ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1996, p. 1014–1016 Vol. 40, No. 4
0066-4804/96/$04.00+0


# Oligonucleotide Probes for Detection of Cephalosporinases among *Bacteroides* Strains

PAOLA MASTRANTONIO,* RITA CARDINES, AND PATRIZIA SPIGAGLIA

*Department of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Rome, Italy*

Received 21 August 1995/Returned for modification 17 November 1995/Accepted 12 January 1996

**Two oligonucleotide probes selected from the sequences of *cepA* and *cfxA* genes, respectively, were used to detect β-lactamase production among strains of the *Bacteroides fragilis* group. By using these probes, colony hybridization was shown to be a specific and rapid method for identifying the more prevalent β-lactamase, CepA, and the rarer CfxA enzyme among *B. fragilis* strains.**

The most common mechanism of resistance to beta-lactams among anaerobes is the production of β-lactamase enzymes (19). Up to now, at least five types of β-lactamase enzymes have been described in the *Bacteroides fragilis* group (1). Most of the enzymes are cephalosporinases, which hydrolyze cephalosporins more rapidly than penicillins. They are divided according to their susceptibilities to clavulanic acid. Penicillinases inhibited by clavulanic acid, penicillinases not inhibited by clavulanic acid, and cloxacillin-hydrolyzing enzymes have been isolated more rarely (17, 20, 21). Moreover, a very limited number of *B. fragilis* strains has been shown to produce a class B $Zn^{2+}$-requiring β-lactamase (3).

The distribution of β-lactamases in *Bacteroides* spp. has been studied mainly by isoelectric focusing and determination of enzymatic substrate profiles and the effects of inhibitors (4, 7, 14, 20). By these methods, a previous study showed a prevalence of 2e caphalosporinases among *B. fragilis* strains isolated from patients in hospitals in Italy (10). This was in agreement with the results obtained by others (2, 5, 12), but a periodic surveillance of the circulation of these enzymes is necessary to detect the emergence of new β-lactamases. To overcome the difficulties linked to the use of isoelectric focusing and substrate profile determinations on a large scale, we used an oligonucleotide probe specific for the detection of the recently sequenced gene *cepA* (16). This gene has been shown to encode the endogenous enzyme belonging to group 2e of the Bush classification scheme. Moreover, to detect the presence of the recently described β-lactamase CfxA capable of cefoxitin degradation (13), an oligonucleotide from the gene sequence was also synthesized. By using clinical isolates of the *B. fragilis* group, the results obtained by the DNA hybridization method were compared with the results obtained by conventional tests.

**Bacterial strains and susceptibility method.** Eighty-seven strains of *B. fragilis*, 8 strains of *B. vulgatus*, 7 strains of *B. distasonis*, and 4 strains of *B. thetaiotaomicron* obtained from clinical isolates were identified with commercial kits (Rapid ID 32A; Biomerieux, Marcy l'Etoile, France). Five *Bacteroides* reference strains (*B. fragilis* ATCC 25285, *B. vulgatus* ATCC 8482, *B. thetaiotaomicron* ATCC 29741, *B. ovatus* ATCC 8483, and *B. distasonis* ATCC 8503) were also used. *Escherichia coli* HB101(pBR322), *E. coli* J53-2(p453), and *E. coli* J53-2(puD18) producing TEM-1, SHV-1, and SHV-3 β-lactamases, respectively, were used to test the specificities of the probes. Patterns of susceptibility to ampicillin, cefoxitin, amoxicillin-clavulanic acid, and imipenem were determined by the E test (AB Biodisk, Solna, Sweden) by using Wilkins-Chalgren agar (Oxoid, Basingstoke, United Kingdom). The breakpoints were 4, 32, 8 and 4, and 8 μg/ml, respectively, as recommended by the National Committee for Clinical Laboratory Standards (15). The inoculum density was adjusted to a 0.5 McFarland turbidity standard. The MICs were read after 24 h of incubation at 37°C under anaerobic conditions.

