ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1990, p. 1501–1504
0066-4804/90/081501-04$02.00/0

Vol. 34, No. 8

Novel β-Lactamase from *Capnocytophaga* sp.

JULIET E. FOWERAKER,[1] PETER M. HAWKEY,[1]* JOHN HERITAGE,[1] AND HERMAN W. VAN LANDUYT[2]

Department of Microbiology, University of Leeds, Leeds LS2 9JT, United Kingdom,[1] and Department of Microbiology, Algemeen Ziekenhuis St Jan, B-8000 Bruges, Belgium[2]

Received 31 October 1989/Accepted 25 April 1990

A novel β-lactamase activity which confers resistance to expanded-spectrum caphalosporins and penicillins has been found in strain IC 5/21 of *Capnocytophaga* spp. Enzyme activity migrated at a molecular size of 38,000 daltons and at an isoelectric point of 3.6, with a minor band at 4.1. Kinetic studies suggested that it belonged to Richmond and Sykes β-lactamase class 1c. Isoelectric focusing could be achieved only if a nonionic detergent was added to the gel, suggesting the presence of a hydrophobic enzyme akin to a membrane-bound β-lactamase of gram-positive bacteria. The location of the gene coding for this β-lactamase is not yet known.

Clinical microbiologists have shown considerable interest in bacteria belonging to the genus *Capnocytophaga* since they were first recognized as a cause of serious sepsis in immunocompromised patients (8). *Capnocytophaga* spp. are gram-negative gliding bacteria, which are microaerophilic and nonsporing. They were thought to belong to the genus *Bacteroides* but are now recognized as members of the class *Flexibacteriae*. The Centers for Disease Control dysgonic fermenter group DF-1 is synonymous with *Capnocytophaga* sp. (13). Recently, members of the genus *Capnocytophaga* have also been shown to cause infection in patients with a normal immune response (13, 21). While infections with *Capnocytophaga* sp. remain relatively uncommon, they do cause a wide range of clinical conditions and may require treatment with an empirical choice of antimicrobial agents. It is therefore important to know the agents to which *Capnocytophaga* spp. are typically susceptible, thus allowing effective treatment. Early work (8, 25) showed all the strains tested to be susceptible to chloramphenicol, clindamycin, penicillins, and tetracycline. Susceptibility to older cephalosporins was variable, but more recent studies (1, 14, 23) have reported that *Capnocytophaga* spp. are susceptible to expanded-spectrum cephalosporins and quinolones. It would seem that either a penicillin or an expanded-spectrum cephalosporin would be the first choice for treating infections caused by *Capnocytophaga* sp. Recent reports of strains of *Capnocytophaga* sp. that produce β-lactamase must give cause for concern (1, 16, 23). We have studied in depth one of the three strains reported by Rummens and colleagues (23) and found it to produce a novel β-lactamase with unusual biochemical properties conferring high-level resistance to a wide range of β-lactam antibiotics.

MATERIALS AND METHODS

Strain IC 5/21 of *Capnocytophaga* sp. was isolated from an oral ulcer in a neutropenic patient by Van Landuyt et al. (23). For the studies described here, it was grown on 5% heated horse blood agar (blood agar base no. 2; Oxoid, Basingstoke, United Kingdom) in 5% CO_2 or in brain heart infusion broth (Oxoid, United Kingdom) by using a shaking incubator. Long-term storage of the strain was in 40% glycerol–3% sodium citrate at −70°C (12). The susceptibility of 120 clinical isolates of *Capnocytophaga* sp. was determined by Rummens et al. and is reported elsewhere (23). The MIC for 50% of strains tested from that study was used in this study as a measure of the typical antibiotic susceptibility of *Capnocytophaga* sp.

