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Chromosome- and Plasmid-Encoded β-Lactamases in *Capnocytophaga* spp.

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Chromosome- and plasmid-encoded CfxA2 and CfxA3 β-lactamases were detected in *Capnocytophaga* spp. from oral sources in France, Norway, and the United States. Unidentified chromosome-encoded β-lactamases were present in *Capnocytophaga sputigena*. Nucleotide sequence analysis of the CfxA3-encoding plasmid from *C. ochracea* revealed an unreported insertion sequence (IS*Coc1*) upstream of the *cfxA* gene.

The genus *Capnocytophaga* includes capnophilic fusiform gram-negative rods that are part of the normal oropharyngeal flora. β-Lactamase-producing *Capnocytophaga* spp. constitute a threat to patients who receive empirical antibiotic therapy (1–3, 5, 12, 13, 15, 17). Two plasmid-encoded extended-spectrum β-lactamase genes, *TEM-17* and *cfxA3* (13, 18), have been identified for *Capnocytophaga ochracea*. The aim of this study was to investigate the β-lactamases of clinical *Capnocytophaga* strains isolated from oral sources in three countries: Norway, France, and the United States.

Twenty-five β-lactamase-producing strains of *Capnocytophaga* spp. were included (Table 1). DNA was isolated by the cetyltrimethylammonium bromide procedure (20). Random amplified polymorphic DNA (RAPD) reactions were performed using a Ready-To-Go RAPD analysis kit (Amersham Biosciences, Cleveland, OH). Pulsed-field gel electrophoresis (PFGE) (6), antimicrobial susceptibility testing, PCRs, DNA sequencing (8, 9, 22), isoelectric focusing (18), and Southern hybridization (21) were performed as described previously. Plasmid DNA was isolated by alkaline lysis with a QIAprep Spin miniprep kit (QIAGEN, GmbH, Germany) and the alkaline lysis method of Ish-Horowicz and Burke (11). For comparative purposes, plasmid DNA was digested with PvuII endonuclease (Amersham Biosciences). A Gene Clean spin kit (Qbiogene, Montreal, Quebec, Canada) was used for gel extraction of plasmid DNA. Dot blotting was performed with Gene Screen Plus membranes (Dupont, NEN Research Products, Boston, MA). Probe DNA (CfxA and TEM PCR products from control strains) was purified by using a QIAquick PCR purification kit (QIAGEN). The plasmids of four strains (321, 595, 616, and 800) were cut at the unique ClaI site, subcloned in the pBC SK+ chloramphenicol resistance vector (Stratagene, La Jolla, CA), and introduced by electroporation into *Escherichia coli* XL1-Blue MRF. Upstream and downstream sequences of the β-lactamase genes were determined by direct sequencing of purified DNA. The plasmid sequence from strain 321 was determined by primer walking (GENOME Express, Meylan, France).

Primer sequences are given in Table 2. For quality controls in susceptibility testing, *C. sputigena* ATCC 33612^T and *C. ochracea* ATCC 27872^T were included. *Haemophilus influenzae* 998/97, provided by the Norwegian Institute of Public Health