**β-Lactamase characterization.** Bacteria grown on Columbia blood agar (Oxoid) supplemented with 0.1% hemin, 0.1% vitamin K, and 5% yeast extract were suspended in 4 ml 0.02 M phosphate buffer (pH 7) and were subjected to three 30-s burst of sonication on ice. The extracts were centrifuged at 27,000 × *g* for 30 min at 4°C. The presence of β-lactamase was determined in crude extracts by hydrolysis of the chromogenic cephalosporin PADAC (0.16 mg/ml; Calbiochem, La Jolla, Calif.) (18). Isoelectric focusing of crude β-lactamase extracts was carried out as described by Matthew et al. (11) by using a pH range of 3.5 to 9.5. The β-lactamase bands were visualized by flooding the gel with PADAC. The molecular masses of the β-lactamases were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of crude extracts by using 12% acrylamide Mini Protean II ready gels (Bio-Rad Laboratories, Richmond, Calif.). β-lactamase activity was detected after renaturing the proteins as described by Foweraker et al. (6). Activity was detected by overlaying the gels with PADAC.

**Oligonucleotide hybridization.** Two sequences were selected for the production of the oligonucleotide probes. The first sequence (On1) was selected from positions 880 to 900 in the coding region of the *cepA* gene (5′-A-T-A-A-G-T-A-T-C-T-T-C-A-T-T-C-A-T-T-G-3′) (16). The second sequence (On2) was selected from positions 635 to 655 in the major open reading frame of the *cfxA* gene (5′-C-G-C-T-C-A-A-A-C-A-G-A-T-A-G-T-T-T-T-A-T-3′) (13). Both sequences were chosen to exclude the highly conservative regions shared with the other class A β-lactamases. The oligonucleotides were synthesized on an Applied Biosystem apparatus. Dephosphorylated oligonucleotides were labeled with T4 polynucleotide kinase (New England Biolabs, Beverley, Mass.) and [γ-$^{32}$P]ATP at 37°C for 30 min. Nylon membranes (Amersham, Arlington Heights, Ill.) were placed over colonies of different bacteria grown on a blood agar plate. After 15 min the filter was removed from plates, placed colony side up on filter paper with lysing solution (0.5 M NaOH, 1.5 M NaCl) for 20 min, treated with neutralization solution (1 M Tris [pH 8], 1.5 M NaCl) for

* Corresponding author. Mailing address: Department of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. Phone: 39-6-49902335. Fax: 39-6-491723.

TABLE 1. Characteristics of β-lactamases produced by 110 *Bacteroides* strains and percentage of hybridization with the oligonucleotide probe

| Strain (antibiotic phenotype)[a] | No. of isolates | No. of β-lactamase producers | Physicochemical characteristics of the enzyme | | % Hybridization with probe CepA |
|---|---|---|---|---|---|
| | | | pI range | Mol wt range | |
| *B. fragilis* (R) | 80 | 80 | 4.9 | 32,000[b] | 100 |
| *B. fragilis* (S) | 7 | 0 | | | 0 |
| *B. vulgatus* (R) | 7 | 7 | 4.0–5.1 | 29,000–40,000 | 14 |
| *B. vulgatus* (S) | 2 | 0 | | | 0 |
| *B. distasonis* (R) | 7 | 7 | 4.3–5.1 | 27,000–48,000 | 28 |
| *B. distasonis* (S) | 1 | 0 | | | 0 |
| *B. thetaiotaomicron* (R) | 5 | 5 | 4.2–4.9 | 30,000–32,000 | 20 |
| *B. ovatus* (R) | 1 | 1 | 5.1 | 32,000 | 0 |

[a] R, strains resistant to ampicillin (breakpoint, 4 μg/ml); S, susceptible strains.
[b] One strain, which was also resistant to cefoxitin (breakpoint, 32 μg/ml), showed a second band of 39,000 and hybridized also to the probe CfxA.