Reagents. Antibiotics were obtained as follows: ampicillin, carbenicillin, cloxacillin, and methicillin, Beecham Pharmaceuticals, Worthing, United Kingdom; penicillin G, Glaxo Pharmaceuticals Ltd., Greenford, United Kingdom; nitrocefin, Oxoid Ltd., Basingstoke, United Kingdom; cefotaxime, Roussel, Uxbridge, United Kingdom; and cephaloridine and cephalothin, Sigma Chemical Co., Poole, United Kingdom. Acrylamide, *N,N'*-methylene bisacrylamide, and ammonium persulfate of electrophoresis grade (electran) were supplied by BDH (Poole, Dorset, United Kingdom); Triton X-100, *p*-chloromercuribenzoate, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), *N,N,N',N'*-tetramethylethylenediamine, and pharmalytes were supplied by Sigma Chemical Co. The agarose used was electrophoresis Z from Pharmacia (LKB Biotechnology AB, Bromma, Sweden). Potassium clavulanate was supplied by Beechams Research Laboratories, Brockham Park, Surrey, United Kingdom.

Antibiotic susceptibility testing. MICs were determined by an agar dilution method as previously described (23). Antibiotic susceptibility disk testing was performed by using a comparative method (15). To test for synergy, disks of either ampicillin (20 μg) or cefotaxime (30 μg) were placed 1 and 2 cm from disks that had been impregnated with 10 μg of potassium clavulanate on a lawn of strain IC 5/21 inoculated onto heated blood agar. The plate was then incubated for 18 h at 37°C.

Preparation of β-lactamase extracts. Crude β-lactamase preparations were made as follows. For detergent lysis, a 100-ml broth culture of *Capnocytophaga* sp. strain IC 5/21 was incubated with shaking for 18 h at 37°C and harvested. The cells were washed in 10 mM HEPES, pH 7.4, and suspended in 4% Triton X-100 to a final concentration of 1.5×10^9 CFU/ml and kept at room temperature for 5 min. For lysis by sonication, cells from 18-h cultures were harvested and washed as above but were resuspended in HEPES buffer at a density of 10^9 CFU/ml. Cells were sonicated for five periods for 20 s, with cooling periods of 30 s, the tube being suspended in ice. Cells lysed by both methods were centrifuged for 5 min at 13,000 × *g* in a microcentrifuge (Micro-Centaur; MSE Instruments, Crawley, England) to pellet cell debris. Extracts either were used fresh (or after overnight dialysis against phosphate-buffered saline with 2% Triton X-100) or were stored at −70°C.

β-Lactamase from standard strains producing TEM-1,

* Corresponding author.

TEM-2, SHV-1, and PSE-4 were extracted by sonication as described by Vecoli et al. (28). β-Lactamase activity from IC 5/21 and standard β-lactamase-producing strains was estimated at each stage by using a semiquantitative nitrocefin assay as follows: 25 μl of extract was added to 75 μg of nitrocefin (50 μg/ml), and the time in seconds was measured for the yellow to red color change, which indicated hydrolysis.

Isoelectric focusing. Methods for isoelectric focusing in agarose and acrylamide were adapted from those described by Matthew et al. (19) and Vecoli et al. (28). It was found that the β-lactamase produced by IC 5/21 would focus only in the presence of Triton X-100. Gels (105 by 105 by 1 mm) were cast from 1% agarose–12% sorbitol–6% ampholines–2% Triton X-100 or from 5% acrylamide–13% glycerol–6% ampholines–2% Triton X-100. For polymerization of acrylamide in the presence of Triton X-100, *N,N,N',N'*-tetramethylethylenediamine at 0.1% in addition to ammonium persulfate at 0.0075% was needed. Isoelectric focusing gels were run at 10 W on a water-cooled flat-bed apparatus (FBE-3000; Pharmacia) connected to a power supply with a volt-hour integrator (VH-1; Pharmacia). Standard enzymes of known pI, TEM-1 (5.4), TEM-2 (5.6), SHV-1 (7.7), and PSE-4 (5.2), and the dye methyl red (3.75) were used. Zymograms were developed by using nitrocefin (10 mg/liter in 0.1 M phosphate buffer, pH 7.0). The positions of bands were marked onto the plastic backing as they developed, as the colored product diffused rapidly in the presence of detergent.

