Multiple Antibiotic Resistance in *Stenotrophomonas maltophilia*

ANA ALONSO AND JOSÉ L. MARTÍNEZ*

Banco de Células, Centro Nacional de Biotecnología (CSIC), Campus UAM, Cantoblanco, 28049-Madrid, Spain

A cryptic multidrug resistance (MDR) system in *Stenotrophomonas maltophilia*, the expression of which is selectable by tetracycline, is described. Tetracycline resistance was the consequence of active efflux of the antibiotic, and it was associated with resistance to quinolones and chloramphenicol, but not to aminoglycosides or β-lactam antibiotics. MDR is linked to the expression of an outer membrane protein (OMP54) both in a model system and in multidrug-resistant clinical isolates.

Multidrug resistance (MDR) is widespread among gram-negative bacteria (6, 12, 15, 20, 22). Indeed, the emergence of new opportunistic pathogenic microorganisms is somehow linked to their multiresistant phenotype (2) that makes them refractory to the antibiotics commonly used in clinical practice. One of these multiresistant opportunistic nosocomial pathogens is *Stenotrophomonas maltophilia* (1, 4, 9, 10, 17, 26). This gram-negative microorganism is increasingly being isolated from patients with different pathologies, and a fatality rate of 44.4% has been reported for patients in critical care units with lower respiratory tract infections (17).

Clinical *S. maltophilia* isolates are often highly resistant to several antibiotics (25), so we tested this species for the presence of an MDR system, as has been described for other gram-negative microorganisms. For this purpose, single-step tetracycline-resistant mutants were selected by plating the antibiotic-susceptible clinical strain D457 onto selective Luria-Bertani agar containing a tetracycline concentration slightly higher than the MIC for the organism. This has been a successful approach, with stepwise selection, in other systems (3). However, we used single-step selection, with the idea that mutations leading to resistance probably correspond to a single genetic event. The frequency of mutation was 1.9×10^{-6}. The MICs of different antibiotics for both the original strain, D457, and the tetracycline-resistant one, D457R, were determined by the E-test (28). Although the only antibiotic present in the selection procedure was tetracycline, the MICs of structurally unrelated antibiotics were also higher in resistant derivative D457R than in parental strain D457 (Table 1). Moreover, the ratio between the MICs of different antibiotics for D457R and D457 was much higher for quinolones (not used during the selection procedure) than for tetracycline. The MDR bacterial systems that have been described so far are associated with overexpression of cryptic efflux pumps. The specificities of the system vary, although all of them accept a broad range of structurally unrelated compounds (6, 15, 22). The mutant used for our study had increasing levels of resistance to tetracycline, chloramphenicol, and quinolones, but the MICs of amikacin, imipenem, and ticarcillin-clavulanate for the mutant did not change appreciably. This phenotype resembles that of the OprK-overexpression mutant of *Pseudomonas aeruginosa*. This type of mutant was also resistant to quinolones, tetracycline, and chloramphenicol, while it retained its susceptibility to β-lactams (8). However, it was hypersusceptible to imipenem. In the case of the mutant studied in the present work, the high

MIC of imipenem is probably the consequence of the expression of a chromosomally encoded β-lactamase with a high level of activity against this antibiotic in *S. maltophilia* (21).

Once we had shown that single-step mutations produced an MDR phenotype in *S. maltophilia*, the presence of new outer membrane proteins (OMPs) in resistant mutant D457R was analyzed as described by Fukuoka et al. (5). Most efflux complexes in gram-negative bacteria are formed by three proteins (6, 22). One is a transporter protein, linked to the cytoplasmic membrane, another is an OMP channel present in the outer membrane, and the last one is a linker protein located between the transporter protein and the OMP channel in the periplasmic space. Therefore, the presence of a new OMP in D457R could be the result of the expression of an efflux pump system linked to the MDR phenotype. As shown in Fig. 1, a new OMP of 54 kDa was detected in D457R. It has been described that *S. maltophilia* is more resistant to gentamicin after culture at 30°C than at 37°C and that this difference is associated with the expression of new OMPs of approximately 55 to 57 kDa (27). Since some efflux pump systems are environmentally regulated (14, 16, 23), we tested whether the MDR phenotype was induced at 30°C. No difference in MICs was observed for D457 or D457R (data not shown), indicating that the mutation in D457R is unrelated to the phenotype of gentamicin resistance displayed by *S. maltophilia* at low temperatures. The fact that the mutation does not affect the MIC of amikacin also favors this hypothesis. Selection for quinolone-resistant mutants has also produced *S. maltophilia* strains with altered OMP profiles (11). Among five different types of mutants, two were resistant not only to quinolones but also to chloramphenicol and expressed new OMPs. Nevertheless, the ratio of MIC for the resistant strain and the MIC for the susceptible parental strain was less than 4, which was much lower than the values ob-

