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Genetic Analysis of an Ambler Class A Extended-Spectrum Beta-Lactamase from *Capnocytophaga ochracea*

Anne Jolivet-Gougeon,[1]* Zohreh Tamanai-Shacoori,[2] Laurent Desbordes,[1] Nathalie Burggraeve,[2] Michel Cormier,[1] and Martine Bonnaure-Mallet[2]

Laboratoire de Microbiologie Pharmaceutique, UPRES-EA 1254, Université de Rennes 1, 35043 Rennes,[1] *and Equipe de Biologie Buccale, UPRES-EA 1256, UFR Odontologie, Université de Rennes 1, 35000 Rennes,*[2] *France*

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A beta-lactamase gene (*cfxA3*, 966 bp) was isolated from a beta-lactam-resistant *Capnocytophaga ochracea* clinical isolate and amplified using primers from the *cfxA* gene of *Bacteroides vulgatus*. The MICs of third-generation cephalosporins were much higher than those of the transconjugant *Escherichia coli* strain. The deduced protein sequence, by comparison with CfxA2 of *Prevotella intermedia*, had a Y239D substitution and possessed the characteristics of a class A, group 2e beta-lactamase.

Capnocytophaga spp. are gram-negative, fusiform, capnophilic bacteria and are part of the normal oral flora in humans. They are found in both immunocompromised and nonimmunocompromised patients (6, 20, 27), are associated with infections of dental origin (11), and can cause various complicated infections (4, 17, 20), including septicemias (7, 14). While clindamycin and amoxicillin-clavulanic acid are the first choices for treating *Capnocytophaga* infections (19) and the susceptibility of *Capnocytophaga* spp. to extended-spectrum cephalosporins has been reported to be variable (2, 10), bacteremias in neutropenic patients are often empirically treated with extended-spectrum cephalosporins before the organism has been identified because *Capnocytophaga* spp. are slow-growing bacteria (19). Beta-lactamase-producing strains are increasingly common (5, 15, 22, 23). It is thus becoming essential to determine which antibiotics are effective against clinical isolates. However, complete characterization of the beta-lactamases has rarely been attempted, except for a plasmid-encoded extended-spectrum TEM-17 beta-lactamase (24). In the beta-lactamase classification scheme of Bush et al. (3), many *Bacteroides* (CfxA from *Bacteroides vulgatus* [21]), *Prevotella* (CfxA2 from *Prevotella intermedia* [15, 31]), and *Capnocytophaga* (5, 23) beta-lactamases belong to group 2e, which includes enzymes with significant activity against cephalosporins rather than penicillins. They have acidic pI values and are susceptible to beta-lactamase inhibitors. The occurrence of beta-lactamases in bacteria in the normal flora is a cause of concern about the spread of resistance to extended-spectrum cephalosporins to more pathogenic bacteria. A clinical strain of *Capnocytophaga ochracea* (E201) was investigated, and a beta-lactamase gene called *cfxA3* was cloned and sequenced.

Jolivet-Gougeon et al. (10) reported that some *Capnocytophaga* strains are highly resistant to broad-spectrum cephalosporins, which makes it important to better understand their resistance mechanisms. Strain E201 was isolated by swabbing the throat of a pediatric cancer patient undergoing chemotherapy at the Department of Pediatric Oncology (Centre Hospitalier Universitaire, Rennes, France) and selected for its ceftazidime resistance. The sample was plated on TBBP agar (Trypticase-blood-bacitracin-polymyxin B) (18), incubated in a 10% CO_2 atmosphere for 48 to 72 h, and identified by conventional methods (12, 13, 28, 32).

The MICs of eight beta-lactam antibiotics alone or in combination with a beta-lactamase inhibitor were determined by the agar dilution method as recommended by Rummens et al. (25) and as already described (10). The plates were incubated for 48 h at 37°C in a 10% CO_2 atmosphere. Strain E201 produced a beta-lactamase that conferred a high level of resistance to a wide range of beta-lactam antibiotics, including amoxicillin, cefalothin, cefuroxime, ceftazidime, and cefotaxime. Strain E201 remained susceptible to imipenem, cefoxitin, aztreonam, and amoxicillin-clavulanic acid. Intrinsic susceptibility to other beta-lactamase inhibitors (tazobactam and sulbactam) was also observed, as already described (10). MIC determinations showed that strain E201 was resistant to extended-spectrum cephalosporins such as cefotaxime and, more particularly, ceftazidime (Table 1). While the *Prevotella* strain was susceptible to cefoxitin, the affinity of CfxA2 for cefazolin was about 10-fold greater. Madinier et al. (15) also reported that the resistance of *B. vulgatus* to cefoxitin might be due to a resistance mechanism other than CfxA production, perhaps a porin mutation.

