ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1996, p. 2288–2290 Vol. 40, No. 10
0066-4804/96/$04.00+0

Multidrug Efflux in Intrinsic Resistance to Trimethoprim and Sulfamethoxazole in *Pseudomonas aeruginosa*

THILO KÖHLER,[1]* MENNO KOK,[1] MEHRI MICHEA-HAMZEHPOUR,[1] PATRICK PLESIAT,[2] NAOMASA GOTOH,[3] TAKESHI NISHINO,[3] LASTA KOCJANCIC CURTY,[1] AND JEAN-CLAUDE PECHERE[1]

Department of Genetics and Microbiology, Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland[1]; Laboratoire de Bactériologie, Hôpital Minjoz, F-25000 Besançon, France[2]; and Department of Microbiology, Kyoto Pharmaceutical University, Yamashina, Kyoto 607, Japan[3]

Received 18 March 1996/Returned for modification 2 May 1996/Accepted 22 July 1996

Pseudomonas aeruginosa **possesses at least two multiple drug efflux systems which are defined by the outer membrane proteins OprM and OprJ. We have found that mutants overexpressing OprM were two- and eightfold more resistant than their wild-type parent to sulfamethoxazole (SMX) and trimethoprim (TMP), respectively. For OprJ-overproducing strains, MICs of TMP increased fourfold but those of SMX were unchanged. Strains overexpressing OprM, but not those overexpressing OprJ, became hypersusceptible to TMP and SMX when *oprM* was inactivated. The wild-type antibiotic profile could be restored in an *oprM* mutant by transcomplementation with the cloned *oprM* gene. These results demonstrate that the *mexABoprM* multidrug efflux system is mainly responsible for the intrinsic resistance of *P. aeruginosa* to TMP and SMX.**

Trimethoprim (TMP) and the sulfonamides are synthetic antibacterial agents that act at separate stages in the pathway of folic acid synthesis (8). The potency of TMP and the sulfonamides against *Pseudomonas aeruginosa* is limited, with MICs typically in the resistance range. The reason for this intrinsic resistance is unknown, but poor affinity for the target enzymes and low outer membrane permeability have been suggested.

Recently, we have shown that OprM (7, 14) and OprK, which is identical to OprJ (21), define two genetically distinct multidrug efflux systems in *P. aeruginosa* (16). We discovered that OprM- and OprJ-overexpressing strains display increased resistance to TMP and sulfamethoxazole (SMX) and demonstrate that the *mexABoprM* efflux system is mainly responsible for the intrinsic resistance of *P. aeruginosa* to these antibiotics.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1.

Chemicals and media. Carbenicillin (SmithKline Beecham Pharmaceuticals, Worthing, Great Britain), chloramphenicol (Sigma, St. Louis, Mo.), norfloxacin (Merck Sharp & Dohme-Chibret, Zürich, Switzerland), pefloxacin (Bellon, Neuilly-sur-Seine, France), TMP, and SMX (F. Hoffmann-La Roche, Basel, Switzerland) were dissolved according to the recommendations of the manufacturers. Other chemicals were of analytical grade. Luria-Bertani medium was used as a rich medium, and M9 (13) was used as a minimal salts medium.

Cloning of the *oprM* gene. A genomic pLAFR3-based cosmid library of strain PAO1 (26) was conjugally transferred from *E. coli* S17-1 into the *oprM* mutant 4098ET, which is hypersusceptible to tetracycline, chloramphenicol, quinolones, and β-lactams. Transconjugants in which this hypersusceptible phenotype was complemented were selected on M9 minimal medium plates supplemented with 0.05% (wt/vol) each proline and methionine, 40 mM citrate as a carbon source, and carbenicillin (40 μg/ml), a specific substrate of the *mexAB oprM* efflux system. When 18 transconjugants were tested on gradient plates containing carbenicillin, pefloxacin, or TMP (2), it was found that the MICs of the antibiotics for all 18 were similar to those for wild-type strain 4098. Plasmid DNA from one of the transconjugants was isolated and introduced into *E. coli* MC1061. The cosmid isolated from *E. coli* was named pOM1. On the basis of the restriction map of the *oprM* DNA region (18), a 7.8-kbp *Eco*RI fragment was cloned into pBR322, yielding plasmid pOMC1. A 3.2-kbp *Pst*I-*Eco*RV fragment containing the entire *oprM* coding region was inserted into the broad-host-range vector pMMB208 cleaved with *Pst*I and *Sma*I, yielding plasmid pOMF6. This plasmid and the corresponding cloning vector pMMB208 were introduced separately into the *oprM* mutant. Transformants were selected on Luria-Bertani medium containing 60 μg of chloramphenicol per ml.

