has been inactivated by the insertion of an Ω-Hg cassette. Poole et al. (18) have designated OprK the gene product of ORFC since the size of the overproduced protein in strain K385 corresponded to the molecular size deduced from the nucleotide sequence of ORFC and since the sequence of a peptide fragment of the purified OprK protein could be matched with the nucleotide sequence of ORFC. The direct proof, however, that OprK was indeed the gene product of ORFC was precluded by the fact that attempts at introducing the mutation from K613 into K385 were unsuccessful (18). Our approach with the transducing phage E79, however, allowed us to correctly transfer the mutation from K613, as demonstrated by Southern hybridization, into all of the strains used in the study. We therefore expected to eliminate the MDR phenotype in the strains of the OprK-like group. To our surprise, this was not the case. Instead, the transduction eliminated the MDR phenotype in the three OprM-like strains. There are at least two possible explanations for this unexpected result: (i) OprK is indeed the gene product of ORFC. In this case, construction of the putative *oprK*::Ω-Hg mutant K613 by gene replacement (18) has resulted for an unknown reason in the generation of an *oprM*::Ω-Hg mutant. (ii) OprM, and not OprK, is the gene product of ORFC, suggesting that the efflux system described by Poole et al. (18) would actually correspond to the *mexC-mexD-oprM* operon. In this case, *oprM* and *oprK* sequences would be expected to exhibit significant DNA homology. Although we cannot clearly distinguish between these two possibilities, our results indicate in any case that the transductants that we obtained are mutated in the gene coding for OprM. We have further demonstrated unequivocally the existence of at least two genetically distinct efflux systems in *P. aeruginosa*, which we designate OprM and OprK-like.

While the transduction experiment showed that overexpression of OprM in the MDR strains of this group results from transcription of the same *oprM*-containing operon, we cannot exclude the possibility that the OprK-like strains overproduce different efflux systems. However, the very similar antibiotic resistance profiles of the three OprK-like strains and the fact that the apparent molecular sizes not only of the OM proteins but also of the IM proteins are indistinguishable strongly suggest that these strains overexpress the same efflux system. On the basis of these observations, we suggest that the OprJ protein overproduced in the OprK-like strain OR01 (13) is probably identical to OprK.

The MIC ratios in Table 3 allow us to make the following conclusions regarding the substrate specificities of the two efflux systems: (i) OprM and OprK seem to be equally effective in exporting chloramphenicol, tetracycline, and all the tested quinolones, (ii) erythromycin is a substrate not only for OprK but also for OprM, as suggested by the 8- to 16-fold decrease in MICs for the transductants of the OprM-like group, and (iii) the MIC ratios of carbenicillin for all OprM-deficient strains (except ERYR) are extremely low (5), suggesting that this β-lactam is specifically exported via OprM and that efflux is the major mechanism of resistance to this antibiotic in the wild-type strain. In addition, we conclude that the lack of the 50-kDa OM component in the transductants of the OprM group seems to be sufficient to cause the collapse of the whole efflux system, suggesting that antibiotics either are not efficiently extruded or can reenter the cytoplasm when they are exported only to the periplasmic space.

What is the physiological role of the efflux systems? Poole et al. (17) have suggested that the OprK-like efflux system is involved in the secretion of the iron chelator molecule pyoverdine. This high-affinity iron-binding protein is synthesized in response to iron starvation and is secreted into the environment, and once it is chelated to iron, it is taken up again through a specific membrane receptor. A second, low-affinity iron-chelating compound called pyochelin is also synthesized in response to iron starvation, secreted, and taken up by a different pyochelin-specific receptor molecule. We therefore suggest that OprM and OprK could function as efflux systems for pyoverdine and pyochelin, with the exact substrate specificity remaining to be determined.

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