ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2001, p. 2386–2389 Vol. 45, No. 8
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.8.2386–2389.2001

Cloning and Biochemical Characterization of a Class A β-Lactamase from *Prevotella intermedia*

I. MADINIER,[1,2] T. FOSSE,[2]* J. GIUDICELLI,[3] AND R. LABIA[4]

Laboratoire de Pathobiologie Orale, Faculté de Chirurgie Dentaire, 06357 Nice Cedex 4,[1] Laboratoire de Bactériologie, Hôpital l'Archet 2, 06202 Nice Cedex 3,[2] Laboratoire de Biochimie, INSERM 145, Faculté de Médecine, 06107 Nice Cedex 2,[3] and CNRS, UMR 175, 29000 Quimper,[4] France

Received 1 August 2000/Returned for modification 10 November 2000/Accepted 11 May 2001

The gene encoding a β-lactamase of *Prevotella intermedia* was cloned and sequenced. This gene, called *cfxA2*, shared 98% identity with *cfxA*, the structural gene of a β-lactamase previously described in *Bacteroides vulgatus*. The deduced protein sequence had a K272E substitution. CfxA2 had the characteristics of class A, group 2e β-lactamases.

Prevotella intermedia, a black-pigmented gram-negative anaerobic rod that is a member of the family *Bacteroidaceae*, is associated with periodontal diseases and infections of dental origin (19). Several studies have reported increasing resistance to antibiotics in gram-negative anaerobes, especially to β-lactam antibiotics, mostly by the production of β-lactamases (3, 4, 8, 13, 14). Only a few genes encoding β-lactamases have been cloned and sequenced in members of the family *Bacteroidaceae (Bacteroides fragilis, Bacteroides vulgatus*, and *Bacteroides uniformis)* but not in *Prevotella* and *Porphyromonas* species (12, 16, 18, 20). A preliminary work concluded that *P. intermedia* and *Prevotella buccae* were the predominant β-lactamase-producing species among anaerobic gram-negative rods isolated from periodontal pockets, with 35 and 42% of these, respectively, β-lactamase-positive strains (9). Biochemical characterization of these β-lactamases is difficult because of fastidious bacterial growth and weak enzymatic activity. The purpose of this work was to clone the β-lactamase gene of a strain of *P. intermedia* for biochemical and genetic analysis.

P. intermedia NI-1187 was a clinical isolate obtained from the subgingival flora of a male adult patient suffering from periodontitis. The wild-type strain showed resistance to penicillin, amoxycillin, tetracycline, and erythromycin and susceptibility to the amoxycillin-clavulanic acid combination. Isoelectric focusing experiments performed with sonified crude extracts of *P. intermedia* did not allow us to visualize the β-lactamase. All of the media and compounds used have been previously described (9). Sequences were determined from both strands of DNA with an Applied Biosystems sequencer (Eurogentec, Herstal, Belgium). Deduced protein sequences and sequence alignments were performed with the National Center for Biotechnology Information, Infobiogen, and ExPaSy suite of programs, and β-lactamase relatedness was investigated by comparison with the GenBank-EMBL-DDBJ databases.

For cloning experiments, chromosomal DNA from *P. intermedia* NI-1187 was obtained with conventional phenol-chloroform extraction methods, restricted with *Eco*RI, ligated in pZErO-2-Kan, and transferred by electroporation in *Escherichia coli* Top 10 (InvitroGen, Leek, The Netherlands) (12, 16, 20). A bank of approximately 10^6 recombinant clones was obtained on kanamycin selective plates and yielded about 10 colonies of β-lactamase-producing *E. coli* transformants on ampicillin selective plates (40 μg/ml). Plasmids from *E. coli* NI-14 transformants presented a 15-kb DNA *Eco*RI insert. After subcloning was done, one clone of *E. coli*, NI-141, was chosen for genetic analysis. It harbored the pNCE-3 plasmid with an *Eco*RI/*Pst*I 4.9-kb insert. The MIC determination was suggestive of a class A, group 2e β-lactamase (Table 1) (2, 7). This phenotype, with penicillinase and cephalosporinase properties and characteristic low resistance to cefotaxime and cefpirome, is a common feature of *P. intermedia* strains producing β-lactamases (4, 9, 23).