Molecular weight determination of β-lactamase activity from *Capnocytophaga* sp. The method of Tai et al. (26) for in situ detection of β-lactamase activity in sodium dodecyl sulfate-polyacrylamide gels was modified as follows. The crude extract from *Capnocytophaga* sp. was incubated with an equal volume of sample buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 50 mM Tris hydrochloride, with bromophenol blue as a tracking dye) at 100°C for 10 min. The treated β-lactamase from approximately 7×10^8 cells was run on an 11% sodium dodecyl sulfate-polyacrylamide gel at 150 mA by using a discontinuous buffer system (17). The gel was washed in 5 mM phosphate buffer, pH 6.0, for 1 h, with one change of buffer, and in 5 mM phosphate buffer, pH 7, for 10 min at 37°C. Enzyme activity was visualized with an overlay of filter paper soaked in nitrocefin as above, and a TEM-1 preparation was also run on the gel as a control.

β-Lactamase kinetics assay. β-Lactamase activity was measured against various substrates in 0.05 M phosphate buffer, pH 7.0, at 30°C by UV spectrophotometric assay (20, 24). The changes in A_{270} for cephaloridine and cephalothin; A_{265} for cefotaxime; A_{235} for ampicillin, benzylpenicillin, and carbenicillin; and A_{305} for methicillin were monitored at 30°C by using a dual beam, temperature controlled, UV spectrophotometer (model PU8800; Pye Unicam, Cambridge, England).

Substrate profiles were determined by using a substrate concentration of 0.1 mM to allow direct comparison of activity rates. V_{max} and K_m for cephalosporinase activity were derived from a Lineweaver-Burke plot by using substrate concentrations of 0.01, 0.02, 0.1, and 0.5 mM. Inhibition studies were done by comparing the rate of hydrolysis of cephaloridine at 0.1 mM after preincubation of the enzyme in buffer at 30°C with the rate after preincubation of enzyme with potassium clavulanate (4 μg/ml), cloxacillin (2 mM), or sodium chloride (10 to 1 mM).

Plasmid analysis and transfer methods. Plasmid DNA was isolated and visualized by using the method of Bennett et al. (3). Transformation of HB101 cells by using plasmid DNA found in IC 5/21 was attempted by using the method of Hanahan (11). Plate matings were by the method of Avila et al. (2); carbenicillin (50 μg/ml) was used for the selection of transconjugants.

TABLE 1. In vitro activity of various antimicrobial agents against *Capnocytophaga* sp. strain IC 5/21 and the MIC for 50% of a collection of 120 strains including IC 5/21 isolated from clinical specimens and reported by Rummens et al. (23) for comparison

Antimicrobial agent	MIC (μg/ml) for:	
	IC 5/21	50% of strains tested
Ampicillin	>128	0.5
Aztreonam	2	0.25
Penicillin	>128	0.5
Temocillin	64	2
Ticarcillin	>128	0.5
Cefazolin	>128	8
Cefotaxime	32	0.12
Cefpirome	32	≤0.03
Ceftazidime	32	0.25
Moxalactam	128 (>128)[a]	1
Amifloxacin	1	0.5
Amikacin	>128	128
Ciprofloxacin	0.25	0.12
Clindamycin	≤0.03	≤0.03
Norfloxacin	2	0.5
Ofloxacin	0.25 (0.5)	0.12
Pefloxacin	0.5	0.5
Ro 15-8074	4	0.25
Ro 17-2301	16	4

[a] Results in parenthesis are those obtained after 5 days of incubation.

