ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1985, p. 943–947
0066-4804/85/060943-05$02.00/0

Vol. 27, No. 6

# Purification and Properties of a Novel β-Lactamase from *Fusobacterium nucleatum*

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Received 15 November 1984/Accepted 25 March 1985

**A strain of *Fusobacterium nucleatum* which produced high levels of beta-lactamase was isolated. The specific activity of the unpurified beta-lactamase was 7.8 U/mg of protein. By Sephacryl S-300 and S-200 column passage and chromatofocusing, the enzyme was purified 450-fold. Sodium dodecyl sulfate-gradient gel electrophoresis revealed a single band. The enzyme hydrolyzed phenoxymethylpenicillin, benzylpenicillin, and ampicillin more rapidly than carbenicillin and piperacillin. Cephaloridine, cefaclor, cephalothin, imipenem, and SCH 34343 had hydrolysis rates of ≤1% that of phenoxymethylpenicillin. The enzyme was inhibited by clavulanic acid and RO-15-1903/001 but not by *p*-mercuribenzoate or cefoxitin. Molecular weight by gel filtration was determined to be 21,000 and by sodium dodecyl sulfate-gradient gel electrophoresis was determined to be 26,000. The amino acids aspartic acid-asparagine, glutamic acid-glutamine, serine, glycine, and lysine dominated the amino acid composition.**

Strains of *Fusobacterium nucleatum* are frequently recovered from infections in the oral cavity and pharynx. Most *F. nucleatum* strains have been considered to be susceptible to penicillins (5). However, we recently found seven *F. nucleatum* strains isolated from tonsillar crypts that were highly resistant to penicillins (K. Tunér, L. Lindqvist, and C. E. Nord, J. Antimicrob. Chemother., in press). This resistance was caused by beta-lactamase production. The aim of this investigation was to purify the beta-lactamase from a strain of *F. nucleatum* (F21) and to study the properties of the enzyme.

## MATERIALS AND METHODS

**Bacterial strain.** *F. nucleatum* F21, a clinical strain obtained from the Otolaryngology Department of Huddinge University Hospital, Huddinge, Sweden, was used. The strain was identified by Gram staining, biochemical tests, and gas-liquid chromatographic analysis by the method of Holdeman et al. (6). Beta-lactamase production was detected with nitrocefin by the method of Kammer et al. (7).

**Production of beta-lactamase.** Cultures in 6 liters of prereduced peptone yeast extract broth were harvested in the late-log phase (20 h). The cells were centrifuged (Sorvall SR5; Du Pont Co., Newton, Conn.) at 10,000 × *g* for 30 min, washed twice in sodium phosphate buffer (10 mM, pH 7.0) at 4°C, and suspended in 1/100 the original volume of the same buffer. The cells were then disrupted in an ultrasonicator for 4 min (Branson B15; Branson Sonic Power Co., Heusenstamm, West Germany) in an ice bath. Cell debris was removed by centrifugation at 13,000 × *g* for 45 min at 4°C, and the supernatant obtained was used for purification.

**Determination of beta-lactamase activity.** Quantitative determination of beta-lactamase activity was assayed spectrophotometrically by the method of O'Callaghan et al. with nitrocefin (100 μM) in 0.05 M sodium phosphate buffer (pH 7.0, 30°C) as the substrate (11). One unit of beta-lactamase was defined as the amount which formed 1.0 μmol of product per min under these conditions.

**Determination of protein.** The protein content was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard. The protein concentrations in samples from the last purification step were calculated from the amino acid analysis.

**Purification of beta-lactamase. (i) Step 1.** The unpurified preparation was passed through a Sephacryl S-300 column (5.0 by 71 cm) at 4°C with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.10 M NaCl as the eluent. The fractions containing beta-lactamase activities were collected and concentrated to 7.5 ml by ultrafiltration (Pellicon Membrane Disc; nominal molecular weight exclusion limit, 10,000; Millipore Corp., Bedford, Mass.).

**(ii) Step 2.** The beta-lactamase active sample from step 1 was applied to a Sephacryl S-200 column (2.6 by 30 cm) with the same conditions as those described for step 1. The fractions containing beta-lactamase were pooled and concentrated to 6.5 ml by ultrafiltration.