The 4.9-kb insert was sequenced and assigned GenBank accession no. AF118110. The β-lactamase gene of *P. intermedia* NI-1187 shared 98% identity with *cfxA*, the structural gene of a class A β-lactamase previously characterized in a cefoxitin-resistant *B. vulgatus* CLA-341 strain, and was provisionally called *cfxA2* (16). The deduced protein sequence called CfxA2 contained 321 amino acids with a K272E substitution (Fig. 1). The flanking sequences revealed transposition genes. The left flanking region contained *mobA* and the partial sequence of a gene related to *TnpA* (98 and 40% homology, respectively) previously described on the *Bacteroides* mobilizable transposon Tn*4555* (11, 22). The right flanking region shared 27% homology with *mobC* and *bfmC*, associated with the pathogenicity island of enterotoxigenic *B. fragilis* strains (10).

For purification purposes, two sets of primers were designed in order to produce the CfxA2 protein with a C- (Set 1, 5′-AAAAAACCATGGAAAAAAACAGAAAAAAACAAATCG-3′ and 5′-AAAAAACTCGAGAGATTTTACTGAAGTTTGCATTAATAAAGAATATAC-3′) and an N-terminal (Set 2, 5′-GGGATCCGAAAAAAACAGAAAAAAACAAATC-3′ and 5′-CGAATCCTTAAGATTTTACTGAAGTTAG-3′) histidine tag. After PCR amplification of *cfxA2*, including the promotor region, *cfxA2* was inserted into pET28 and cloned into *E. coli* BL21(DE3) (TA cloning kit; Novagen, Madison,

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Hôpital l'Archet 2, 151, route de Saint Antoine Ginestière, 06202 Nice Cedex 3, France. Phone: (33) (4) 92 03 62 14. Fax: (33) (4) 90 03 65 49. E-mail: fosse@unice.fr.

TABLE 1. MICs of 10 β-lactam antibiotics alone in *E. coli* Top 10 host strains and alone and in combination with β-lactamase inhibitors (clavulanic acid and tazobactam) in *E. coli* NI-141 cloned with the *cfxA2* β-lactamase gene of an oral strain of *P. intermedia* (DNA insert, 4.9-kb)

Substrate	MIC (μg/ml)			
	E. coli Top 10 (antibiotic alone)	*E. coli* NI-141 coding for CfxA2 β-lactamase		
		Antibiotic alone	Combination with clavulanic acid[a]	Combination with tazobactam[b]
Amoxycillin	4[c]	1,024	16	16
Ticarcillin	4	256	8	4
Cephalothin	4	32	8	8
Cefuroxime	8	64	8	8
Cefoxitin	4	4	4	4
Cefotaxime	0.06	1	0.125	0.125
Ceftazidime	0.5	2	ND[d]	ND
Aztreonam	0.125	0.125	0.125	0.125
Cefpirome	0.03	1	0.125	0.125
Imipenem	0.06	0.125	ND	ND

[a] Clavulanic acid at a fixed concentration of 2 μg/ml.
[b] Tazobactam at a fixed concentration of 4 μg/ml.
[c] Amoxycillin and clavulanic acid, MIC <2 μg/ml.
[d] ND, not done.