RESULTS

The susceptibility of IC 5/21 to a variety of antibiotics is shown in Table 1. Disk testing, in addition, showed synergy between potassium clavulanate and both ampicillin and cefotaxime. The enzyme kinetics data are shown in Table 2. Extracts from *Capnocytophaga* sp. strain IC 5/21 produced by sonication or lysis in Triton X-100 with or without overnight dialysis all showed the same pattern of activity. There was cephalosporinase activity but no detectable penicillinase activity. It was noted that penicillinase activity could be detected when whole cells were crushed onto indicator paper containing penicillin, starch, and iodine. Hydrolysis of penicillin and ampicillin was easily demonstrated by extracts of standard TEM-1-producing strains by

TABLE 2. Hydrolysis of β-lactam antibiotics by cell extract of *Capnocytophaga* sp. strain IC 5/21

Antibiotic	Relative hydrolysis rate[a]	Relative V_{max}[b]	K_m[c]	Relative V_{max}/K_m[b]
Cephaloridine	100	100	47	100
Cephalothin	32	53	23	107.9
Cefotaxime	21	46	560	3.8
Penicillins[d]	<6	ND[e]	ND	ND

[a] Percentage of rate relative to cephaloridine (100%) with 0.1 mM substrate and an enzyme specific activity of 0.1085 μmol/min per 10^8 cells.
[b] Percent relative to cephaloridine (100%).
[c] K_m in μmol.
[d] Benzylpenicillin, ampicillin, carbenicillin, and methicillin.
[e] ND, Not detected.

TABLE 3. Inhibition of a β-lactamase activity from *Capnocytophaga* sp. strain IC 5/21

Antimicrobial agent	Concn	Time of preincubation (min)	% Inhibition[a]
p-Chloromercuribenzoate	1 mM	20	94.8
Cloxacillin	2 mM	5	89.5
NaCl	1–10 mM	20	0
Clavulanate	4 μg/ml (0.006 mM)	15	98.1

[a] By comparison with a control of preincubation of same amount of enzyme with buffer for 20 min.

using the spectrophotometric assay. Cephalosporinase activity was sensitive to inhibition by clavulanate, cloxacillin, and *p*-chloromercuribenzoate but was not influenced by NaCl (1 to 10 mM) (Table 3).

Isoelectric focusing of the capnocytophaga β-lactamase by conventional methods was impossible. The enzyme activity remained at the application site wherever it was located across the pH gradient. The use of Triton X-100 produced cell extracts with good β-lactamase activity, and by incorporating Triton X-100 at 2% into an agarose gel, the β-lactamase could be focused into a major band corresponding to a pI of 3.6 to 3.75 and a very minor band at pI 4.1 to 4.2. The extract from 3×10^8 cells was the minimum necessary for a band to be stained by the nitrocefin. Focusing took 2,500 V · h and was reproducible; the best results were achieved with fresh extracts. Even after many modifications, it was not possible to focus the enzyme by using a 2% Triton X-100 polyacrylamide gel.

TEM-1 and the capnocytophaga β-lactamase were successfully run on 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the enzyme activity could be visualized to give a molecular size for TEM-1 of 28,000 daltons and for the capnocytophaga β-lactamase of 38,000 daltons. A single band was seen for the capnocytophaga enzyme.

Plasmid analysis showed IC 5/21 to contain a single plasmid of 9.0 kilobases. All attempts at transfer of the plasmid by using transformation and conjugation were unsuccessful.

DISCUSSION

There have been few studies of the mechanism of resistance to antibiotics in bacteria of the genus *Capnocytophaga*. A number of susceptibility surveys (7, 14, 23, 24) have shown that such strains are resistant to aminoglycosides, trimethoprim, polymyxin, and vancomycin. Occasional strains of capnocytophaga have been reported to be resistant to erythromycin (7), chloramphenicol, clindamycin, and tetracycline (10). The strain described by Guiney and Davis (10) contained a conjugative plasmid (pGD10) which encoded chloramphenicol, streptomycin, and tetracycline resistance. This plasmid did not encode ampicillin resistance; the β-lactamase produced by the bacterium was presumed to be chromosomally encoded. Another report of β-lactamase production by Kinder and colleagues (16) identified seven strains, all of which were positive for β-lactamase production by the nitrocefin test. Arlet et al. (1) have also reported a β-lactamase-positive strain which produces an enzyme with little activity against expanded-spectrum cephalosporins. None of these groups attempted a full characterization of these β-lactamases.

