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Capnocytophaga ochracea: Characterization of a Plasmid-Encoded Extended-Spectrum TEM-17 β-Lactamase in the Phylum *Flavobacter-Bacteroides*

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A plasmid-encoded extended-spectrum TEM β-lactamase with a pI of 5.5 was detected in a *Capnocytophaga ochracea* clinical isolate. The *bla* gene was associated with a strong TEM-2 promoter and was derived from bla_{TEM-1a} with a single-amino-acid substitution: Glu_{104}→Lys, previously assigned to TEM-17, which is thus the first TEM β-lactamase to be reported in the phylum *Flavobacter-Bacteroides*.

The dissemination of antibiotic resistance is a clear example of gene exchange between distantly related bacteria (13). However, this phenomenon is rare enough that the presence of a TEM β-lactamase in *Capnocytophaga ochracea*, a *Cytophagale* belonging to the phylum *Flavobacter-Bacteroides* (14), is of note, this type of enzyme being previously described only in the very phylogenetically distant *Proteobacteria* (4, 17, 22). *C. ochracea* is a capnophilic gram-negative fusiform rod with gliding motility. It is part of the normal gingival flora in humans and causes gingivitis and periodontitis. It may cause systemic infections in immunocompromised and neutropenic patients (11). Antibiotic treatment was originally based on its susceptibility to penicillins, including benzylpenicillin (7). The organism was later shown to be susceptible to extended-spectrum cephalosporins, making it possible to prescribe these drugs (2). Enzymatic resistance to β-lactams, including extended-spectrum cephalosporins, was reported as early as 1986 in clinical isolates of *C. ochracea* (3, 8, 18, 19). The genetic basis of resistance was not determined for any of the resistant strains. We report for the first time a plasmid-encoded TEM extended-spectrum β-lactamase in a clinical isolate of *C. ochracea*.

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C. ochracea CIP 105321 was isolated in 1996 at Bretonneau Hospital Tours, France, from a culture of blood from an 11-year-old child with acute myeloid leukemia, fever (39°C), severe neutropenia, and oral mucositis who had received an empiric antibiotic treatment, including ceftazidime. The β-lactamase reaction in the nitrocefin-disk test (Cefinase; bioMérieux, Marcy l'Etoile, France) was positive. The disk-agar diffusion test performed on chocolate agar containing 1% PolyVitex (bioMérieux) showed that strain CIP 105321 was resistant to penams (amoxicillin, ticarcillin, and piperacillin) and cephems (cephalothin, cefoperazone, cefuroxime, cefotaxime, ceftazidime, ceftriaxone, cefpirome, and cefepime), but susceptible to cefoxitin, aztreonam, piperacillin-tazobactam, and imipenem. There was extensive synergy between amoxicillin-clavulanic acid and β-lactam disks, including those for all extended-spectrum cephalosporins tested. The strain was also resistant to gentamicin, polymyxin B, and cotrimoxazole, as commonly reported in the genus *Capnocytophaga* (7, 19). MICs were determined by agar dilution on Wilkins-Chalgren agar (Difco, Detroit, Mich.) with an inoculum of 10^4 CFU per spot (3, 18). The MICs of the two β-lactamase inhibitors, clavulanic acid and sulbactam, were found to be 0.6 and 0.8 μg/ml, respectively. To prevent an intrinsic antibacterial effect of clavulanate and sulbactam, a concentration of 0.1 μg/ml (if combined with a β-lactam) was then used for all plates. The strain was resistant to amoxicillin (MIC, 512 μg/ml), had intermediate resistance to piperacillin (MIC, 32 μg/ml), and was more resistant to ceftazidime (MIC, 64 μg/ml) than to cefpirome, cefepime, and cefotaxime (MIC, 16 μg/ml). It was susceptible to imipenem and cefoxitin, as was the β-lactamase-negative control strain, CIP 103448. Clavulanic acid and sulbactam partly restored the activity of amoxicillin, cefotaxime, and ceftazidime (Table 1).

Three types of crude β-lactamase extract were prepared. Lysis by sonication and by Triton treatment was performed as previously described (8). For lysis by both sonication and detergent, 4% Triton X-100 was added to the sonicated cells. The β-lactamase activity of the supernatants was estimated by the iodine procedure in gels containing benzylpenicillin, by comparing the decolorized zones obtained with that for a sonicated extract containing TEM-1 β-lactamase (6). β-Lactamase activity was not detected in a 24-h broth culture or a Triton extract. Weak activity was detected in a sonicated extract, and strong activity was obtained if Triton was added after sonication. The analytical isoelectric focusing method used was adapted from that described by Foweraker et al. (8), with polyacrylamide gels containing ampholines (Pharmacia, Uppsala, Sweden) with a pH range of 3.5 to 9.5. Focusing of the enzyme could only be achieved for extracts containing Triton and if 4% Triton was included in the gel. This was previously reported to be the case for two other β-lactamases in *Capnocytophaga*, possibly due to enzyme binding to membrane components (8, 18). The β-lactamase migrated as a single band with an estimated pI of 5.5 in comparisons with known β-lactamases (TEM-1, pI 5.4; TEM-3, pI 6.3; and TEM-4, pI 5.9).