MIC determination. Susceptibility to antimicrobial agents was assayed in Mueller-Hinton broth by the microdilution method (25).

Dihydrofolate reductase (DHFR) activity. Cell extracts of *P. aeruginosa* 4098, 4098T, and 4098E were prepared from 100-ml overnight cultures. The cells were centrifuged and resuspended in 2 ml of 100 mM Tris (pH 8.0) containing 2 mM EDTA, 1 M sucrose, and 0.1 mg of lysozyme per ml; 4 ml of ice-cold water was added, and the cell suspension was slightly agitated on ice for 20 min. The suspension was sonicated three times for 20 s each at 70 W in a Branson sonicator, $MgCl_2$ was added to a 5 mM final concentration, and the membrane fraction was removed by centrifugation at 200,000 × *g* for 1 h at 4°C.

DHFR activity was measured as described by Bacanari and Joyner (1) in 25 mM potassium glutamate buffer with 1 mM dithiothreitol (pH 7.4) at 25°C, in the presence of 10 μM NADPH and 4 μM dihydrofolate. The enzyme and cofactor were premixed in a volume of 0.9 ml and incubated at 25°C for 2 min. Subsequently, 100 μl of substrate dilution was added and cofactor oxidation was monitored at 340 nm in a Kontron 340 spectrophotometer. Enzyme activities were calculated by using a molar extinction coefficient of 12.3×10^3 cm^{-1} for the reaction (1).

The 50% inhibitory concentration of TMP was determined in a protein fraction enriched in DHFR obtained by precipitation of the cell extract with 30 to 80% ammonium sulfate, adsorption on methotrexate agarose, and then elution with a 100 mM potassium borate buffer (pH 9.0) containing 2 mM EDTA, 2 mM dithiothreitol, and 5 mM folate. The partially purified enzyme preparation was extensively dialyzed against a solution containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 2 mM dithiothreitol. The 50% inhibitory concentration of TMP was determined as described above, the inhibitor being added to the substrate dilution. The residual enzyme activity was measured as a function of inhibitor concentration, and the 50% inhibitory concentration was determined by interpolation.

Outer membrane preparations and Western blotting (immunoblotting). Outer membranes were prepared from exponentially grown cells in Luria-Bertani medium as described previously (16). Outer membrane proteins were analyzed by electrophoresis with a Protean II slab electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) and either stained with Coomassie blue solution or electrotransferred onto nitrocellulose membranes. After being blocked with 10% skim milk, the membranes were hybridized with the murine monoclonal immunoglobulin M antibody TM002 directed against OprM and were developed with peroxidase conjugated to an anti-mouse antibody.

RESULTS AND DISCUSSION

MICs. Relative to that of the wild-type strain, resistance to TMP, but not to SMX, increased eightfold in the OprM-over-

* Corresponding author. Mailing address: Department of Genetics and Microbiology, CMU, 9, av. de Champel, CH-1211 Geneva 4, Switzerland. Phone: 41-22-7025655. Fax: 41-22-7025702. Electronic mail address: kohler@cmu.unige.ch.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
MC1061	F$^-$ *araD139* Δ(ara-leu)7696 *galE15 galK16* Δ(lac)X74 *rpsL thi*	Laboratory collection
S17-1	*thi pro hsdR recA* chr::RP4-2	24
P. aeruginosa		
PAO1	Wild type	Laboratory collection
PAO4098	FP$^-$ *met-9020 pro-9024 blaP9208*	10
PAO4098T	PAO4098 *oprM*::ΩHg	16
PAO4098E	MDR mutant overproducing OprM; selected on ciprofloxacin and cefsulodin	10
PAO4098ET	PAO4098E *oprM*::ΩHg	16
PAO1TETR	MDR mutant overexpressing OprM; selected on tetracycline	16
PAO1TETRT	PAO1TETR *oprM*::ΩHg	16
PAO1OCR03	MDR mutant overproducing OprM; selected on ofloxacin and cefsulodin	15
PAO1OCR03T	PAO1OCR03 *oprM*::ΩHg	16
PAO1ERYR	MDR mutant overproducing OprJ; selected on erythromycin	16
PAO1ERYRT	PAO1ERYR *oprM*::ΩHg	16
PAO1OR01	MDR mutant overproducing OprJ; selected on ofloxacin	15
PAO1OR01T	PAO1OR01 *oprM*::ΩHg	16
K372	PAO6609 derivative deficient in production of pyoverdin, pyochelin, and the ferripyochelin receptor	23
K385	MDR mutant of K372 overproducing OprJ; selected on dipyridyl	22
K385T	K385 *oprM*::ΩHg	16
Plasmids		
pLAFR3	Cosmid vector harboring genomic library; Tc^r	5
pMMB208	Broad-host-range expression vector; Cm^r	17
pOM1	*oprM*-containing pLAFR3-based cosmid; Tc^r	This study
pOMC1	7.8-kbp *Eco*RI fragment of pOM1 harboring *oprM* inserted into *Eco*RI-cleaved pBR322; Ap^r	This study
pOMF6	3.2-kbp *Pst*I-*Eco*RV fragment of pOMC1 harboring *oprM* inserted into *Pst*I-*Sma*I-cleaved pMMB208; Cm^r	This study

