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Identification of a Carbenicillin-Hydrolyzing β-Lactamase in *Alcaligenes denitrificans* subsp. *xylosoxydans*

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Eleven strains of *Alcaligenes denitrificans* subsp. *xylosoxydans* produced a β-lactamase with a pI of 5.7 with kinetic data characteristic of a PSE-1-type enzyme. A CARB-type enzyme was identified by using an intragenic DNA probe of bla_{CARB}. Hybridization of genomic DNA after *Xba*I restriction and pulsed-field electrophoresis suggested a chromosomal location for the gene.

Alcaligenes denitrificans subsp. *xylosoxydans*, formerly *Achromobacter xylosoxidans*, is an aerobic, nonfermentative, oxidase-positive, gram-negative bacillus found in aqueous environmental sources and isolated from a wide range of clinical samples, e.g., ear discharge, blood, urine, peritoneal fluid, and spinal fluid. This organism is today recognized as an opportunistic pathogen responsible for serious infections (4, 23). In addition, a number of hospital outbreaks due to solutions or medical equipment contaminated with this organism have been described previously (5, 18, 19). *A. denitrificans* subsp. *xylosoxydans* has been shown to display significant resistance to most antimicrobial agents including β-lactams, aminoglycosides, and quinolones (8, 16, 20). Resistance to broad-spectrum penicillins was initially related to the production of cephalosporinases (12) and penicillinases (7, 17) and in one case to the overproduction of a broad-spectrum clavulanate-sensitive β-lactamase (6). This report describes the characteristics of a β-lactamase produced by 11 ticarcillin-resistant clinical isolates of *A. denitrificans* subsp. *xylosoxydans.*

Among 46 clinical strains of *A. denitrificans* subsp. *xylosoxydans* isolated in our hospital since 1986, 11 strains were found to be resistant to broad-spectrum penicillins (e.g., ticarcillin and piperacillin) by the standard agar diffusion method (1). The MICs of the β-lactams were determined in the presence or absence of clavulanic acid (SmithKline-Beecham, Paris, France) by a dilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France), with a multi-inoculator device (Dynatech AM 80). Plates inoculated with 10^5 to 10^6 CFU/ml were incubated at 37°C for 18 h. The MICs of the 10 β-lactam antibiotics are reported in Table 1. All clinical isolates and two reference strains were resistant to cefuroxime, cefoxitin, and cefotaxime (MICs ranging from 32 to ≥128 μg/ml) but susceptible to moxalactam and imipenem (MICs ranging from 0.5 to 4 μg/ml); MICs of ceftazidime ranged from 4 to 16 μg/ml. The distribution of MICs of broad-spectrum penicillins (ticarcillin, piperacillin) and of cefoperazone demonstrated that the strains could be divided into two groups. The 11 ticarcillin-resistant strains exhibited high resistance to ticarcillin and piperacillin (MICs ranging from 64 to ≥256 μg/ml). Susceptibility to ticarcillin, piperacillin, and cefoperazone was partially restored in the presence of clavulanic acid (2 μg/ml) while susceptibility to ceftazidime remained unchanged.

β-Lactamase activities from cell extracts were detected with the iodine-iodide starch system, including benzylpenicillin (1 mM) as substrate, and expressed in terms of the diameter (millimeters) of the discoloration zone after 6 and 18 h of incubation at room temperature (16). Clavulanic acid and cloxacillin (1 mM) were used to specifically inhibit penicillinase and cephalosporinase activities, respectively; inhibition of enzymatic activity was expressed as the decrease in discoloration zone size (millimeters) compared with a control without inhibitor. β-Lactamase activities of the ticarcillin-susceptible strains (35 clinical isolates and 2 reference strains) were inhibited by cloxacillin but not by clavulanic acid: after 18 h of incubation, the reduction (millimeters) in discoloration zone size ranged from 14.5 ± 2.4 (control without inhibitor) to 14.8 ± 2.1 and ≤9 in the presence of clavulanic acid and cloxacillin, respectively. A previous report suggested a putative chromosomal β-lactamase in *A. denitrificans* subsp. *xylosoxydans* (16). By contrast, β-lactamase activities of the 11 ticarcillin-resistant strains were inhibited by clavulanic acid and very poorly inhibited by cloxacillin: the reduction in the discoloration zone size ranged from 24 ± 1 (control) to 16 ± 1.8 and 22.3 ± 1 in the presence of clavulanic acid and cloxacillin, respectively. These results were consistent with the production of a penicillinase.