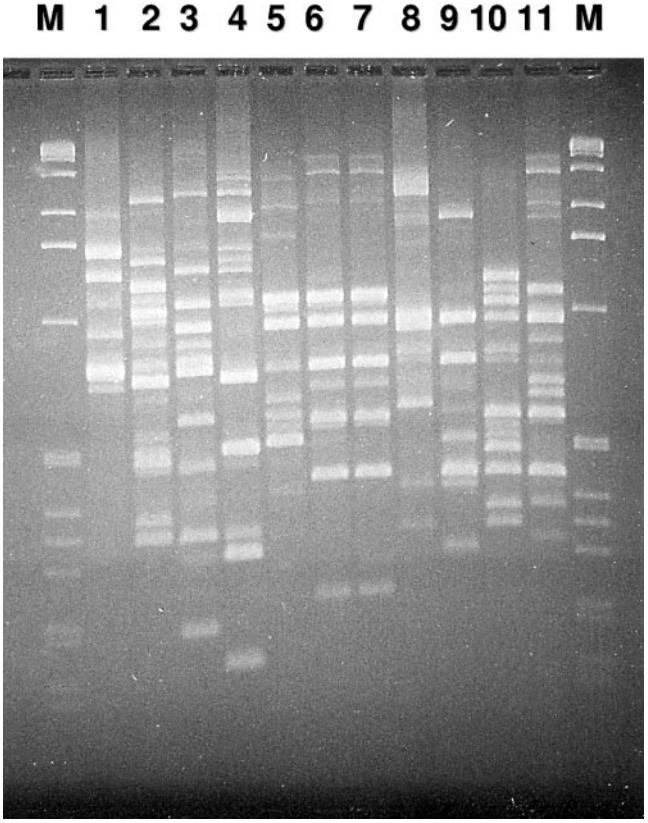

FIG. 1. RAPD profiles of *Capnocytophaga* spp. by use of primer 1. Lanes 1 to 11, isolates 507, 161, 149, 136, 10-02B, 10-02C, 10-02A, 7-02, 2-02, 11-01, and 8-01; lanes M, molecular size markers (100-bp ladder).

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TABLE 1. Antibiotic susceptibilities and β-lactamases characterized for oral *Capnocytophaga* isolates[a]

Isolate	Identity (16S rRNA)	Source	Country of origin	Etest MIC (μg/ml) of[a]:										β-Lactamase identity
				AC	XL	CT	FX	XM	EM	TC	CM	TZ	TZ-TZL	
372	*C. granulosa*	Periodontitis	France	2	0.032	32	0.38	32	32	0.094	256	1.2	<0.064	CfxA3[e]
45	*C. sputigena*	NUG[b]	France	>256	0.19	2	0.75	>256	0.125	>256	0.016	2	<0.064	CfxA3
62	*C. sputigena*	Periodontitis	France	64	0.125	4	1	32	>256	1.5	>256	1	<0.064	CfxA2[e]
176	*C. sputigena*	Dental abscess	France	2	0.25	16	0.75	12	256	1.5	>256	4	0.01	CfxA3
315	*C. sputigena*	Periodontitis	France	>256	0.25	8	1	64	0.5	0.5	<0.016	32	<0.064	ND[c]
456	*C. sputigena*	Periodontitis	France	24	0.064	64	0.38	>256	0.125	0.125	<0.016	0.5	<0.064	ND
507	*C. sputigena*	Periodontitis	France	32	0.125	4	1	64	>256	0.38	>256	256	<0.064	ND
516	*C. sputigena*	Periodontitis	France	32	0.125	32	1	>256	>256	0.38	>256	24	<0.064	ND
581	*C. sputigena*	Periodontitis	France	64	0.047	2	0.38	12	0.19	0.064	<0.016	2	<0.064	ND
568	*C. sputigena*	Periodontitis	France	>256	0.25	4	0.75	32	>256	0.19	256	3	0.03	CfxA3
301[d]	*C. sputigena*	Periodontal lesion	U.S.	0.19	0.19	0.125	0.5	0.25	0.125	0.19	0.016	1.5	<0.064	—[f]
528	*C. ochracea*	Periodontitis	France	>256	0.75	256	2	128	0.125	0.125	0.023	256	<0.064	CfxA3
321[d]	*C. ochracea*	Blood	France	>256	0.5	256	1	>256	1.5	0.25	0.064	>256	<0.064	CfxA3
595	*C. ochracea*	Periodontitis	France	>256	0.125	64	1	256	256	0.38	>256	256	<0.064	CfxA3
616	*C. ochracea*	Periodontitis	France	>256	0.125	>256	1	>256	0.064	0.19	0.064	64	<0.064	CfxA3
800	*C. ochracea*	Periodontitis	France	>256	8	>256	2	>256	>256	0.38	>256	>256	<0.064	CfxA3
136	*C. ochracea*	Periodontitis	Norway	2	0.047	0.19	0.50	6	0.125	0.125	<0.016	0.125	<0.064	CfxA2
161	*C. ochracea*	Periodontitis	Norway	2	0.19	0.25	0.25	2	0.125	0.38	<0.016	0.047	<0.064	CfxA2
448[d]	*C. ochracea*	Oral cavity	U.S.	0.25	0.19	0.19	0.38	0.5	0.25	0.125	0.016	1.5	<0.064	—
140	*C. gingivalis*	Dental abscess	France	>256	1	256	1	>256	0.125	0.25	0.023	32	<0.064	CfxA3
8-01	*C. gingivalis*	Periodontitis	U.S.	2	0.064	3	2	32	0.75	0.25	<0.016	1.5	<0.064	CfxA3
11-01	*C. gingivalis*	Periodontitis	U.S.	256	0.75	256	0.5	256	0.125	0.25	<0.016	>256	<0.064	CfxA3
2-02	*C. gingivalis*	Periodontitis	U.S.	4	0.094	8	0.75	256	0.38	0.125	<0.016	4	<0.064	CfxA2
10-02A	*C. gingivalis*	Periodontitis	U.S.	1	0.032	6	0.25	24	0.047	1.5	<0.016	4	<0.064	CfxA2
10-02B	*C. gingivalis*	Periodontitis	U.S.	0.50	0.064	1	0.75	3	0.094	1	<0.016	1.5	<0.064	CfxA2
149	*C. gingivalis*	Periodontitis	Norway	32	0.032	256	0.75	256	0.125	0.125	<0.016	8	<0.064	CfxA2
7-02	*Capnocytophaga* sp.	Periodontitis	U.S.	2	0.064	0.125	0.125	0.75	0.094	0.125	<0.016	0.064	<0.064	CfxA2