20 min, and air dried (9). The solution for prehybridization and hybridization consisted of 6 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% SDS, and 100 μg of sonicated salmon sperm DNA per ml (8). The filters were preincubated for 2 h at 40°C prior to the addition of the labeled probe and were then incubated overnight at 40°C. After hybridization, the filters were washed once in 2 × SSC for 30 min at the same temperature and once in 0.1 × SSC–0.1% SDS for 30 min at the same temperature. After air drying, the filters were exposed to X-ray film at −70°C in the presence of an intensifying screen. The film was developed after 16 to 20 hours. Strong hybridization, as indicated by a distinct darkening of the film, was considered a positive result and was always obtained with the reference strain *B. fragilis* ATCC 25285, which was used as a control. The absence of darkening, as obtained with the control strains *E. coli* ATCC 25922 and *B. distasonis* ATCC 8503, was considered a negative result.

Analysis of the results obtained by the E test showed incidences of resistance to ampicillin of *B. fragilis* and *B. fragilis* group strains of 92 and 87%, respectively. All isolates were susceptible to imipenem and amoxicillin-clavulanate, and all but one *B. fragilis* strain was susceptible to cefoxitin. This last strain was highly resistant to this antibiotic (MIC, ≥128 μg/ml). A detectable amount of β-lactamase by the PADAC test was produced by all of the resistant strains (80 *B. fragilis* strains and 20 strains belonging to the *B. fragilis* group). The crude extracts of β-lactamases were characterized by analytical isoelectric focusing and molecular weight (Table 1). The range of the isoelectric points obtained was between 4.0 and 5.1, and the molecular weights of the enzymes ranged from 32,000 to 39,000. All resistant *B. fragilis* strains, including the reference strain *B. fragilis* ATCC 25285, produced a β-lactamase with a pI of 4.9 and a molecular weight of 32,000. The strain also resistant to cefoxitin also showed by SDS-PAGE a second band with a molecular weight of 39,000. All ampicillin-resistant *B. fragilis* strains hybridized with the probe On1 obtained from the sequence of the *cepA* gene (Table 1). No susceptible strain had positive results, suggesting the absence of a silent gene in the chromosome. The only *B. fragilis* strain resistant to cefoxitin also hybridized to probe On2, obtained from the sequence of the *cfxA* gene. These results are in agreement with the physicochemical characteristics of the β-lactamases produced by the *B. fragilis* strains tested, suggesting a specificity of the probe On1 in detecting the 2e cephalosporinase with a pI of 4.9 and a molecular weight of 32,000 and the specificity of the probe On2 in detecting the rarer CfxA enzyme. *E. coli* strains with β-lactamases different from the CepA or CfxA enzymes did not hybridize to our probes. As far as strains belonging to the *B. fragilis* group are concerned, probe On1 hybridized only to 20% of the resistant strains. The β-lactamases produced by these strains showed a pI of 4.9 and a molecular weight of 32,000, as was the case for the typical 2e cephalosporinase produced by *B. fragilis* strains, CepA. All the other resistant strains, which were negative by the colony hybridization assay, produced β-lactamase enzymes with pIs of 4.0, 4.2, 4.5, and 5.1 and molecular weights ranging between 27,000 and 48,000. In particular, each of 10 of these strains had a β-lactamase with molecular weight of 32,000, which was the same as that of the CepA enzyme, but the β-lactamases had different pIs. The percentage of strains hybridizing with the On1 probe suggests a lower level of circulation of the CepA enzyme in the *B. fragilis* group compared with that among *B. fragilis* species. The following *Bacteroides* reference strains did not hybridize to probes On1 and On2: *B. vulgatus* ATCC 8482 and *B. distasonis* ATCC 8503 were susceptible strains, and *B. ovatus* ATCC 8483 and *B. thetaiotaomicron* ATCC 29741 produced β-lactamases each with a molecular weight of 32,000 and pIs of 5.1 and 4.5, respectively. As shown in a previous work (16), they had no sequences that hybridized to the probe specific for CepA.

Until now, the circulation and the prevalence of the β-lactamases in *Bacteroides* spp. have mainly been studied by conventional methods (4, 7, 20). However, by this approach only a limited number of clinical isolates may be tested each time, and information about the molecular correlation of enzymes is missing. The colony hybridization assay showed a satisfactory specificity of the oligonucleotide On1 in detecting the widespread 2e cephalosporinase of *B. fragilis*: the CepA enzyme. The use of the oligonucleotide On2 was useful for demonstrating the parallel production of the two β-lactamases (CepA and CfxA) in the only *B. fragilis* strain that we found that was also resistant to cefoxitin. Furthermore, the molecular method is able to detect genes encoding enzymes even when they are phenotypically masked by the presence of another β-lactamase.