Isoelectric focusing was performed on polyacrylamide gels (pI range of 3.5 to 9.5) as previously described (6). The pI values were estimated by comparison with the pIs of reference β-lactamases: TEM-1 (pIP1100, pI 5.4), PSE-1 (RPL11, pI 5.7), and SHV-1 (p453, pI 7.6). A major band with a pI of 5.7 was detected in the 11 ticarcillin-resistant strains (Fig. 1); in addition, minor bands with a pI greater than 8.0, probably related to the chromosomal β-lactamase, were detected later.

β-Lactam hydrolytic activity was evaluated by a microacidimetric method (6). Values of V_{max} for various substrates were expressed relative to the V_{max} for benzylpenicillin, defined arbitrarily as 100. Inhibition of β-lactamase activities by cloxacillin (1 mM), clavulanic acid (10 μM), imipenem (1 mM), and chloride ions (100 mM) was expressed as the percentage of inhibition of the rate of benzylpenicillin hydrolysis; clavulanic acid was preincubated with the sample for 10 min at 30°C before the rate of benzylpenicillin hydrolysis was tested. The enzymatic properties of the β-lactamase with a pI of 5.7, analyzed for two ticarcillin-resistant isolates (Adx 3 and Adx 21),

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TABLE 1. MICs (micrograms per milliliter) of β-lactam antibiotics for 46 clinical isolates and 2 reference strains (ATCC 27061 and ATCC 27063) of *A. denitrificans* subsp. *xylosoxydans* in the presence or absence of clavulanic acid[a]

β-Lactam	Ticarcillin-resistant isolates (n = 11)			Ticarcillin-susceptible isolates and reference strains (n = 37)		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Amoxicillin	≥256	≥256	≥256	4–32	8	32
Amoxicillin + CA	32–64	32	64	2–16	4	16
Ticarcillin	≥256	≥256	≥256	0.25–4	1	2
Ticarcillin + CA	32–64	32	32	0.25–2	1	2
Piperacillin	64–128	128	128	0.125–1	0.25	0.5
Piperacillin + CA	1–4	1	2	0.125–0.5	0.25	0.5
Cefuroxime	≥128	≥128	≥128	≥128	≥128	≥128
Cefoxitin	≥128	≥128	≥128	64–≥128	≥128	≥128
Cefoperazone	32–128	64	64	0.5–2	2	2
Cefoperazone + CA	2–4	4	4	0.5–2	2	2
Cefotaxime	128	128	128	32–128	64	64
Ceftazidime	4–16	8	16	4–16	8	8
Ceftazidime + CA	4–16	8	16	4–8	8	8
Moxalactam	0.5–2	1	2	0.5–4	2	2
Imipenem	0.5–2	1	2	0.5–2	1	1

[a] MIC_{50}, MIC for 50% of isolates tested; MIC_{90}, MIC for 90% of isolates tested; CA, clavulanic acid (2 μg/ml).

are reported in Table 2, in which they are compared with the corresponding properties of PSE-1 (or CARB-2) β-lactamase determined by two different methods (11, 13). They exhibited significant hydrolysis against carbenicillin, ticarcillin, and other penicillins such as amoxicillin and mezlocillin, unlike cephaloridine, and were strongly inhibited by clavulanic acid (10 μM) but not by chloride ions (100 mM) or imipenem (1 mM). These results are consistent with a PSE-1-type β-lactamase as originally suggested (17). By contrast, this enzyme clearly differs from other clavulanate-sensitive β-lactamases reported for *A. denitrificans* subsp. *xylosoxydans*, notably the penicillinase with a pI of 7.4, which exhibits a significant rate of hydrolysis of oxacillin (695%) and piperacillin (218%) and is strongly inhibited by chloride ions, imipenem, and clavulanic acid (17).

Colony hybridization was carried out with a specific DNA probe obtained by amplification of an internal 587-nucleotide fragment of the bla_{CARB} gene of RPL11 from *Pseudomonas aeruginosa* (2). The following reference β-lactamase-producing