Wis.) (17, 21). The transformed bacteria were grown in 1 liter of Luria-Bertani broth (kanamycin [50 μg/ml]) for 5 h at 37°C, followed by IPTG (isopropyl-β-D-thiogalactopyranoside) (0.3 mM) induction for 4 h at 30°C, centrifugation, and disruption by two passages through a French pressure cell. The tagged proteins were then purified by affinity chromatography through nickel-coated Sepharose beads with an imidazole elution buffer (20 to 500 mM, pH 7.4) according to the manufacturer's recommendations (HiTrap chelating column and GradiFrac; Pharmacia Biotec, Uppsala, Sweden). β-Lactamase activity was monitored with the chromogenic nitrocefin (482 nm) (Uvikon 820 spectrophotometer; Kontron Instruments, Zürich, Switzerland). The C-terminally tagged CfxA2 protein from clone NI-124 (33 to 35 kDa) was obtained in a pure but inactive form and was resistant to thrombin hydrolysis (21). The N-terminally tagged protein (clone NI-142) was highly purified in an active form. After an affinity purification step, the β-lactamase was purified about 50-fold compared to crude homogenate supernatant. Active fractions were pooled, extensively dialyzed, thrombin treated, and stored at −70°C until use. Protein concentrations were determined by the method of Bradford, with bovine serum albumin used as a standard (protein assay; Bio-Rad Laboratories GmbH, Munich, Germany). Repeated isoelectric focusing experiments with crude extracts were necessary to visualize a discrete reactive band with a pI of 5.4. The pI reported for *Bacteroides* species was 5.8, but CfxA2 differed from CfxA by a glutamic acid (acid) replacing a lysine (base) in position 272 (12, 16). The hydrolysis of β-lactams was monitored at 37°C in sodium phosphate buffer (0.05 M; pH 7.0) with 20 μg of β-lactamase in a 500-μl reaction mixture. Kinetic parameters were estimated for at least three different assays, and substrate inhibition was confirmed with antibiotic concentrations above 50 μM (23). The apparent K_m and relative V_{max} values were calculated from Eadie-Hofstee plots (Table 2), with V_{max} values relative to that of benzylpenicillin, which was set as 100 as previously described (1, 17). The kinetic parameters of CfxA2 are characterized by K_m values ranging from 12 to 38 μM for all of the β-lactams tested, except for cefoxitin, which was not hydrolyzed. The inhibitory kinetic parameters (K_i) of CfxA2 with cefazolin as a substrate were as follows: cefoxitin, 10 nM, and clavulanic acid, 200 nM. Inhibitors were preincubated with the enzyme for 10 min at 37°C before the rate of cefazolin inhibition was tested.

In order to determine whether the K272E substitution affects the kinetic parameters towards representative β-lactam substrates, and particularly resistance to cefoxitin (6), we compared the kinetic properties of the original CfxA (*B. vulgatus* CLA-341 [kindly provided by J. C. Smith]) and CfxA2 with substitutions (*B. vulgatus* NI-2869 [a clinical laboratory strain]) produced in wild-type *B. vulgatus* strains. For comparison, (i) wild-type β-lactamase genes were sequenced for identification purposes (PCR amplification); (ii) susceptibility profiles and MICs were determined, and (iii) kinetic parameters were calculated from partially purified β-lactamase crude extracts as previously described (23). *B. vulgatus* CLA-341 was resistant to benzylpenicillin, amoxycillin, cefoxitin, and moxalactam and susceptible to the amoxycillin-clavulanic acid combination, piperacillin, and imipenem. No synergy was observed between cefoxitin and amoxycillin-clavulanic acid. In comparison, *B. vulgatus* NI-2869 was susceptible to moxalactam and showed decreased susceptibility to cefoxitin. Amoxycillin, amoxycillin-clavulanic acid, cefoxitin, and cefuroxime MICs were as follows: 256, 0.125, 256, and 256 μg/ml (CLA-341) and 4, 0.94, 12, and 1 μg/ml (NI-2869), respectively. Comparison of CfxA and CfxA2 kinetic parameters showed that the K272E substitution has no significant influence on their catalytic properties towards benzylpenicillin, ampicillin, cefotaxime, cephalothin (hydrolyzed), and cefoxitin (not hydrolyzed) but increases CfxA2 affinity for cefazolin about 10-fold (Table 3). The high level of resistance of *B. vulgatus* CLA-341 towards cefoxitin should be attributed to another resistance mechanism than CfxA production, such as a porin mutation (16).

In order to determine the location of enzymatic activity, cultures of *E. coli* NI-142 were fractionated according to the osmotic-shock method (16). Cell membranes were finally treated with 2% polyoxyethylene 10–tridecyl ether, a detergent which does not inhibit enzyme activity (Emulphogene-BC-720; Sigma, St. Louis, Mo.). Cefazolin (50 μM) was used as a substrate, and no enzymatic activity could be detected in extracellular, periplasmic, or cytoplasmic fractions, while 52% of enzymatic activity was recovered in the pellet after the French press treatment. Eighty-three percent of this membrane activ-

TABLE 2. Steady-state kinetic parameters of highly purified CfxA2 β-lactamase cloned in *E. coli* NI-142

Substrate	Relative V_{max}[a]	K_m (μM)	Relative V_{max}/K_m
Benzylpenicillin	100	20.7	4.8
Ampicillin	160	38.0	4.2
Cefazolin	300	12.3	24.4
Cefuroxime	1,500	60.6	24.8
Cefotaxime	600	12.9	46.5
Cephalothin	40	24.7	1.6
Cefoxitin	<0.01		

[a] Values relative to that of benzylpenicillin, which was set at 100.