This research was partially supported by a grant (grant 93.01113.70) from C.N.R. Target Project "Biotechnology and Bioinstrumentation."

## REFERENCES

1. **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.
2. **Cornick, N. A., G. J. Cuchural, D. R. Snydman, N. V. Jacobus, P. Iannini, G. Hill, T. Cleary, J. P. O'Keefe, C. Pierson, and S. M. Finegold.** 1990. The antimicrobial susceptibility patterns of the *Bacteroides fragilis* group in the United States, 1987. J. Antimicrob. Chemother. **25:**1011–1019.
3. **Cuchural, G. J., Jr., M. H. Malamy, and F. P. Tally.** 1986. β-Lactamase-mediated imipenem resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **30:**645–648.

4. **Edwards, R., and D. Greenwood.** 1992. An investigation of β-lactamases from clinical isolates of Bacteroides species. J. Med. Microbiol. **36:**89–95.
5. **Eley, A., and D. Greenwood.** 1986. Characterization of β-lactamases in clinical isolates of Bacteroides. J. Antimicrob. Chemother. **18:**325–333.
6. **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.
7. **Leung, T., and J. D. Williams.** 1978. β-Lactamases of subspecies of *Bacteroides fragilis*. J. Antimicrob. Chemother. **4:**47–54.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Mass, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10:**296–298.
10. **Mastrantonio, P., P. Spigaglia, and A. Sebastianelli.** 1994. Susceptibility patterns and characterization of β-lactamases in clinical isolates of *Bacteroides fragilis*. Eur. J. Clin. Microbiol. Infect. Dis. **13:**475–480.
11. **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.
12. **Nord, C. E., and B. Olsson-Liljequist.** 1981. Resistance to β-lactam antibiotics in Bacteroides species. J. Antimicrob. Chemother. **8:**33–42.
13. **Parker, A. C., and C. J. Smith.** 1993. Genetic and biochemical analysis of novel Ambler class A β-lactamase responsible for cefoxitin resistance in *Bacteroides* species. Antimicrob. Agents Chemother. **37:**1028–1036.
14. **Rasmussen, B. A., K. Bush, and F. P. Tally.** 1993. Antimicrobial resistance in Bacteroides. Clin. Infect. Dis. **16**(Suppl. 4)**:**S390–S400.
15. **Rodloff, A. C., P. C. Appelbaum, and R. J. Zabransky.** 1991. Cumitech 5A, Practical anaerobic bacteriology. Coordinating ed., A. C. Rodloff. American Society for Microbiology, Washington, D.C.
16. **Rogers, M. B., A. C. Parker, and C. J. Smith.** 1993. Cloning and characterization of the endogenous cepholosporinase gene, *cepA*, from *Bacteroides fragilis* reveals a new subgroup of Ambler class A β-lactamases. Antimicrob. Agents Chemother. **37:**2391–2400.
17. **Sato, K., Y. Matsuura, M. Inoue, and S. Mitsuhoshi.** 1982. Properties of a new penicillinase type produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **22:**579–584.
18. **Schindler, P., and G. Huber.** 1990. Use of PADAC, a novel chromogenic β-lactamase substrate for the detection of β-lactamase producing organisms and assays of β-lactamase inhibitors/inactivators, p. 169–176. *In* V. Brodbeck (ed.), Enzyme inhibitors. Verlag Chemie, Weinheim, Germany.
19. **Tally, F. P., and G. J. Cuchural, Jr.** 1988. Antibiotic resistance in anaerobic bacteria. J. Antimicrob. Chemother. **22:**63–71.
20. **Timewell, R., E. Taylor, and I. Phillips.** 1981. The β-lactamases of Bacteroides species. J. Antimicrob. Chemother. **7:**137–146.
21. **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.