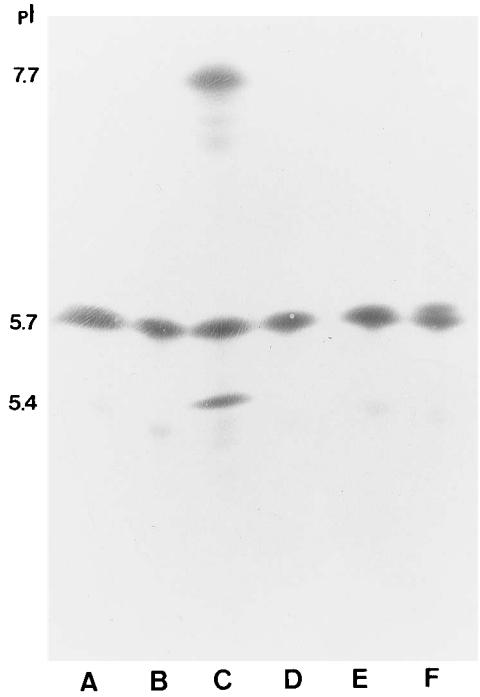

FIG. 1. Comparative analytical isoelectric focusing patterns of five penicillin-resistant strains of *A. denitrificans* subsp. *xylosoxydans* and reference β-lactamases. Lanes: A, Adx 1; B, Adx 3; C, TEM-1 (R111; pI, 5.4), PSE-1 (RPL11; pI, 5.7), and SHV-1 (p453, pI, 7.7); D, Adx 11; E, Adx 21; and F, Adx 24.

TABLE 2. Comparative enzymatic properties of PSE-1 β-lactamase from *P. aeruginosa* and two β-lactamases produced by two ticarcillin-resistant strains of *A. denitrificans* subsp. *xylosoxydans*

Property	β-Lactamase[a]			
	Adx 3	Adx 21	PSE-1[b]	CARB-2[c]
pI	5.7	5.7	5.7	5.7
Substrate profile[d]				
Amoxicillin	110	98	90[e]	93[e]
Carbenicillin	320	310	97	340
Ticarcillin	100	95	ND[f]	130
Mezlocillin	78	74	ND	83
Cloxacillin	<1	<1	<2	0.5
Cephaloridine	29	27	18	16
Cephalothin	2.7	2	<2	0.8
Cefuroxime	<1	<1	<2	ND
Cefotaxime	<1	<1	ND	ND
Ceftazidime	<1	<1	ND	ND
Moxalactam	<1	<1	ND	ND
Imipenem	<1	<1	ND	ND
Inhibition profile[g]				
Clavulanic acid (10 μM)	89	91	S[h]	ND
Cloxacillin (1 mM)	43	45	ND	ND
Chloride ions (100 mM)	0	0	ND	ND
Imipenem (1 mM)	0	0	ND	ND

[a] Sonicated extracts.
[b] Iodometric procedure from reference 13.
[c] Microacidimetric method from reference 11.
[d] Expressed as V_{max} relative to that of benzylpenicillin set at 100.
[e] Ampicillin.
[f] ND, not determined.
[g] Percentage of inhibition relative to that of benzylpenicillin set at 100.
[h] S, sensitive (spectrophotometric method).

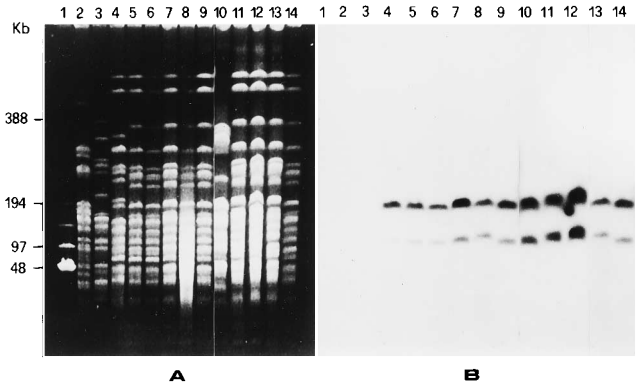

FIG. 2. (A) Pulsed-field gel electrophoresis of *A. denitrificans* subsp. *xylosoxydans* strains after *Xba*I restriction. Lanes: 1, Lambda DNA concatemers (molecular size markers); 2, reference strain ATCC 27063; 3, Adx 25 (ticarcillin-susceptible strain); 4 to 14, ticarcillin-resistant strains (Adx 1, Adx 2, Adx 3, Adx 11, Adx 12, Adx 17, Adx 18, Adx 19, Adx 21, Adx 22, Adx 24). (B) The corresponding autoradiograph after transfer onto a nylon membrane and hybridization with the DNA probe of bla_{CARB}.