**(iii) Step 3.** For chromatofocusing experiments, the solvent was changed to piperazine-HCl buffer (25 mM, pH 6.8) by gel filtration on Sephadex G-25 columns (PD 10). This last purification step was carried out with a MonoP (Pharmacia) prepacked HR 5/20 column by the Pharmacia fast protein liquid chromatography system. The column was preequilibrated with piperazine-HCl buffer (25 mM, pH 6.3), and the pH gradient was developed by elution with Polybuffer-74 (10% [vol/vol], pH 4.5). The most active fractions (1.0 ml each) from this step were either freeze-dried (after extensive dialysis against deionized water) or stored at −70°C for later use.

**Substrate profile.** For kinetic experiments, purified enzyme diluted in 0.05 M sodium phosphate buffer (pH 7.0) containing 1% gelatin was used. To determine $K_m$ for the antibiotics, the hydrolysis rate of nitrocefin was measured with and without the antibiotics (1). The antibiotic concentration was varied in the following ratios of antibiotic to nitrocefin: 0:1, 1:1, 2:1, 3:1, 4:1, and 5:1, each antibiotic together with nitrocefin at six different concentrations between 8 and 100 μM.

For the estimation of $V_{max}$, the activity of the enzyme was assayed by a direct spectrophotometric method based on the difference in light absorption ($\Delta\epsilon$) between substrate and

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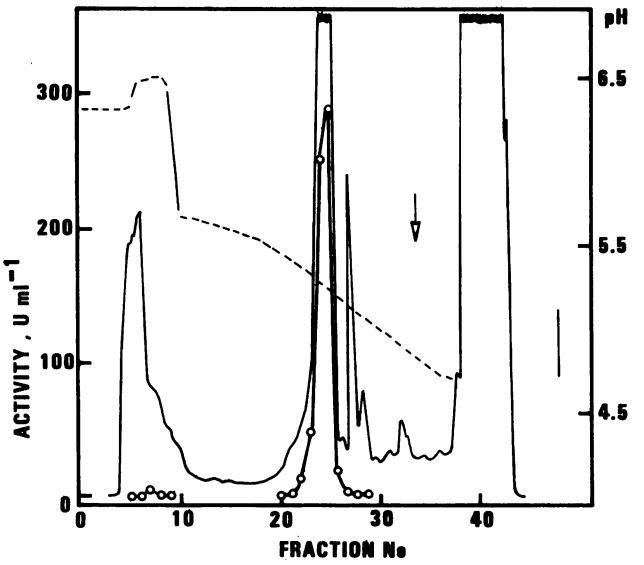


FIG. 1. Last step in purification of beta-lactamase by chromatofocusing on MonoP. One-fifth of the material from the second gel filtration (1.5 ml) was applied. The pH gradient was formed with polybuffer-74 (pH 4.5), and the effluent was collected in 1.0-ml fractions (flow rate, 0.5 ml/min). The arrow marks the addition of acetic acid (2 ml, 70%) to the column. Beta-lactamase activity was determined with nitrocefin as the substrate and is expressed in units per milliliter (○). The bar represents 0.02 absorbance unit at 280 nm (——) and pH = (– – –).

product at a specified wavelength (3, 14, 20). The $\Delta\epsilon$'s for cephaloridine (wavelength, 260 nm), SCH 34343 (322 nm), and cefaclor (265 nm) were determined to be 10.0, 4.3, and 7.2 $mM^{-1}$ $cm^{-1}$, respectively. $V_{max}$ was calculated from the Henri-Michaelis-Menten equation by using the initial rates obtained from hydrolyses of ampicillin, benzylpenicillin, and phenoxymethylpenicillin at final concentrations of 500 μM, piperacillin at 200 μM, carbenicillin at 400 μM, and cephalothin, cefaclor, cephaloridine, imipenem, and SCH 34343 at 100 μM. The relative maximal hydrolysis rate was obtained by setting $V_{max}$ for benzylpenicillin to 100. The turnover number ($k_3$) was calculated from $V_{max}$ assuming a molecular weight of 26,000.