[a] Hg, mercury; Tc, tetracycline; Cm, chloramphenicol; Ap, ampicillin.

expressing strains and fourfold in the OprJ-overexpressing strains (Table 2). While OprM-deficient derivatives of wild-type strains and of OprM-overexpressing strains were 25-fold more susceptible to TMP and SMX than were wild-type strains, the resistance to these antibiotics was not affected in OprM-deficient derivatives of the OprJ-overexpressing strains (Table 2).

TABLE 2. MICs for *P. aeruginosa* MDR and *oprM* mutant strains[a]

Strain	Relevant phenotype	MIC (μg/ml)		
		TMP	SMX	NOR
PAO1	wt	100	125	0.25
4098	wt	100	125	0.25
TETR	OprM$^+$	800	250	2
OCR03	OprM$^+$	1,600	250	2
4098E	OprM$^+$	800	250	2
TETRT	OprM$^-$	4	4	0.03
OCR03T	OprM$^-$	4	8	0.015
4098ET	OprM$^-$	4	4	0.03
ERYR	OprJ$^+$	400	125	4
OR01	OprJ$^+$	400	125	4
K385	OprJ$^+$	400	250	4
ERYRT	OprJ$^+$ OprM$^-$	400	125	8
OR01T	OprJ$^+$ OprM$^-$	400	125	4
K385T	OprJ$^+$ OprM$^-$	400	200	4
4098T	OprM$^-$	4	8	0.06
4098T(pOMF6)	OprM$^-$	100	125	0.25
4098T(pMMB208)	OprM$^-$	8	15	0.06

[a] Abbreviations: wt, wild type; NOR, norfloxacin.

Determination of DHFR activity. In wild-type strain 4098, the *oprM* mutant 4098T, and the OprM overproducer 4098E, the mean specific activities of DHFR ± standard errors of the means were 4.7 ± 1.2, 3.1 ± 1.0, and 1.9 ± 0.6 μmol/min/mg of protein, respectively, and the mean TMP concentrations which reduced the enzyme activity in vitro to 50% were 0.041 ± 0.004, 0.046 ± 0.005, and 0.042 ± 0.004 μM, respectively.

Complementation analysis. We next cloned a DNA fragment containing the *oprM* gene of *P. aeruginosa* as described in Materials and Methods and used this clone, designated pOMF6, to complement OprM-deficient strain 4098T. A protein expressed from this construct hybridized with a monoclonal OprM antibody and showed an electrophoretic mobility identical to that of the chromosomally expressed OprM protein in the wild-type strain. The protein levels obtained with plasmid pOMF6 were comparable to those of the wild type but were lower than those of the multidrug-resistant (MDR) strain (data not shown). The cloned *oprM* gene restored wild-type levels of resistance to TMP and SMX as well as to norfloxacin, which was included as an additional control (Table 2).

Recently (16) we have classified six MDR strains of *P. aeruginosa* into two groups on the basis of genetic and phenotypic characterization. Members of the first group, strains 4098E, OCR03, and TETR, overexpressed the outer membrane protein OprM, resulting in increased resistance to quinolones, tetracycline, chloramphenicol, and β-lactams. The second group was composed of the OprK-overexpressing strains K385 and ERYR and the OprJ-overexpressing strain OR01. As suggested previously (16), OprK and OprJ are the same protein (21). This group was characterized by increased resis-

tance to quinolones, tetracycline, chloramphenicol, and erythromycin.

We show here that the broad-specificity efflux system encoded by the *mexABoprM* operon (11, 22) plays a major role in the intrinsic resistance of *P. aeruginosa* to both TMP and SMX. This can be concluded from the following observations: (i) mutants deficient in the outer membrane protein OprM were hypersusceptible; (ii) transcomplementation with the cloned *oprM* gene restored the wild-type antibiotic phenotype; and (iii) the expression and affinity of DHFR, the target enzyme of TMP, for the wild-type strain were comparable to those for the OprM-overproducing and OprM-deficient strains. Interestingly, both TMP and SMX were also substrates of the multidrug efflux system encoded by the *mexCDoprJ* operon (21), since mutation of the *oprM* gene in OprJ-overexpressing strains did not affect their resistance phenotype.