strains were used as positive controls: *P. aeruginosa* Dalgleish-(pMG19, PSE-4), PAO303(pUD12, CARB-4), PAO303(pUD14, CARB-2), PAO303(pUD15, CARB-2), and PAO38(RPL11, PSE-1). *P. aeruginosa* PAO38(R151, PSE-2) and Ming (pMG39, OXA-6) and *Escherichia coli* K-12(RP4, TEM-2), K-12(p453, SHV-1), K-12(R46, OXA-2), K-12(pMG202, OXA-7), K-12(RGN238, OXA-1), K-12(R55, OXA-3), and K-12 (pIP1100, TEM-1) were used as negative controls. All strains producing the β-lactamase with a pI of 5.7 (Adx 1, Adx 2, Adx 3, Adx 11, Adx 12, Adx 17, Adx 18, Adx 19, Adx 21, Adx 22, and Adx 24) hybridized with the intragenic DNA probe of bla_{CARB}, whereas none of the 22 ticarcillin-susceptible strains tested hybridized with the probe.

Carbenicillin-hydrolyzing enzymes were initially and mostly reported for clinical isolates of *P. aeruginosa* and rarely for members of the family *Enterobacteriaceae* (14). Conjugation experiments were performed by mixing equal volumes (1 ml) of exponentially growing cultures (10^9 cells per ml) of the ticarcillin-resistant isolates and *E. coli* K-12 J53-2 F^- *met pro rpoB* in Luria broth containing dextrose or *P. aeruginosa* PAO38 *leu* in nutrient broth containing KNO_3 at 37°C for 24 h (6). Transconjugants were selected on minimal agar plates containing methionine (30 mg/liter), proline (50 mg/liter), leucine (100 mg/liter), rifampin (100 mg/liter), or ticarcillin (512 mg/liter). Ticarcillin resistance of *A. denitrificans* subsp. *xylosoxydans* was not transferred to *E. coli* K-12 J53-2 or *P. aeruginosa* PAO38. The PSE-1 β-lactamase, once thought to be restricted to *P. aeruginosa*, has been found in enterobacteria. PSE-1 β-lactamase production by plasmids of enteric origin is determined by 12- to 14-kb transposons (10, 15). In addition, failure to demonstrate plasmids, or their transfer, in *P. aeruginosa* isolates producing PSE-1 is common (9) and might indicate that the PSE-1 gene has become integrated in the chromosome. Among the closely related variants of the PSE-type enzymes, the PSE-4 determining transposon was reported to be integrated at a specific site in the *Pseudomonas* chromosome (21).

In order to determine the chromosomal location of the bla_{CARB} gene, hybridization was carried out after the transfer of *Xba*I restriction fragments of genomic DNA separated by pulsed-field electrophoresis (22). Hybridization revealed two fragments of ca. 150 and 200 kb (Fig. 2) which were consistent with the single *Xba*I restriction site in the bla_{CARB} sequence (3). Of the 11 strains tested, 9 corresponded to a probable epidemic strain on the basis of the same pulsed-field electrophoresis profile; however, the presence of two identical bands for the two strains showing different profiles (lanes 4 and 10) suggests that the gene has the same location of insertion.

A. denitrificans subsp. *xylosoxydans* is generally found to be resistant to most antimicrobial agents, including β-lactams. The patterns of β-lactam susceptibilities of 46 clinical strains isolated in our hospital from 1986 to 1990 confirm previous reports. Two susceptibility patterns could be characterized, of which the ticarcillin-sensitive one was predominant (76%). Eleven ticarcillin-resistant strains produced a β-lactamase with a pI of 5.7. The enzyme exhibited enzymatic properties typical of a PSE-1-type β-lactamase. The carbenicillin-hydrolyzing β-lactamase produced by these strains was confirmed by colony hybridization with a specific DNA probe and, finally, hybridization of genomic DNA after *Xba*I restriction suggested a chromosomal location for the bla_{CARB} gene in this bacterial species.