**Inhibition studies.** Inhibition of beta-lactamase activity was determined with nitrocefin as the substrate.

The $K_i$ values were determined without preincubation of inhibitor-enzyme. In contrast, to investigate the binding degree of clavulanic acid, RO-15-1903/001 (RO-15) and *p*-mercuribenzoate, various amounts of enzyme were preincubated together with the inhibitor for 15 min at 30°C before activity determination.

**Determination of molecular weight.** Sephacryl S-200 was used to determine the molecular weight of the purified enzyme by gel filtration chromatography.

**SDS-gradient gel electrophoresis.** The sample was dissolved in 10 mM Tris-hydrochloride (pH 8.0) with 1 mM EDTA, 5% beta-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) and then heated for 6 min at 100°C. Electrophoresis was carried out in 0.04 M Tris–0.02 M sodium acetate–2 mM EDTA–0.2% SDS (pH 7.4) with commercially obtained gradient gels PAA 4/30.

**Determination of amino acid composition.** The amino acid composition of the purified freeze-dried enzyme was determined in a Stein-Moore amino acid analyzer at the Central Amino Acid Analysis Laboratory, University of Uppsala, Sweden.

**Chemicals.** Sephacryl S-300, Sephacryl S-200, Sephadex G-25, Polybuffer-74, and the polyacrylamide gradient gels PAA 4/30 were purchased from Pharmacia, Uppsala, Sweden; and molecular weight markers were from Sigma Chemical Co., St. Louis, Mo., and from Pharmacia. Benzylpenicillin, phenoxymethylpenicillin, ampicillin, carbenicillin, clavulanic acid, and cloxacillin were obtained from Astra, Södertälje, Sweden. Piperacillin was received from Lederle, Wayne, N.J., and cefoxitin and imipenem were from Merck Sharp & Dohme, Rahway, N.J. Cephalothin, cefaclor, and moxalactam were from Lilly, Fegersheim, France; RO-15 was from Hoffmann-La Roche, Basel, Switzerland; cephaloridine and nitrocefin were from Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom; and sulbactam was from Pfizer Inc., Groton, Conn. Constituents for buffers were obtained from Merck AG, Darmstadt, Federal Republic of Germany.

## RESULTS

**Beta-lactamase formation.** Qualitative detection of beta-lactamase with nitrocefin revealed that the strain was a high producer of beta-lactamase. In the supernatant of disrupted cells, the specific activity of the beta-lactamase was 7.8 U/mg. Approximately 80% of the beta-lactamase produced was found intracellular, and the rest was found extracellular. The production of beta-lactamase was constant when

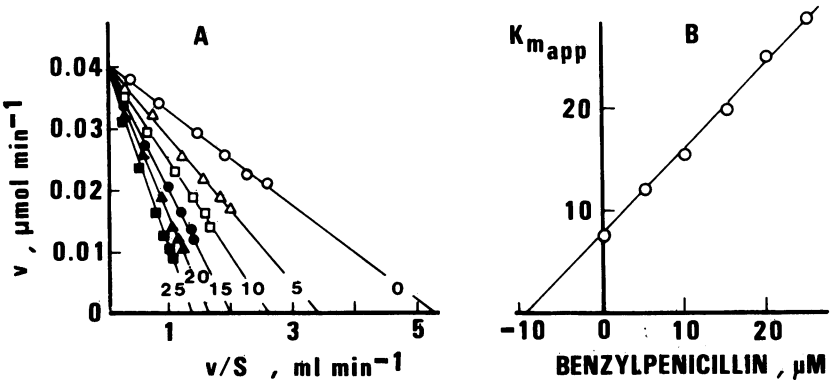


FIG. 2. Inhibition of beta-lactamase activity (substrate [S], nitrocefin) by benzylpenicillin. (A) Woolf-Augustinsson-Hofstee plot with benzylpenicillin concentration (0 to 25 μM) varied as marked. (B) Replot of the apparent $K_m$ value for nitrocefin (micromolar) obtained from the plot in (A) (slope = negative apparent $K_m$ [$K_{mapp}$]) versus the concentration of benzylpenicillin. Intercept on the concentration axis = $-K_m$ for the antibiotic. The final enzyme concentration was 0.14 nM.