The substrate profile (cephalosporinase), molecular weight, pI, and susceptibility to clavulanic acid and *p*-chloromercuribenzoate suggest that a β-lactamase from IC 5/21 may belong to the Richmond and Sykes class 1c (22), or as classified by Bush, as 2e (4–6), as, for example, are some of the *Bacteroides* spp. β-lactamases (27, 29). The minor band on the isoelectric focusing gel could represent a second β-lactamase, although the presence of a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis would make it more likely that protein microheterogeneity or denaturation is being seen.

Penicillinase activity was shown in IC 5/21 by crushing cells onto penicillin starch iodine paper and could be inferred from the synergy between ampicillin and clavulanic acid seen on disk testing. No penicillinase activity could, however, be shown in the crude cell extract. This could be due to one of two reasons. The penicillinase activity may occur only in the cellular environment or it may be that the K_m for the penicillin is low and the V_{max} is below the sensitivity of the method, giving very efficient catalysis at low antibiotic concentrations, as has been shown for the class C cephalosporinases (5, 9).

The β-lactamase is solubilized by detergent and therefore hydrophobic and presumably membrane bound. The only previous report of a membrane-associated β-lactamase in a gram-negative bacterium is in *Pseudomonas pseudomallei* (18). The *P. pseudomallei* enzyme differs from ours in its pI and substrate profile and in that it requires a lower concentration of Triton X-100 for isoelectric focusing. It is interesting to speculate on the origins of such a membrane-associated β-lactamase, particularly whether it is a variation of a known enzyme from a gram-negative bacterium or whether it could have been acquired from a gram-positive one.

This enzyme does not appear to be located on the 9.0-kilobase plasmid found in this strain. However, our data are not totally conclusive and further work is required to determine the precise location of the genes. It is an interesting enzyme and of some clinical significance since it confers resistance to expanded-spectrum cephalosporins which are used for empirical treatment of infection in patients likely to be infected with capnocytophaga. A survey of the occurrence of β-lactamases in clinical isolates of capnocytophaga would be of considerable interest.

A capnocytophaga β-lactamase may be one of a new group of β-lactamases associated with the membrane of gram-negative bacteria. There have been reports of β-lactamases that could not be focused by using existing methods (27); perhaps those were similar hydrophobic enzymes. The use of the methods described in this paper may reveal the presence of similar β-lactamases in gram-negative bacteria.

LITERATURE CITED

1. **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.
2. **Avila, P., F. de la Cruz, E. Ward, and J. Grinsted.** 1984. Plasmids containing one inverted repeat of Tn21 can fuse with other plasmids in the presence of Tn21 transposase. Mol. Gen. Genet. **195**:288–293.
3. **Bennett, P. M., J. Heritage, and P. M. Hawkey.** 1986. An ultra-rapid method for the study of antibiotic resistance plasmids. J. Antimicrob. Chemother. **18**:421–424.
4. **Bush, K.** 1989. Classification of β-lactamases: groups 1, 2a, 2b, and 2b′. Antimicrob. Agents Chemother. **33**:264–270.
5. **Bush, K.** 1989. Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob. Agents Chemother. **33**:271–276.
6. **Bush, K., and R. B. Sykes.** 1986. Methodology for the study of