[a] AC, amoxicillin; XL, amoxicillin-clavulanic acid; CT, cefotaxime; FX, cefoxitin; XM, cefuroxime; EM, erythromycin; TC, tetracycline; CM, clindamycin; TZ, ceftazidime; TZ-TZL, ceftazidime–ceftazidime-clavulanic acid.
[b] NUG, necrotizing ulcerative gingivitis.
[c] ND, not detected in PCR experiments with the CfxA primer.
[d] Reference strains: 301, *C. sputigena* ATCC 33612^T; 321, *C. ochracea* CIP 105321; 448, *C. ochracea* ATCC 27872^T.
[e] Amino acids substitutions: from CfxA (*Bacteroides vulgatus*) to CfxA2, E72K; from CfxA to CfxA3, E72K and D239Y (16, 19).
[f] —, β-lactamase negative.

in Norway, and *E. coli* pNCE-3 (14) were used as positive controls for PCR with TEM- and CfxA-specific primers, respectively.

The species identities and antibiotic resistance phenotypes of the isolates are shown in Table 1. A great diversity of *Capnocytophaga* isolates was demonstrated by RAPD analyses (Fig. 1), in agreement with a previous study (23). PFGE showed equally different patterns for each strain, suggesting that expanding resistance in *Capnocytophaga* spp. (1–3, 5, 12, 13, 15, 17) is more likely due to gene transfer than clonal dissemination.

TABLE 2. PCR primers used in this study

Amplicon	Primer sequence (5′–3′)	Reference or source
TEM	GTATGGATCCTCAACATTTCCGTGTCG ACCAAAGCTTAATCAGTGAGGCA	16
CfxA	GCAAGTGCAGTTTAAGATT CGTAGTTTTGATTATAGCT	4
RAPD	GGTGCGGGAA GTTTCGCTCC AAGAGCCCGT	This study
Tn*1* Tn*4*	GATGAAAATGCAAACTAAAGC TGTAGTATATTCTTTATTAATGC	This study

High MICs for amoxicillin were generally seen among *Capnocytophaga* spp. However, some strains presented low MICs (0.5 to 1 μg/ml). Most strains were highly resistant to the cephalosporins, with the exception of cefoxitin. Good synergy with clavulanic acid was generally observed for amoxicillin and ceftazidime. Eight strains were resistant to erythromycin and clindamycin. Only one isolate (*C. sputigena* strain 45) was resistant to tetracycline.