TABLE 1. Kinetic parameters of the purified β-lactamase from *F. nucleatum* F-21 with different β-lactam antibiotics and the chromogenic substrate nitrocefin[a]

| Substrate or antibiotic | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $V_{max}$ (relative) | $k_3$ ($s^{-1}$) | $k_3/K_m$ ($\mu M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|
| Phenoxymethylpenicillin | 19 | 16,800 | 480 | 7,280 | 383 |
| Benzylpenicillin | 9.2 | 3,500 | 100 | 1,520 | 165 |
| Ampicillin | 86 | 14,700 | 420 | 6,370 | 74 |
| Piperacillin | 21 | 1,340 | 38.3 | 581 | 28 |
| Carbenicillin | 135 | 1,760 | 50.3 | 763 | 5.7 |
| Cloxacillin | 390 | ND | | | |
| Cephalothin | 6.1 | 8.7 | 0.25 | 3.8 | 0.63 |
| Cefaclor | 600 | 109 | 3.11 | 47 | 0.08 |
| Cefaloridine | 3,500 | 173 | 4.94 | 75 | 0.02 |
| Imipenem | 4.4 | 0.90 | 0.026 | 0.39 | 0.09 |
| SCH 34343 | 2.4 | 0.29 | 0.008 | 0.13 | 0.05 |
| Nitrocefin | 8.0 | 3,820 | 109 | 1,660 | 207 |

[a] The activity with each substrate was determined in 50 mM sodium phosphate buffer (pH 7.0) at 30°C. The final enzyme concentration used for determination of $V_{max}$ was 0.43 nM (phenoxymethylpenicillin, benzylpenicillin, and ampicillin), 1.3 nM (piperacillin and carbenicillin), 26 nM (cephalothin, cefaclor, cephaloridine, and imipenem), and 65 nM (SCH 34343). ND, Not determined.

benzylpenicillin was added to the medium at concentrations from 0.004 μg/ml to the MIC of the strain (256 μg/ml). The strain was highly resistant to phenoxymethylpenicillin (1,024 μg/ml), ampicillin (128 μg/ml), piperacillin (1,024 μg/ml), and cloxacillin (32 μg/ml) but susceptible to cefaclor (1.0 μg/ml), cephaloridine (0.25 μg/ml), imipenem (0.064 μg/ml), and SCH 34343 (0.004 μg/ml).

**Purification.** In step 1, Sephacryl S-300 chromatography, the enzyme was separated from the main high-molecular-weight components. The fractions containing beta-lactamase were further purified by chromatography on Sephacryl S-200 before the final chromatofocusing step (Fig. 1). From steps 1 and 2, the enzyme was purified about 15-fold. The yield (78%) after the chromatofocusing step contained 0.82 mg of homogenous enzyme purified 454-fold.

**Substrate profile.** Figure 2A shows the inhibition of the activity on nitrocefin by benzylpenicillin. A second plot of the $K_m$ obtained for nitrocefin versus antibiotic concentration shows the $K_m$ values for the antibiotics (Fig. 2B). The kinetic parameters for the antibiotics hydrolyzed by the purified beta-lactamase are summarized in Table 1. The maximal rate of hydrolysis of the different beta-lactam antibiotics related to the maximal rate of hydrolysis of benzylpenicillin was in the following order: phenoxymethylpenicillin, 480; ampicillin, 420; benzylpenicillin, 100; carbenicillin, 50.3; piperacillin, 38.3; cephaloridine, 4.94; cefaclor, 3.11; cephalothin, 0.25; imipenem, 0.026; SCH 34343, 0.008. For nitrocefin the relative rate of hydrolysis was 109. $V_{max}$ (micromoles per minute per milligram) and turnover ($k_3$, per second) values as well as the ratio $k_3/K_m$ also are shown in Table 1.