Plasmid DNA isolated by alkaline lysis was about 9 kb in size. Plasmid curing was done by culture with ethidium bromide and replica plating (6). The cured clones were highly susceptible to all β-lactams tested (Table 1) and did not pro-

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TABLE 1. In vitro susceptibility of *C. ochracea* strains to antibiotics

Strain	β-Lactamase	MIC (μg/ml)[a]												
		Amoxicillin			Cefoxitin			Cefotaxime			Ceftazidime			Imipenem alone
		Alone	+CA	+SU	Alone	+CA	+SU	Alone	+CA	+SU	Alone	+CA	+SU	
CIP 105321 (wild-type plasmid)	+	512	64	128	2	2	2	16	2	8	64	8	16	0.125
CIP 105321 (cured)	−	0.125	0.125	0.125	1	1	1	0.015	0.015	0.015	0.03	0.03	0.03	0.125
CIP 103448	−	0.125	0.125	0.125	1	1	1	0.03	0.03	0.03	0.03	0.03	0.03	0.125

[a] CA, clavulanic acid (0.1 μg/ml); SU, sulbactam (0.1 μg/ml).

duce β-lactamase detectable by the nitrocefin test on colonies. Resistance to other antimicrobial agents was not affected.

PCR was performed with plasmid DNA as the template and a pair of primers (Eurogentec, Seraing, Belgium), 1079 [5′-d(GGTCTGACAGTTACCAATGC)-3′] and 6 [5′-d(GAAGACGAAAGGGCCTCGTG)-3′], internal to bla_{TEM-1}, with nucleotide positions given according to the numbering of Sutcliffe (21). Both strands of the PCR product were sequenced by automated fluorescent sequencing with an ABI Prism 377 sequencer (Perkin-Elmer), by using Thermo Sequenase dye terminator cycle sequencing premixed version 2.0 (Amersham, Les Ulis, France). Analysis of the nucleotide sequence (Table 2) showed that the gene encoding *C. ochracea* β-lactamase differed from the bla_{TEM-1a} gene by four silent mutations (positions 469, 682, 863, and 985) and that there was a Glu_{104}→Lys amino acid substitution in the protein according to the numbering scheme of Ambler et al. (1, 9). Lys_{104} substitution is common in extended-spectrum β-lactamases derived from TEM-1 or TEM-2 enzymes and is always associated with one to three additional amino acid changes at positions 21, 153, 164, 182, 237, 238, 240, and 265 (15; http://www.lahey.org/studies/webt.htm). The presence of single mutation Glu_{104}→Lys has already been suspected on the basis of colony hybridization with specific oligonucleotide probes (oligotyping) in a single strain of *Klebsiella pneumoniae*. The enzyme was called TEM-17 (12). After sequencing of the corresponding gene, the deduced amino acid sequence was identical to that of TEM-15, and the enzyme was therefore redesignated TEM-15 (10). Because the β-lactamase of *C. ochracea* CIP 105321 presents the single Glu_{104}→Lys amino acid substitution, we assigned it the number TEM-17 (http://www.lahey.org/studies/webt.htm). As for most of these enzymes, the bla_{TEM-17} gene was associated with a strong TEM-2 promoter with a C-to-T substitution at nucleotide 32 that increases the amount of enzyme (5).

TABLE 2. Nucleotide and amino acid differences between bla_{TEM-1a}, bla_{TEM-2}, and bla_{TEM-17}

Nucleotide no.[a]	Amino acid no.[b]	Nucleotide (amino acid)[c] in:		
		bla_{TEM-1a}	bla_{TEM-2}	bla_{TEM-17}
Promoter region				
32		C	T	T
Coding region				
317	39	C (Gln)	A (Lys)	C
469	89	G (Glu)	G	A
512	104	G (Glu)	G	A (Lys)
682	160	T (Thr)	C	C
863	221	C (Leu)	C	T
985	261	C (Ile)	C	T

[a] Numbering according to Sutcliffe (21).
[b] Numbering according to Ambler et al. (1).
[c] The encoded amino acid was indicated when there was a point mutation leading to an amino acid change relative to the TEM-1 sequence.

It is not clear how *Capnocytophaga* acquired a bla_{TEM} gene. Given the probable emergence in 1986 of the first isolates of *Capnocytophaga* producing extended-spectrum β-lactamases and the small size of the plasmids involved, the donating strain probably transferred the extended-spectrum β-lactamase gene via a transposon. However, *Capnocytophaga* may have acquired the bla_{TEM-1} gene via a transposon or small plasmid and then modified the bla_{TEM-1} gene by point mutation to give bla_{TEM-17}, possibly due to selection pressure exerted by the extended-spectrum cephalosporins used to treat neutropenic patients. The ecological niche of *Capnocytophaga* and the small size of the plasmid involved suggest that the donating strain was of the genus *Haemophilus* or *Neisseria*, in which TEM extended-spectrum β-lactamases have never been reported. The TEM-1 β-lactamase should therefore also be present in *Capnocytophaga* strains.