Isoelectric focusing analysis revealed that the β-lactamases of different *Capnocytophaga* spp. migrated with a main band with a pI of around 5.6. DNA sequencing of PCR products showed that both *cfxA2* and *cfxA3* genes were prominent in *Capnocytophaga* spp. (Table 1). The CfxA2/CfxA3 division was not related to species differentiation or MICs. Five strains, all identified as *C. sputigena*, were CfxA negative, but two of them (507 and 581) were positive with the CfxA probe, suggesting the presence of a gene coding for an unidentified chromosomal β-lactamase related to the CfxA family.

Six of the 25 strains harbored a 9-kb plasmid (*C. ochracea* 321, 595, 616, and 800 and *C. gingivalis* 140 and 11-01) with the *cfxA3* gene, and for one strain (*C. gingivalis* 10-02A), the β-lactamase gene (*cfxA2*) was located on a 4-kb plasmid. PvuII restriction profiles of plasmids from *C. ochracea* strains were identical but differed from those of the *C. gingivalis* strains. In the *C. gingivalis* strains harboring either the 9-kb or the 4-kb plasmid, the *cfxA* gene (*cfxA3* or *cfxA2*, respectively) was

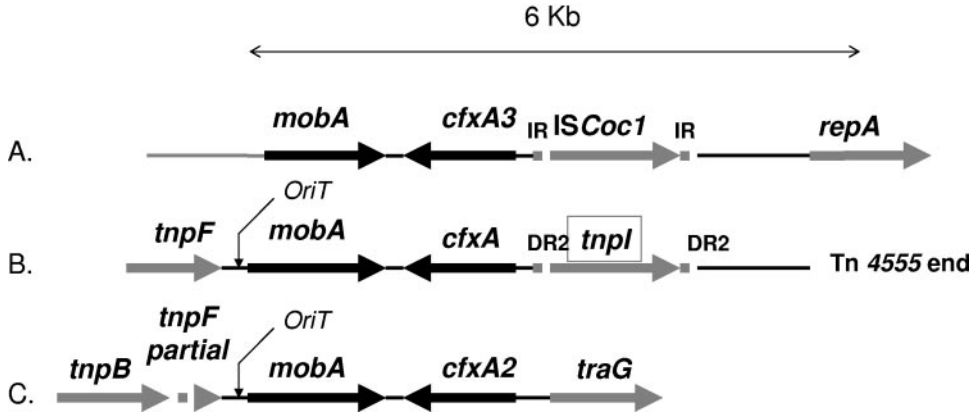

FIG. 2. Schematic comparison of the genetic environment of bla_{cfxA} of (A) *C. ochracea* strain 321 (CfxA3) (GenBank accession no. AY860640) with that of (B) *Bacteroides fragilis* (CfxA) and (C) *Prevotella intermedia* (CfxA2). The arrangements of the common region *mobA-cfxA* are based upon updated sequences (GenBank accession no. U75371 and AF118110, respectively). IR, inverted repeat; DR2, direct repeat 2.

present on the 1.2-kb PvuII fragment. Thus, it is likely that the plasmid-mediated CfxA resistance in *C. gingivalis* was acquired independently by these strains. Interestingly, in a different strain of *C. gingivalis* from the same patient (strain 10-02B), the *cfxA2* gene was chromosome encoded, as demonstrated by Southern hybridization studies. PCR experiments with purified plasmid DNA were positive with specific CfxA primers and negative with TEM primers for all strains, including the *C. ochracea* reference strain (strain 321) previously described by Rosenau et al. (18) to harbor a TEM-17 plasmid-encoded β-lactamase. This strain possessed, however, the 9-kb plasmid with the *cfxA* gene, as did other *C. ochracea* strains.

Hybridization of plasmid DNA and total DNA with the CfxA probe indicated that the *cfxA* gene was located on the plasmid and not on the chromosome. CfxA-positive, plasmid-negative strains were all positive by dot blotting, but no hybridization was detected between 50- and 300-kb bands after PFGE migration, suggesting that the *cfxA* gene is located at a chromosomal band of larger size.