**Inhibition studies.** The inhibitory effects of clavulanic acid, moxalactam, and sulbactam were initially (<1 min) competitive, with $K_i$ values of 0.22, 8.2, and 0.80 mM, respectively. Cefoxitin did not inhibit the enzyme, even at a concentration 1,000 times that of the substrate. When the beta-lactamase (6 nM) was preincubated with clavulanic acid (10 μM) for up to 30 min (30°C), the activity slowly decreased to 15% that of the control (Fig. 3A). With various amounts of enzyme (0.12 to 0.60 pmol) preincubated for 15 min with 1.2 nmol of clavulanic acid, the degree of inhibition varied between 98 and 44% (Fig. 3B). The rate of reaction between the compound RO-15 and beta-lactamase is also shown in Fig. 3A. In the presence of 0.15 μM RO-15 the beta-lactamase lost 100% activity within 15 min. After 15 min of preincubation, 1.8 pmol of RO-15 inactivated 0.16 pmol of beta-lactamase (Fig. 3B). The enzyme activity was not inhibited by *p*-chloromercuribenzoate (1 mM) after a preincubation time of 15 min.

**Molecular weight.** Gel filtration indicated a molecular weight of 21,000. Gradient gel electrophoresis of SDS-treated and reduced beta-lactamase revealed a single band which corresponded to approximately 26,000 (Fig. 4).

**Amino acid composition.** The amino acid composition of the purified beta-lactamase is shown in Table 2. The amino acids aspartic acid-asparagine, glutamic acid-glutamine, serine, glycine, and lysine dominate the composition (60 mol%). The beta-lactamase contained 0.87 mol% half-cystine, the lowest value for the amino acids present. Assuming a

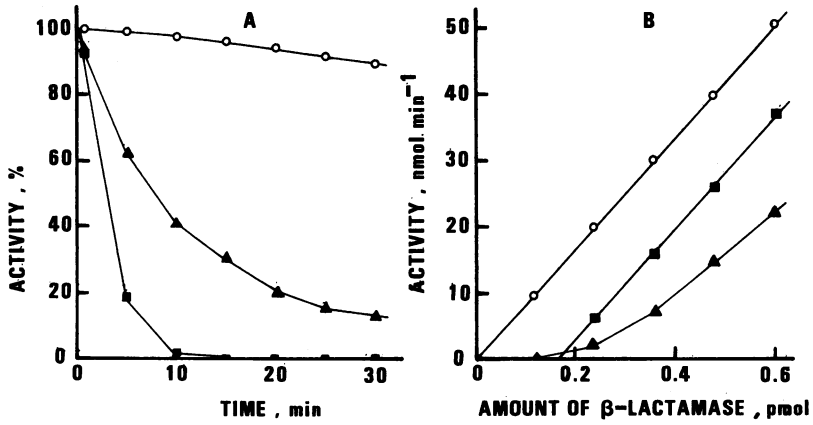


FIG. 3. Effect of clavulanic acid and RO-15 on β-lactamase activity. (A) Rates of β-lactamase (6.0 nM) inactivation by reaction with 10 μM clavulanic acid (▲) and 0.15 μM RO-15 (■) at pH 7.0 and 30°C. Activity of β-lactamase without inhibitor (○). (B) Relationship between activity and amount of β-lactamase without (○) and with (▲) 1.2 nmol of clavulanic acid or 1.8 pmol of RO-15 (■) after preincubation for 15 min at pH 7.0 and 30°C.

molecular weight of 26,000, the beta-lactamase consisted of 239 amino acid residues.

## DISCUSSION

Beta-lactamases from both gram-positive and gram-negative bacteria have been studied extensively during recent years. Beta-lactamases play a major role in the resistance in both aerobic and anaerobic bacteria to beta-lactam antibiotics (10). The properties of beta-lactamases from different bacteria vary to a great extent, which makes investigating these properties very important.

The beta-lactamase from *F. nucleatum* was purified in three steps to homogeneity with a high yield (78%). These favorable results were mainly due to the last step, chromatofocusing. We used this technique as a preparative step in the purification procedure because chromatofocusing is a rapid method for the separation of proteins. In a previous study, chromatofocusing of unpurified beta-lactamases from seven different *F. nucleatum* strains showed elution at a pH of about 5.2, somewhat higher than the pI value of 4.8 obtained by density gradient electrofocusing (Tunér et al., in press).

$V_{max}$ for the different antibiotics revealed that the beta-lactamase from *F. nucleatum* hydrolyzed penicillins much more rapidly than cephalosporins, imipenem, and SCH 34343 when the substrate concentration was higher than $K_m$ (the rate of hydrolysis is equal to $k_3$). However, when the substrate concentration was lower than $K_m$, the hydrolysis rate depended on the ratio $k_3/K_m$ and the substrate concentration.