TABLE 1. Susceptibilities of *S. maltophilia* D457 and its tetracycline-resistant derivative D457R to antibiotics

Antimicrobial agent	MIC (μg/ml)		Fold change
	D457	D457R	
Tetracycline	6	16	2.7
Chloramphenicol	1.5	12	8
Ciprofloxacin	0.75	16	21.3
Amikacin	24	16	0.7
Norfloxacin	6	64	10.7
Imipenem	200	200	1
Ticarcillin-clavulanate	4	6	1.5
Nalidixic acid	8	128	16

* Corresponding author. Phone: 34-1-5854551. Fax: 34-1-5854503. E-mail: jmartinez@samba.cnb.uam.es.
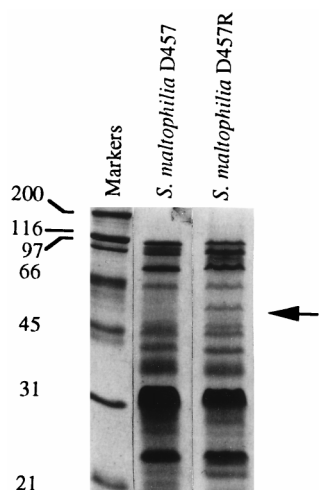
FIG. 1. Outer membrane profiles of *S. maltophilia* D457 and D457R. Outer membrane profiles of the tetracycline-susceptible strain D457 and its tetracycline-resistant derivative D457R were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The new OMP (molecular size, 54 kDa) present in D457R is marked with an arrow.

served in our work. Furthermore, the sizes of the OMPs associated with the mutations were slightly lower than that of OMP54 (48 and 50.5 kDa).

Antibiotic efflux pump systems (15, 22) are energy dependent, so that antibiotic accumulation by bacterial cells increases in the presence of proton motive uncoupler agents (13). To test whether or not this occurs for *S. maltophilia*, the effect of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on the kinetics of tetracycline accumulation by D457 and D457R was tested as described previously (13). As shown in Fig. 2, steady-state accumulation of the drug was achieved at 5 min after treatment for both strains. Treatment with CCCP significantly increased tetracycline accumulation by D457R, although this accumulation was not observed for D457. This result indicates that the mutation leading to an MDR phenotype is associated with the expression of an energy-dependent efflux pump system in *S. maltophilia*. Mutations leading to MDR phenotypes can be the consequence of mutations either in the promoter sequences of the operon encoding for the synthesis of the system or in a master regulatory locus. Since in this last case single-step mutations can affect the expression of multiple genes, we cannot be assured that the efflux pump system that we detected is the only one in which the expression is affected in D457R.

Nine unrelated clinical isolates of *S. maltophilia* were also analyzed for the presence of OMP54. The OMP profiles of these strains are presented in Fig. 3. It is noteworthy that all six strains for which tetracycline MICs were greater than 10 μg/ml presented an OMP with a molecular mass of 54 kDa (Fig. 3A), whereas those for which MICs used less than 10 μg/ml lacked this protein (Fig. 3B). The MICs of different antibiotics for these clinical strains were also determined. As indicated in Table 2, for all strains expressing OMP54 the MICs of not only tetracycline but also chloramphenicol and quinolones were greater than those for strains which lacked this OMP. These data reinforce the idea that OMP54 expression is linked to an MDR phenotype in clinical *S. maltophilia* strains.

All of these data are consistent with the idea that *S. maltophilia* possesses at least one MDR system, similar to those described previously for other gram-negative bacteria (6, 15),
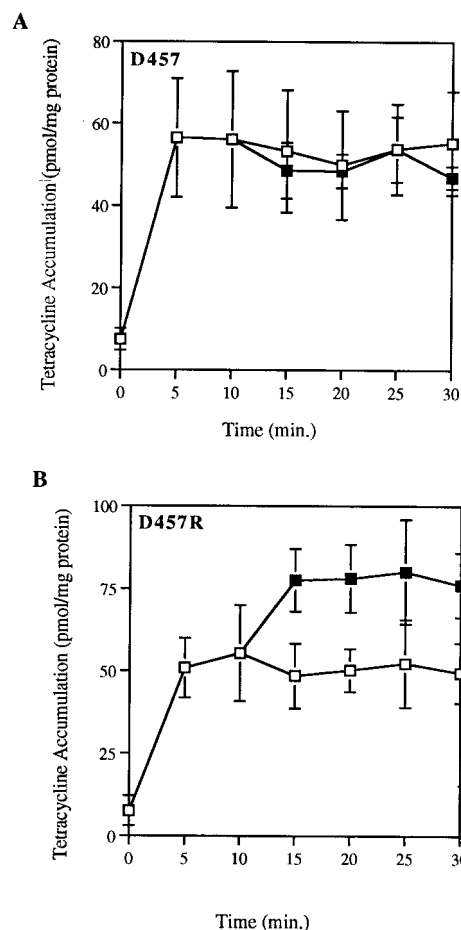