Nucleotide sequence analysis of the plasmid from strain 321 (Fig. 2) (GenBank accession no. AY860640) revealed identity with the *mobA-cfxA* region of Tn*4555* (CfxA of *Bacteroides vulgatus*) (19), but without the origin of transfer (*oriT*) of the transposon. A new insertion sequence (IS*Coc1*) was found upstream of the *cfxA* gene (Fig. 3). IS*Coc1* had a size of 1,038 bp with two 25-bp terminal inverted repeats. The deduced protein sequence of the transposase showed homology, although low, with a transposase-like protein of the rumen bacterium *Mannheimia succiniciproducens* (10). Upstream, the plasmid sequence showed a characteristic *repA* region with an

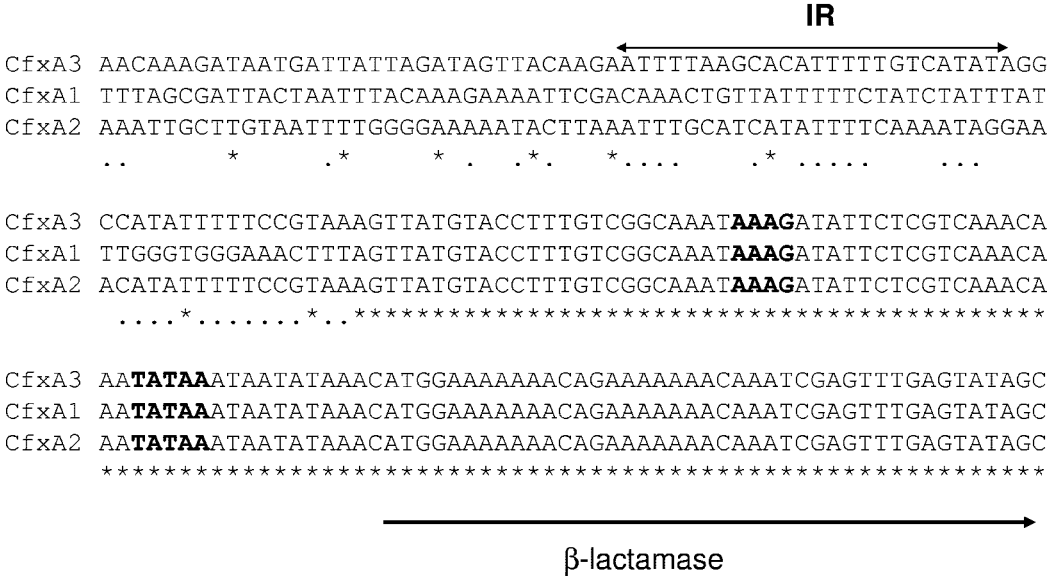

FIG. 3. Comparison of the upstream nucleotide sequences of the *cfxA3* gene of strains 321, 595, 616, and 800, along with the partial sequence upstream of *cfxA1* and *cfxA2* (GenBank accession no. U75371 and AF118110, respectively). Sequence identity among the *cfxA* genes is indicated with asterisks. Sequence identity between bla_{cfxA2} and bla_{cfxA3} is indicated with dots. The −35 and −10 regions of putative promoter sequences of the *cfxA* genes are shown in boldface. The horizontal arrow indicates an inverted repeat (IR) of 25 bp found upstream of bla_{cfxA3}.

origin of replication, containing three iterons. The *repA* region was similar to the *B. vulgatus* pIP417 plasmid gene (7), but with relatively low amino acid identities (47%) for replication proteins. The *cfxA* flanking regions from the three other *C. ochracea* strains (595, 616, and 800) were identical. The *mobA-cfxA* region could represent the minimum DNA sequence responsible for the mobility of the *cfxA* gene in *Bacteroidaceae*. To verify this hypothesis, further sequence studies on the *cfxA*-surrounding region are needed, particularly for *C. sputigena* strains where the *cfxA* gene had a chromosomal location.

In summary, *cfxA2* and *cfxA3* were the genes responsible for the extended-spectrum resistance to β-lactam antibiotics in 80% of *Capnocytophaga* spp. However, several undescribed β-lactamase genes seem to be present in these bacteria.

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