Primers cfxA1 plus cfxA4, cfxA5 plus cfxA6, and cfxAZ1 plus cfxAZ2 (Genset SA, Paris, France) were selected from the *cfxA* sequence of *B. vulgatus*, (locus BVU38243, GenBank Accession no. U38243) (21) (Table 2) by using primer3.Output software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The following amplification conditions were used: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35 cycles). Two fragments (580 and 507 bp) were amplified by PCR, and a 966-bp consensus sequence of *cfxA3* was determined.

For the cloning experiments, total DNA from the *C. ochracea* E201 *cfxA3* gene was amplified with composite primers cfxAZ1 and cfxAZ2 (Table 2), digested with *Eco*RI and

* Corresponding author. Mailing address: Laboratoire de Microbiologie Pharmaceutique, UPRES-EA 1254, Université de Rennes 1, 2 avenue du Professeur Léon Bernard 35 043 Rennes, France. Phone: (33) 2 23 23 49 05. Fax: (33) 2 23 23 49 13. E-mail: anne.gougeon@univ-rennes1.fr.

TABLE 1. MICs of antimicrobial agents for the *C. ochracea* E201 clinical isolate, transconjugant *E. coli* TrE201, and *E. coli* DH5α

Antimicrobial agents	MIC (μg/ml) for:		
	C. ochracea E201	*E. coli* TrE201	*E. coli* DH5α
Amoxicillin	>256	>256	2
Amoxicillin-clavulanic acid	0.25	8	2
Cephalothin	>256	8	1
Cefuroxime	>256	4	4
Cefoxitin	2	2	2
Cefotaxime	32	0.03	<0.03
Ceftazidime	>256	0.12	0.06
Aztreonam	0.5	0.12	0.12
Imipenem	0.12	0.25	0.12

*Hin*dIII, ligated in pET-22b$^+$ (the *bla* gene was replaced by a Kan^r gene, which was kindly supplied by Dr. Blanco from the Université de Rennes 1, Rennes, France), and inserted by electroporation into *E. coli* DH5α, which harbors the recombinant vector with a 0.970-kb *Eco*RI-*Hin*dIII insert (TrE201 strain).

The sequences of both strands of DNA were determined using a Perkin-Elmer 310 Genetic Analyzer sequencer and assigned GenBank accession no. AF472622 (Banklt 442706). Deduced protein sequences, sequence alignments, and beta-lactamase relatedness were determined using the BLAST similarity search programs of the National Center for Biotechnology Information (NCBI).

Analysis of the nucleotide sequence showed that the gene coding for the *C. ochracea* beta-lactamase differed from the *cfxA* gene of *B. vulgatus* (21) by two amino acid substitutions (K272E and Y239D) and from the *cfxA2* gene of *P. intermedia* (15) by one amino acid substitution (Y239D), according to the numbering scheme of Ambler et al. (1) (Table 3). The *cfxA3* gene had the four conserved elements of class A beta-lactamases as per Ambler et al. (1): the Ser-X-X-Lys consensus active-site serine residue at Ser70, the SDN loop at Ser130, Glu166, and the KTG sequence at Lys234. As with the *cfxA2* gene (15), the K272E substitution had no significant influence on the catalytic properties of the enzyme with respect to cefoxitin but increased the affinity of CfxA3 for first-generation cephalosporins. The Y239D substitution might not be linked to the extension of the beta-lactamase spectrum to broad-spectrum cephalosporins, especially ceftazidime, because the MICs of both cefotaxime and ceftazidime were in the susceptible range of the *E. coli* transformant. CfxA had some homology to other beta-lactamases at the active-site serine of Ambler class A beta-lactamases, although it appeared to have diverged significantly, as exemplified by the substitution at position 13 of 25 amino acid residues previously described as being invariant in class A beta-lactamases.

TABLE 2. PCR primers and composite primers from the *B. vulgatus cfxA* gene sequence

Primer	Position[a]	Sequence (5′ → 3′)
cfxA1	97	CTTTGTCGGCAAATAAAGAT
cfxA3	167	AAAACAAATCGTAGTTTTGAGTATAGC
cfxA4	677	TGAACGAGGAATGAGTGTGG
cfxA5	624	TGGTTAATGTCGCTCAAACA
cfxA6	1131	TCAAAGCAAGTGCAGTTTAAGA
cfxAZ1	*Eco*RI-153	GGAATTC-GAAAAAAACAGAAAAAAAC AAATCGTAGTTTTGAG
cfxAZ2	*Hin*dIII-1112	GGGAAGCTT-AGATTTTACTGAAGTTTG CATTAATAAAG

[a] Position on the *cfxA* sequence of *B. vulgatus* (locus BVU38243, GenBank accession no. U38243) (21).