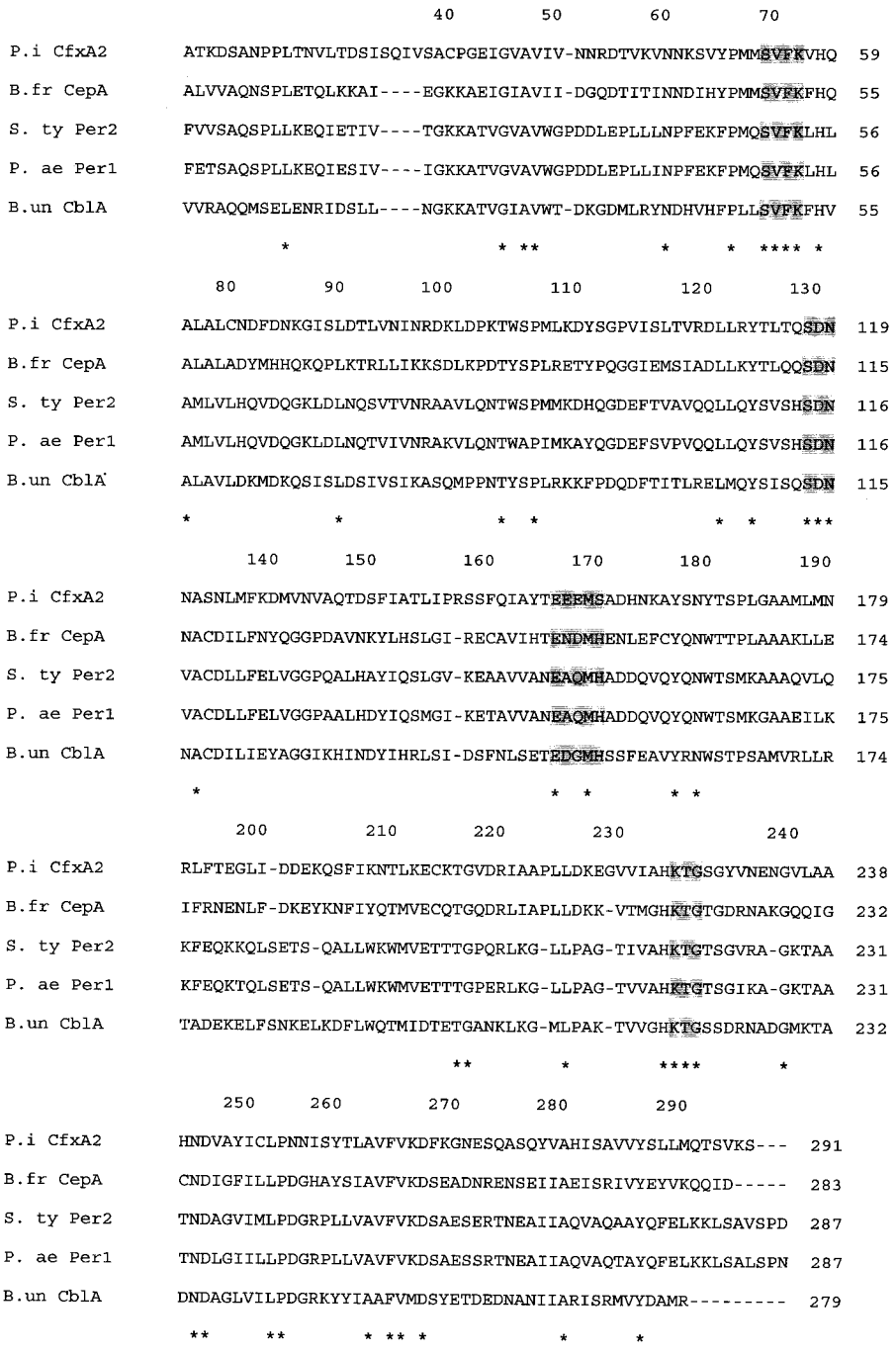

FIG. 1. Multiple sequence alignment of the amino acid sequences of CfxA2 and related β-lactamases. Amino acids that are identical in all five sequences are marked with asterisks. The amino acid numbering for the class A β-lactamases is used (2). The main conserved amino acid motifs of class A β-lactamases are shaded. Abbreviations: P.i, *P. intermedia* NI-1187; B.fr, *B. fragilis* (17); S. ty, *S. enterica* serovar Typhimurium (4); P. ae, *P. aeruginosa* (14); B.un, *B. uniformis* (19). CfxA2 differs from CfxA of *B. vulgatus* by a K272E substitution (16).

ity could be solubilized after detergent treatment. These results in *E. coli* suggest a cytoplasmic membrane localization of CfxA β-lactamases. However, interference of the N-terminal histidine tag cannot be excluded (16).

Phylogenetic analysis revealed homologies with other β-lactamases of anaerobes (CfxA of *B. vulgatus*, >99%; CepA of *B. fragilis*, 35%; CblA of *B. uniformis*, 28%) and with extended-spectrum β-lactamases of aerobic rods (Per-1 of *Pseudomonas aeruginosa*, 27%; Per-2 of *Salmonella enterica* serovar Typhimurium, 