REFERENCES

1. **Acar, J. F., and F. W. Goldstein.** 1985. The disc susceptibility test, p. 27–63. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
2. **Arlet, G., and A. Philippon.** 1991. Construction by polymerase chain reaction and use of intragenic DNA probes for three main types of transferable β-lactamases (TEM, SHV, CARB). FEMS Microbiol. Lett. **82:**19–26.
3. **Boissinot, M., and R. C. Levesque.** 1990. Nucleotide sequence of the PSE-4 carbenicillinase gene and correlations with the *Staphylococcus aureus* PC1 beta-lactamase crystal structure. J. Biol. Chem. **265:**1225–1230.
4. **Chandrasekar, P. H., E. Arathoon, and D. P. Levine.** 1986. Infections due to *Achromobacter xylosoxidans*. Case report and review of literature. Infection **14:**279–282.
5. **Cheron, M., E. Abachin, E. Guerot, M. El-Bez, and M. Simonet.** 1994. Investigation of hospital-acquired infections due to *Alcaligenes denitrificans* subsp. *xylosoxydans* by DNA restriction fragment length polymorphism. J. Clin. Microbiol. **32:**1023–1026.
6. **Decré, D., G. Arlet, C. Danglot, J. C. Lucet, G. Fournier, E. Bergogne-Bérézin, and A. Philippon.** 1992. A β-lactamase-overproducing strain of *Alcaligenes denitrificans* subsp. *xylosoxydans* isolated from a case of meningitis. J. Antimicrob. Chemother. **30:**769–779.
7. **Fujii, T., K. Sato, M. Inoue, and S. Mitsuhashi.** 1985. Purification and properties of a β-lactamase from *Alcaligenes denitrificans* subsp. *xylosoxydans*. J. Antimicrob. Chemother. **16:**297–304.
8. **Glupczynski, Y., W. Hansen, J. Freney, and E. Yourassowsky.** 1988. *In vitro* susceptibility of *Alcaligenes denitrificans* subsp. *xylosoxidans*. J. Antimicrob. Chemother. **32:**276–278.
9. **Jacoby, G. A.** 1986. Resistance plasmids of *Pseudomonas*, p. 265–293. *In* J. R. Sokatch (ed.), The bacteria, vol. X. The biology of *Pseudomonas*. Academic Press, Orlando, Fla.
10. **Katsu, K., M. Inoue, and S. Mitsuhashi.** 1982. Transposition of the carbenicillin-hydrolyzing beta-lactamase gene. J. Bacteriol. **150:**482–489.
11. **Labia, R., M. Guionie, M. Barthélémy, and A. Philippon.** 1981. Properties of three carbenicillin-hydrolysing β-lactamases (CARB) from *Pseudomonas aeruginosa*: identification of a new enzyme. J. Antimicrob. Chemother. **7:**49–56.
12. **Levesque, R., P. H. Roy, R. Letarte, and J. C. Péchère.** 1982. A plasmid-mediated cephalosporinase from *Achromobacter* species. J. Infect. Dis. **145:** 753–761.
13. **Matthew, M., and R. B. Sykes.** 1977. Properties of the beta-lactamase specified by the *Pseudomonas* plasmid RPL11. J. Bacteriol. **132:**341–345.
14. **Medeiros, A. A.** β-Lactamases. 1984. Br. Med. Bull. **40:**18–27.
15. **Medeiros, A. A., R. W. Hedges, and G. A. Jacoby.** 1982. Spread of a "*Pseudomonas*-specific" β-lactamase to plasmids of enterobacteria. J. Bacteriol. **149:** 700–707.
16. **Mensah, K., A. Philippon, C. Richard, and P. Névot.** 1990. Susceptibility of *Alcaligenes denitrificans* subsp. *xylosoxydans* to β-lactam antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **9:**405–409.
17. **Philippon, A., K. Mensah, G. Fournier, and J. Freney.** 1990. Two resistance phenotypes to β-lactams of *Alcaligenes denitrificans* subsp. *xylosoxydans* in relation to β-lactamase types. J. Antimicrob. Chemother. **25:**698–700.
18. **Reina, J., M. Antich, B. Siquier, and P. Alomar.** 1988. Nosocomial outbreak of *Achromobacter xylosoxidans* associated with a diagnosis contrast solution. J. Clin. Pathol. **41:**920–921.
19. **Reverdy, M. E., J. Freney, J. Fleurette, M. Coulet, M. Surgot, D. Marmet,**

and C. Ploton. 1984. Nosocomial colonization and infection by *Achromobacter xylosoxidans*. J. Clin. Microbiol. **19:**140–143.
20. **Rolston, K. V. I., and M. Messer.** 1990. The *in vitro* susceptibility of *Alcaligenes denitrificans* subsp. *xylosoxidans* to 40 antimicrobial agents. J. Antimicrob. Chemother. **26:**857–860.
21. **Sinclair, M. I., and B. W. Holloway.** 1982. A chromosomally located transposon in *Pseudomonas aeruginosa*. J. Bacteriol. **151:**569–579.
22. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. **155:**449–467.
23. **Spear, J. B., J. Fuhrer, and B. D. Kirby.** 1988. *Achromobacter xylosoxidans* (*Alcaligenes xylosoxidans* subsp. *xylosoxidans*) bacteremia associated with a well-water source: case report and review of literature. J. Clin. Microbiol. **26:**598–599.