P. aeruginosa mutants overexpressing efflux operons have been selected by various agents, including quinolones, tetracycline, erythromycin, and the iron chelator dipyridyl (Table 1). We have also generated mutants of PAO1 by selection for resistance to TMP (unpublished results). Interestingly, these mutants did not show an MDR profile, suggesting that TMP does not preferentially select an MDR phenotype in *P. aeruginosa*.

Efflux-mediated resistance to TMP might also be relevant in other bacterial species. Recently, George et al. (6) cloned a DNA fragment from *Klebsiella pneumoniae* containing the *ramA* locus, which is involved in multiple antibiotic resistance. A susceptible *E. coli* laboratory strain harboring the *ramA* gene showed increased resistance to nine structurally unrelated antibiotics, including tetracycline, chloramphenicol, norfloxacin, puromycin, and TMP. RamA shows weak homology to MarA (4), a transcriptional activator also involved in regulation of the multidrug efflux operon *acrAB* of *E. coli* (20). However, in a *K. pneumoniae ramA* mutant and in an *E. coli mar* mutant, the levels of the major porin OmpF were significantly decreased, suggesting that reduced antibiotic influx might contribute to TMP resistance in these organisms.

More recently, Burns et al. (3) have identified in *Burkholderia cepacia* an outer membrane protein gene, OpcM, which has significant homology to OprM of *P. aeruginosa*. A strain from which the DNA region comprising *opcM* has been deleted was hypersusceptible to TMP, chloramphenicol, and ciprofloxacin.

These findings support the current hypothesis that antibiotic efflux (9, 12, 19) plays a major role in the MDR phenotype of a number of clinically relevant bacterial pathogens.

ACKNOWLEDGMENTS

This work was supported by a grant from the Fonds National Suisse pour la Recherche Scientifique and from a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

We thank L. Serino, University of Lausanne, for providing the genomic library of *P. aeruginosa*, and we appreciate the skillful technical assistance of A. Kahr, C. Cherbuliez, and K. Hiraki.

REFERENCES

1. **Bacanari, D. P., and S. S. Joyner.** 1981. Dihydrofolate reductase hysteresis and its effect on inhibitor binding analyses. Biochemistry **20:**1710–1716.
2. **Bryson, L., and W. Szybalski.** 1952. Microbial selection. Science **116:**45–51.
3. **Burns, J. L., C. D. Wadsworth, J. J. Barry, and C. P. Goodall.** 1996. Nucleotide sequence analysis of a gene from *Burkholderia* (*Pseudomonas*) *cepacia* encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. Antimicrob. Agents Chemother. **40:**307–313.
4. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
5. **Friedman, I. M., S. R. Long, S. E. Brown, W. S. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium meliloti* mutants. Gene **18:**289–296.
6. **George, A. M., R. M. Hall, and H. W. Stokes.** 1995. Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. Microbiology (Reading) **141:**1909–1920.
7. **Gotoh, N., N. Itoh, H. Tsujimoto, J.-I. Yamagishi, Y. Oyamada, and T. Nishino.** 1994. Isolation of OprM-deficient mutants of *Pseudomonas aeruginosa* by transposon insertion mutagenesis: evidence of involvement in multiple antibiotic resistance. FEMS Microbiol. Lett. **122:**267–274.
8. **Huovinen, P., L. Sundström, G. Swedberg, and O. Sköld.** 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother. **39:**279–289.
9. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
10. **Li, X.-Z., D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. **38:**1732–1741.
11. **Li, X.-Z., H. Nikaido, and K. Poole.** 1995. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1948–1953.
12. **Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in Gram-negative bacteria. Trends Microbiol. **2:**489–493.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Masuda, N., and S. Ohya.** 1992. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1847–1851.
15. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
16. **Michea Hamzehpour, M., J.-C. Pechere, P. Plesiat, and T. Köhler.** 1995. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**2392–2396.
17. **Morales, V. M., A. Bäckman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39–47.
18. **Morshed, S. R. M., Y. Lei, H. Yoneyama, and T. Nakae.** 1995. Expression of genes associated with antibiotic extrusion in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **210:**356–362.
19. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
20. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
21. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, and T. Nishino.** Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB* type multidrug resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol., in press.
22. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
23. **Poole, K., S. Neshat, and D. Heinrichs.** 1991. Pyoverdin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. FEMS Microbiol. Lett. **78:**1–5.
24. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–790.
25. **Thornsberry, C., J. Anhalt, A. L. Barry, E. H. Gerlach, J. Hossom, R. N. Jones, J. M. Matsen, R. C. Moellering, and R. Norton.** 1983. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, p. 48–56. National Committee for Clinical Laboratory Standards, Villanova, Pa.
26. **Visca, P., A. Ciervo, and N. Orsi.** 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N^5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**1128–1140.