28%) (Fig. 1) (5, 6, 15, 16, 18, 20). From genetic data, Nordmann and Naas proposed a novel subgroup of class A β-lactamases for CfxA and Per-1 (15). Per-2 and CfxA2 would be new members of this subgroup, but CfxA (*B. vulgatus* and *P. intermedia*) is not an extended-spectrum β-lactamase. Additional work is necessary to characterize the mobilization genes associated with *cfxA2* and its spread among *Prevotella* species.

TABLE 3. Comparison of kinetic parameters of partially purified CfxA and CfxA2 β-lactamases obtained from wild-type strains (CfxA, *B. vulgatus* CLA-341; CfxA2, *B. vulgatus* NI-2869)

Substrate	Relative V_{max}[a]		K_m (μM)	
	CfxA	CfxA2	CfxA	CfxA2
Benzylpenicillin	100	100	30.0	20.0
Ampicillin	35	130	40.0	40.0
Cefazolin	740	170	133.3	10.2
Cefotaxime	90	670	40.0	8.0
Cefoxitin	<0.01	<0.01		

[a] Values relative to that of benzylpenicillin, which was set at 100.

We thank C. J. Smith, Department of Microbiology and Immunology, East Carolina University, who sent us *Bacteroides vulgatus* CLA-341, and A. Galmiche, INSERM 452, Faculté de Médecine de Nice, Nice, France, for helpful assistance in protein purification.