β-lactamases. Antimicrob. Agents Chemother. **30**:6–10.
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. **Forlenza, S. W., M. G. Newman, A. I. Lipsey, S. E. Siegel, and U. Blachman.** 1980. Capnocytophaga sepsis: a newly recognised clinical entity in granulocytopenic patients. Lancet **i**:567–568.
9. **Galleni, M., and J.-M. Frere.** 1988. A survey of the kinetic parameters of class C β-lactamases. Penicillins. Biochem. J. **255**:119–122.
10. **Guiney, D. G., and C. E. Davis.** 1978. Identification of a conjugative R plasmid in *Bacteroides ochraceus* capable of transfer to *Escherichia coli*. Nature (London) **274**:181–182.
11. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning—a practical approach, vol. 1. IRL Press, Oxford.
12. **Hawkey, P. M., P. M. Bennett, and C. A. Hawkey.** 1984. Cryptic plasmids in hospital isolates of *Providencia stuartii*. J. Med. Microbiol. **18**:177–284.
13. **Hawkey, P. M., H. Malnick, S. Glover, N. Cook, and J. A. Watts.** 1984. *Capnocytophaga ochracea* infection: two cases and a review of published work. J. Clin. Pathol. **37**:1066–1070.
14. **Hawkey, P. M., S. D. Smith, J. Haynes, H. Malnick, and S. W. Forlenza.** 1987. In vitro susceptibility of *Capnocytophaga* species to antimicrobial agents. Antimicrob. Agents Chemother. **31**:331–332.
15. **Holt, H. A., and D. F. J. Brown.** 1989. Antimicrobial susceptibility testing, p. 170–180. *In* P. M. Hawkey, and D. A. Lewis (ed.), Medical bacteriology—a practical approach. Oxford University Press, Oxford.
16. **Kinder, S. A., S. C. Holt, and K. S. Korman.** 1986. Penicillin resistance in the subgingival microbiota associated with adult periodontitis. J. Clin. Microbiol. **23**:1127–1133.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Livermore, D. M., P. Y. Chau, A. I. W. Wong, and Y. K. Leung.** 1987. β-Lactamase of *Pseudomonas pseudomallei* and its contribution to antibiotic resistance. J. Antimicrob. Chemother. **20**:313–321.
19. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88**:169–178.
20. **O'Callaghan, C. H., P. W. Muggleton, and G. W. Ross.** 1969. Effects of β-lactamase from gram-negative organisms on cephalosporins and penicillins, p. 57–63. Antimicrob. Agents Chemother. 1968.
21. **Parenti, D. M., and D. R. Snydman.** 1985. *Capnocytophaga* species: infection in non-immunocompromised and immunocompromised hosts. J. Infect. Dis. **151**:140–147.
22. **Richmond, M. H., and R. B. Sykes.** 1973. The β-lactamase of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. **9**:31–38.
23. **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.
24. **Samuni, A.** 1975. A direct spectrophotometric assay and determination of Michaelis constants for the β-lactamase reaction. Anal. Biochem. **63**:17–26.
25. **Sutter, V. L., D. Pyeatt, and Y. Y. Kwok.** 1981. In vitro susceptibility of *Capnocytophaga* strains to 18 antimicrobial agents. Antimicrob. Agents Chemother. **20**:270–271.
26. **Tai, P. C., N. Zyk, and N. Citri.** 1985. In situ detection of β-lactamase activity in sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. **144**:199–203.
27. **Timewell, R., E. Taylor, and I. Phillips.** 1981. The β-lactamases of *Bacteroides* species. J. Antimicrob. Chemother. **7**:137–146.
28. **Vecoli, C., F. E. Prevost, J. J. Ververis, A. A. Medeiros, and G. P. O'Leary, Jr.** 1983. Comparison of polyacrylamide and agarose gel thin-layer isoelectric focusing for the characterization of β-lactamases. Antimicrob. Agents Chemother. **24**:186–189.
29. **Yotsuji, A., S. Minami, M. Inoue, and S. Mitsuhashi.** 1983. Properties of novel β-lactamase produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **24**:925–929.