In conclusion, the emergence of such inactivating enzymes in the *Flavobacter-Bacteroides* phylum at the same time as in the very distant *Proteobacteria* phylum (16, 20) requires further molecular investigation to trace the movements of the bla_{TEM} gene.

Nucleotide sequence accession number. The nucleotide sequence of bla_{TEM-17} has been assigned accession no. Y14574 in the EMBL Nucleotide Sequence Database.

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REFERENCES

1. **Ambler, R. P., A. F. W. Coulson, J. M. Frère, J. M. 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:**269–272.
2. **Arlet, G., M. J. Sanson-Le Pors, I. M. Casin, M. Ortenberg, and Y. Perol.** 1987. In vitro susceptibility of 96 *Capnocytophaga* strains, including a β-lactamase producer, to new β-lactam antibiotics and six quinolones. Antimicrob. Agents Chemother. **31:**1283–1284.
3. **Arlet, G., M. J. Sanson-Le Pors, S. Castaigne, and Y. Perol.** 1987. Isolation of a strain of β-lactamase-producing *Capnocytophaga ochracea*. J. Infect. Dis. **155:**1346.
4. **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.
5. **Chen, S.-T., and R. C. Clowes.** 1987. Variations between the nucleotide sequences of Tn*1*, Tn*2*, and Tn*3* and expression of β-lactamase in *Pseudomonas aeruginosa* and *Escherichia coli*. J. Bacteriol. **169:**913–916.
6. **Courvalin, P., F. Goldstein, A. Philippon, and J. Sirot (ed.).** 1985. L'antibiogramme. MPC-Vidéom, Paris, France.
7. **Forlenza, S. W., M. G. Newman, A. L. Horikoshi, and U. Blachman.** 1981. Antimicrobial susceptibility of *Capnocytophaga*. Antimicrob. Agents Chemother. **19:**144–146.
8. **Foweraker, J. E., P. M. Hawkey, J. Heritage, and H. W. Van Landuyt.** 1990. Novel β-lactamase from *Capnocytophaga* sp. Antimicrob. Agents Chemother. **34:**1501–1504.
9. **Goussard, S., and P. Courvalin.** 1991. Sequence of the genes *blaT-1B* and *blaT-2*. Gene **102:**71–73.
10. **Goussard, S., and P. Courvalin.** 1999. Updated sequence information for

TEM β-lactamase genes. Antimicrob. Agents Chemother. **43:**367–370.
11. **Kristensen, B., H. C. Schønheyder, N. A. Peterslund, S. Rosthøj, N. Clausen, and W. Frederiksen.** 1995. *Capnocytophaga* (*Capnocytophaga ochracea* group) bacteremia in hematological patients with profound granulocytopenia. Scand. J. Infect. Dis. **27:**153–155.
12. **Mabilat, C., and P. Courvalin.** 1990. Development of "oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in members of the family *Enterobacteriaceae*. Antimicrob. Agents Chemother. **34:** 2210–2216.
13. **Maiden, M. C. J.** 1998. Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. Clin. Infect. Dis. **27:**S12–S20.
14. **Nakagawa, Y., and K. Yamasato.** 1996. Emendation of the genus *Cytophaga* and transfer of *Cytophaga agarovorans* and *Cytophaga salmonicolor* to *Marinilabilia* gen. nov.: phylogenetic analysis of the *Flavobacterium-Cytophaga* complex. Int. J. Syst. Bacteriol. **46:**599–603.
15. **Palzkill, T., and D. Botstein.** 1992. Identification of amino acid substitutions that alter the substrate specificity of TEM-1 β-lactamase. J. Bacteriol. **174:** 5237–5243.
16. **Paul, G. C., G. Gerbaud, A. Bure, A. M. Philippon, B. Pangon, and P. Courvalin.** 1989. TEM-4, a new plasmid-mediated β-lactamase that hydrolyzes broad-spectrum cephalosporins in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **33:**1958–1963.
17. **Philippon, A., S. Ben Redjeb, G. Fournier, and A. Ben Hassen.** 1989. Epidemiology of extended spectrum β-lactamases. Infection **17:**347–354.
18. **Roscoe, D. L., S. J. V. Zemcov, D. Thornber, R. Wise, and A. M. Clarke.** 1992. Antimicrobial susceptibilities and β-lactamase characterization of *Capnocytophaga* species. Antimicrob. Agents Chemother. **36:**2197–2200.
19. **Rummens, J. L., B. Gordts, and H. W. Van Landuyt.** 1986. In vitro susceptibility of *Capnocytophaga* species to 29 antimicrobial agents. Antimicrob. Agents Chemother. **30:**739–742.
20. **Sirot, D., J. Sirot, R. Labia, A. Morand, P. Courvalin, A. Darfeuille-Michaud, R. Perroux, and R. Cluzel.** 1987. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J. Antimicrob. Chemother. **20:**323–334.
21. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
22. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.