REFERENCES

1. **Ahamed, J., and M. Kundu.** 1999. Molecular characterization of the SHV-11 β-lactamase of *Shigella dysenteriae*. Antimicrob. Agents Chemother. **43:** 2081–2083.
2. **Ambler, R. P., A. F. W. Coulson, J. Frere, J. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
3. **Andres, M. T., W. O. Chung, M. C. Roberts, and J. F. Fierro.** 1998. Antimicrobial susceptibilities of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* spp. isolated in Spain. Antimicrob. Agents Chemother. **42:**3022–3023.
4. **Appelbaum, P. C., S. K. Spangler, and M. R. Jacobs.** 1990. β-Lactamase production and susceptibilities to amoxycillin, amoxycillin-clavulanate, ticarcillin, ticarcillin-clavulanate, cefoxitin, imipenem, and metronidazole of 320 non-*Bacteroides fragilis Bacteroides* isolates and 129 fusobacteria from 28 U.S. centers. Antimicrob. Agents Chemother. **34:**1546–1550.
5. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, P. Pangold, S. Amann, E. Akalin, O. Ang, C. Bal, and J. M. Casellas.** 1996. Characterization of β-lactamase gene *bla*$_{PER-2}$, which encodes an extended-spectrum class A β-lactamase. Antimicrob. Agents Chemother. **40:**616–620.
6. **Bouthors, A. T., N. Dagoneau-Blanchard, T. Naas, P. Nordmann, V. Jarlier, and W. Sougakoff.** 1998. Role of residues 104, 164, 166, 238 and 240 in the substrate profile of PER-1 beta-lactamase hydrolyzing third-generation cephalosporins. Biochem. J. **15:**1443–1449.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
8. **Edwards, R., D. Thirlwell, and D. Greenwood.** 1996. Changes in beta-lactam antibiotic susceptibility and beta-lactamase production of clinical isolates of *Bacteroides* and *Prevotella* species over a 9 year period. J. Antimicrob. Chemother. **37:**636–638.
9. **Fosse, T., I. Madinier, C. Hitzig, and Y. Charbit.** 1999. Prevalence of β-lactamase producing strains among 149 anaerobic Gram negative rods isolated from periodontal pockets. Oral Microbiol. Immunol. **14:**352–357.
10. **Franco, A. A., R. K. Cheng, G. T. Chung, S. Wu, H. B. Oh, and C. L. Sears.** 1999. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. J. Bacteriol. **181:**6623–6633.
11. **Guiney, D. J., and P. Hasegawa.** 1992. Transfer of conjugal elements in oral black-pigmented *Bacteroides (Prevotella)* spp. involves DNA rearrangements. J. Bacteriol. **174:**4853–4855.
12. **Hedberg, M., L. Lindqvist, T. Bergman, and C. E. Nord.** 1995. Purification and characterization of a new β-lactamase from *Bacteroides uniformis*. Antimicrob. Agents Chemother. **39:**1458–1461.
13. **Könonen, E., M. Saarela, A. Kanervo, J. Karjalainen, S. Asikainen, and H. Jousimies-Somer.** 1995. β-Lactamase production and benzylpenicillin susceptibility among different ribotypes of *Prevotella melaninogenica* simultaneously colonizing the oral cavity. Clin. Infect. Dis. **20**(Suppl. 2)**:**S364–S366.
14. **Medeiros, A. A.** 1997. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. **24**(Suppl. 1)**:** S19–S45.
15. **Nordmann, P., and T. Naas.** 1994. Sequence analysis of PER-1 extended-spectrum β-lactamase from *Pseudomonas aeruginosa* and comparison with class A β-lactamases. Antimicrob. Agents Chemother. **38:**104–114.
16. **Parker, A. C., and C. J. Smith.** 1993. Genetic and biochemical analysis of a novel Ambler class A β-lactamase responsible for cefoxitin resistance in *Bacteroides* species. Antimicrob. Agents Chemother. **37:**1028–1036.
17. **Poirel, L., T. Naas, M. Guibert, C. El Bachir, R. Labia, and P. Nordmann.** 1999. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum β-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob. Agents Chemother. **43:**573–581.
18. **Rodgers, M. B., A. C. Parker, and C. J. Smith.** 1993. Cloning and characterization of the endogenous cephalosporinase gene, CEP-A, from *Bacteroides fragilis* reveals a new subgroup of Ambler class A β-lactamases. Antimicrob. Agents Chemother. **37:**2391–2400.
19. **Shah, H. N., and S. E. Gharbia.** 1995. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the *Bacteroides*. Int. J. Syst. Bacteriol. **40:**5426.
20. **Smith, C. J., T. K. Bennett, and A. C. Parker.** 1994. Molecular and genetic analysis of the *Bacteroides uniformis* cephalosporinase gene, CBL-A, encoding the species-specific β-lactamase. Antimicrob. Agents Chemother. **38:** 1711–1715.
21. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
22. **Tribble, G. D., A. C. Parker, and C. J. Smith.** 1999. Transposition genes of the *Bacteroides* mobilizable transposon Tn4555: role of a novel targeting gene. Mol. Microbiol. **34:**385–394.
23. **Valle, G., L. M. Quiros, M. T. Andrés, and J. F. Fierro.** 1998. A β-lactamase belonging to group 2e from oral clinical isolates of *Prevotella intermedia*. FEMS Microbiol. Lett. **158:**191–194.