FIG. 2. Kinetics of tetracycline accumulation by D457 and D457R. The kinetics of tetracycline accumulation by D457 and D457R in the presence of CCCP are shown. In both cases, the antibiotic was added to the cells at time zero, and the proton motive uncoupler CCCP was added 10 min later. Filled squares, accumulation in the presence of CCCP; open squares, control cells without CCCP. (A) Tetracycline accumulation by D457. (B) Tetracycline accumulation by D457R. The data in the figure represent the means and standard deviations of three independent experiments performed in duplicate.

and that this system is selectable by low concentrations of tetracycline. The system is effective for quinolones, tetracycline, and chloramphenicol but not for aminoglycosides or β-lactams. It is also notable that a modest increase in the MIC of tetracycline was associated with a much higher increase in the MICs of quinolones (Table 1). Quinolones are one of the

TABLE 2. Susceptibilities of clinical isolates of *S. maltophilia* to antibiotics

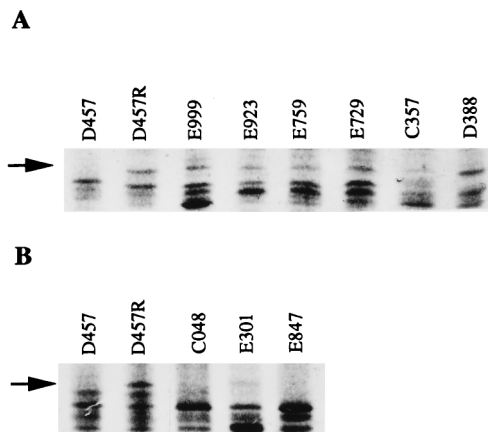| Antimicrobial agent	MIC (μg/ml)								
	C357	E759	E923	E999	E729	D388	C048	E301	E847
Tetracycline	32	16	32	16	32	12	4	8	4
Chloramphenicol	96	19	64	12	16	6	1.5	3	0.75
Ciprofloxacin	>32	2	8	2	>32	2	0.38	0.50	0.50
Amikacin	4	24	12	>256	16	6	6	2	48
Norfloxacin	256	8	32	16	>256	8	3	2	3
Imipenem	250	250	325	150	150	200	250	150	150
Ticarcillin-clavulanate	24	>256	96	1.5	1	16	0.75	4	0.75

A

B

FIG. 3. Outer membrane profiles of clinical isolates of *S. maltophilia*. (A) Outer membrane profiles of antibiotic-resistant clinical isolates of *S. maltophilia*. (B) Outer membrane profiles of antibiotic-susceptible clinical isolates of *S. maltophilia*. In both panels, strains D457 (lane 1) and D457R (lane 2) were used as controls. OMP54 is marked with an arrow. Note that all resistant isolates show an OMP of the same molecular size as that of OMP54, whereas none of the susceptible ones expressed such an OMP.

few families of antibiotics to which *S. maltophilia* is moderately susceptible (11, 24, 25), although the number of high-level-resistant strains has increased in recent years. In fact, the MIC of ciprofloxacin at which 90% of isolates are inhibited increased from 16 µg/ml between 1981 and 1988 and reached 64 µg/ml in 1992 (25). It has recently been suggested that in *Escherichia coli* low-level resistance to fluoroquinolones resulting from *mar* locus expression might favor the emergence of strains with high-level resistance, which would be of clinical relevance (7). In the case of *S. maltophilia*, the expression of OMP54 is associated with clinically relevant resistance to quinolones. This conclusion is based not only on the MICs of quinolones for D457 and D457R but also on the MICs of quinolones for clinical isolates whether or not they expressed OMP54. Probably, some other factors such as intrinsic permeability of cellular envelopes (19) and mutations in the gyrase (18) should contribute to the final level of resistance; however, our data are consistent with a relevant role of OMP54 in the resistant phenotype. Altogether our data indicate that the selection of MDR by the use of a single antibiotic may have severe implications for the future therapy of infections caused by *S. maltophilia*.