TABLE 3. Nucleotide and amino acid substitution in CfxA, CfxA2, and CfxA3

Position (nucleotide or amino acid)[a]	Substitution at position in[b]:		
	CfxA	CfxA2	CfxA3
775	TAT	TAT	**G**AT
239	Tyr	Tyr	**Asp**
886	AAA	**G**AA	**G**AA
272	Lys	**Glu**	**Glu**

[a] Nucleotide numbering scheme according to reference 29; amino acid numbering scheme according to reference 1.
[b] Boldface type indicates the nucleotide or amino acid substitution.

The recombinant *Escherichia coli* strain harboring the *cfxA3* gene was susceptible to ceftazidime and cefotaxime, suggesting a difference in the level of beta-lactamase production, which may be linked to the promoter (33), or alterations in the porins (16). It is also possible that another beta-lactamase with a pI of 5.6, which is close to that of other extended-spectrum beta-lactamases, might be present in the clinical isolate (3, 23). Phylogenetic analyses revealed homologies to other beta-lactamase genes of anaerobic bacteria (NCBI, BLAST N): *cfxA* of *B. vulgatus* (locus BVU38243; GenBank accession no. U38243), 99%; *cfxA2* of *P. intermedia* (GenBank accession no. AF118110), 99%; *cepA* of *B. fragilis* (GenBank accession no. L13472), 39%; and *cblA* of *B. uniformis* (locus BNRCBLAX; GenBank accession no. L08472), 29%. Homologies to extended-spectrum beta-lactamases of aerobic bacilli were also found: TLA-1 of *E. coli* (GenBank accession no. AF148067), 31%; Per-1 of *Pseudomonas aeruginosa* (locus PAPER1A; GenBank accession no. Z21957), 30%; and Per-2 of *Salmonella enterica* serovar Typhimurium (locus STBLAPER2, EMBL accession no. X93314), 32%.

CfxA3 differed from CfxA2 by having an aspartic acid instead of a tyrosine at position 239 and from CfxA by having a glutamic acid instead of a lysine at position 272. The pIs reported for CfxA (*B. vulgatus*) and CfxA2 (*P. intermedia*) were 5.8 and 5.4, respectively. Isoelectric focusing experiments with the CfxA3 beta-lactamase revealed only one reactive band with a pI of 5.6 (data not shown). In the beta-lactamase classification scheme of Bush et al. (3), many *Capnocytophaga* beta-lactamases belong to group 2e, including enzymes that display strong activity against cephalosporins rather than penicillins. They also have acidic pI values and are susceptible to beta-lactamase inhibitors. The Van1 *Capnocytophaga* beta-lactamase described by Roscoe et al. (23) efficiently hydrolyzes a wide range of beta-lactams, including extended-spectrum cephalosporins. However, their substrate affinities are different despite their closely related MICs and similar pls. Rummens et al. (25) described a cephalosporinase, which was studied by Foweraker et al. (5). It had an isoelectric point of 3.6 (with a minor band at 4.1), conferred resistance to extended-spectrum beta-lactams, and was presumed to be chromosomally en-

coded. However, a full genetic characterization of the strain has never been performed.

Plasmid DNA from *C. ochracea* E201 was extracted using the alkaline lysis method of Ish-Horowicz and Burke (9). Plasmids and total DNA were separated on 0.8% agarose gels, transferred to nylon membranes (Amersham), and hybridized with a 510-bp digoxigenin-labeled CfxA3 probe synthesized using the cfxA3 and cfxA4 primers (Table 2) (8) as previously described (26).

Strain E201 contained three plasmids of approximately 3.5, 5, and 9 kb. Specific hybridization experiments with the cfxA3 probe indicated that the *cfxA3* gene was located on the 9-kb plasmid, which also carried a mobilizable transposon. Tribble et al. (30) reported the presence of such a transposon in *Bacteroides*. The spontaneous loss of plasmids during storage made it possible to recover a susceptible phenotype.

Capnocytophaga spp. cause septicemias in compromised hosts with underlying malignancies complicated by severe granulocytopenia. Since multidrug-resistant strains have been described, the importance of detecting beta-lactamase producers is now well established. The occurrence of a gene coding for an extended-spectrum beta-lactamase on a transposable element in a clinical *Capnocytophaga* strain is potentially a cause for concern about the spread of resistance to extended-spectrum cephalosporins. Additional work is needed to characterize the mobilization genes associated with *Capnocytophaga* spp. and their spread to other oral bacteria.

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