REFERENCES

1. **Arpi, M., M. A. Victor, J. K. Moller, V. Jonsson, M. M. Hansen, N. A. Peterslund, and B. Bruun.** 1994. Changing etiology of bacteremia in patients with hematological malignancies in Denmark. Scand. J. Infect. Dis. **26:**157–162.
2. **Bergogne Berezin, E., D. Decre, and M. L. Joly Guillou.** 1993. Opportunistic nosocomial multiply resistant bacterial infections: their treatment and prevention. J. Antimicrob. Chemother. **32**(Suppl. A):39–47.
3. **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: de-

creased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
4. **Denton, M., N. J. Todd, and J. M. Littlewood.** 1996. Role of anti-pseudomonal antibiotics in the emergence of *Stenotrophomonas maltophilia* in cystic fibrosis patients. Eur. J. Clin. Microbiol. Infect. Dis. **15:**402–405.
5. **Fukuoka, T., N. Masuda, T. Takenouchi, N. Sekine, M. Iijima, and S. Ohya.** 1991. Increase in susceptibility of *Pseudomonas aeruginosa* to carbapenem antibiotics in low-amino-acid media. Antimicrob. Agents Chemother. **35:**529–532.
6. **George, A. M.** 1996. Multidrug resistance in enteric and other gram-negative bacteria. FEMS Microbiol. Lett. **139:**1–10.
7. **Goldman, J. D., D. G. White, and S. B. Levy.** 1996. Multiple antibiotic resistance (*mar*) locus protects *Escherichia coli* from rapid cell killing by fluoroquinolones. Antimicrob. Agents Chemother. **40:**1266–1269.
8. **Hamzehpour, M. M., J. C. Pechere, P. Plesiat, and T. Kohler.** 1995. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**2392–2396.
9. **Heath, T., and B. Currie.** 1995. Nosocomial and community-acquired *Xanthomonas maltophilia* infection in tropical Australia. J. Hosp. Infect. **30:**309–313.
10. **Karpati, F., A. S. Malmborg, H. Alfredsson, L. Hjelte, and B. Strandvik.** 1994. Bacterial colonisation with *Xanthomonas maltophilia*: a retrospective study in a cystic fibrosis patient population. Infection **22:**258–263.
11. **Lecso Bornet, M., J. Pierre, D. Sarkis Karam, S. Lubera, and E. Bergogne Berezin.** 1992. Susceptibility of *Xanthomonas maltophilia* to six quinolones and study of outer membrane proteins in resistant mutants selected in vitro. Antimicrob. Agents Chemother. **36:**669–671.
12. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. **19:**119–123.
13. **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.
14. **Lomovskaya, O., K. Lewis, and A. Matin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.
15. **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.
16. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
17. **Maningo, E., and C. Watanakunakorn.** 1995. *Xanthomonas maltophilia* and *Pseudomonas cepacia* in lower respiratory tracts of patients in critical care units. J. Infect. **31:**89–92.
18. **Neu, H. C.** 1992. Quinolone antimicrobial agents. Annu. Rev. Med. **43:**465–486.
19. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
20. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
21. **Paton, R., R. S. Miles, and S. G. Amyes.** 1994. Biochemical properties of inducible beta-lactamases produced from *Xanthomonas maltophilia*. Antimicrob. Agents Chemother. **38:**2143–2149.
22. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. **60:**575–608.
23. **Seoane, A. S., and S. B. Levy.** 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*. J. Bacteriol. **177:**3414–3419.
24. **Smit, W. J., A. L. Boquest, J. E. Geddes, and F. A. Tosolini.** 1994. The antibiotic susceptibilities of *Xanthomonas maltophilia* and their relation to clinical management. Pathology **26:**321–324.
25. **Vartivarian, S., E. Anaissie, G. Bodey, H. Sprigg, and K. Rolston.** 1994. A changing pattern of susceptibility of *Xanthomonas maltophilia* to antimicrobial agents: implications for therapy. Antimicrob. Agents Chemother. **38:**624–627.
26. **Vartivarian, S. E., K. A. Papadakis, and E. J. Anaissie.** 1996. *Stenotrophomonas (Xanthomonas) maltophilia* urinary tract infection. A disease that is usually severe and complicated. Arch. Intern. Med. **156:**433–435.
27. **Wilcox, M. H., T. G. Winstanley, and R. C. Spencer.** 1994. Outer membrane protein profiles of *Xanthomonas maltophilia* isolates displaying temperature-dependent susceptibility to gentamicin. J. Antimicrob. Chemother. **33:**663–666.
28. **Yao, J. D., M. Louie, L. Louie, J. Goodfellow, and A. E. Simor.** 1995. Comparison of E test and agar dilution for antimicrobial susceptibility testing of *Stenotrophomonas (Xanthomonas) maltophilia*. J. Clin. Microbiol. **33:**1428–1430.