The inhibition studies indicated that RO-15 and clavulanic acid acted mainly as irreversible inhibitors (8). Both substances were also progressive inhibitors, i.e., a greater effect of the inhibitor was seen with increasing time. Cefoxitin, which is considered to be a powerful beta-lactamase inhibitor (21), did not inhibit the enzyme.

Microorganisms in the oropharynx that produce beta-lactamases mainly belong to the species *Bacteroides melaninogenicus*, *Bacteroides oralis*, and *Bacteroides ruminicola* (19). These strains produce generally smaller amounts of beta-lactamases that are more active on penicillins than on cephalosporins (16, 18). *Bacteroides fragilis*, mainly found in the large intestine and female genital tract, produces cephalosporinases (12, 13, 17). However, Sato et al. recently reported a strain of *B. fragilis* with high penicillinase production (15). In contrast to our findings, the beta-lactamase from the *B. fragilis* strain was inhibited by cefoxitin, and the isoelectric point was less acidic (15) than that of the beta-lactamase from *F. nucleatum*.

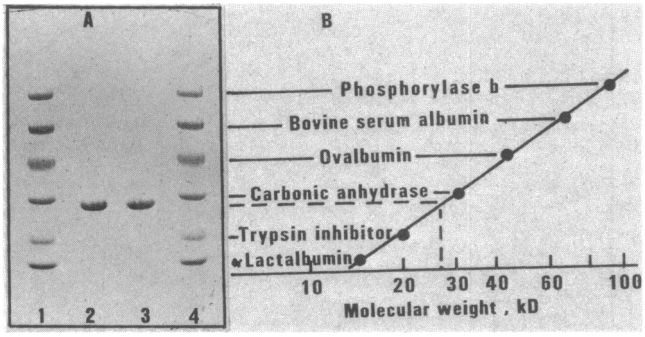


FIG. 4. (A) SDS-polyacrylamide gradient gel electrophoresis of the purified beta-lactamase (1.5 μg) of *F. nucleatum* (lanes 2 and 3) and low-molecular-weight calibration kit proteins (each ca. 3 μg) (lanes 1 and 4). Electrophoresis: 125 V (constant voltage) for 2 h at 12°C. Staining: 18 h (0.02% Coomassie brilliant blue, 7% acetic acid). Destaining: 24 h (30% ethanol, 10% acetic acid). (B) Calibration curve established by plot of migration distance against molecular weight (log scale) for the calibration kit proteins.

TABLE 2. Amino acid composition of the purified β-lactamase from *F. nucleatum* F-21

| Amino acid | Mol% | Residues/26,000[a] (nmol/nmol) |
|---|---|---|
| Asx | 10.60 | 25 |
| Thr | 3.66 | 9 |
| Ser | 11.44 | 27 |
| Glx | 14.22 | 34 |
| Pro | 3.40 | 8 |
| Gly | 12.02 | 29 |
| Ala | 5.37 | 13 |
| Val | 4.07 | 10 |
| Met | 1.36 | 3 |
| Ile | 4.06 | 10 |
| Leu | 5.26 | 13 |
| Tyr | 3.55 | 8 |
| Phe | 3,97 | 10 |
| His | 1.34 | 3 |
| Lys | 10.96 | 26 |
| Arg | 2.62 | 6 |
| Trp | 1.22 | 3 |
| Cys[b] | 0.87 | 2 |

[a] The number of amino acid residues was calculated assuming a molecular weight of 26,000. The total number of residues is 239.
[b] Determined after performic acid oxidations.

The molecular weight of 26,000 of the purified beta-lactamase from *F. nucleatum* corresponds to those of beta-lactamases from gram-negative microorganisms which range from 25,000 to 30,000 (2). The molecular weight obtained by gel filtration was somewhat lower than the molecular weight calculated by gradient gel electrophoresis. Determinations of molecular weights of beta-lactamases by gel filtration methods are reported to give lower values than are those by other techniques (2).

Both cysteine and tryptophan residues were found in the beta-lactamase of *F. nucleatum*, which differentiated this beta-lactamase from that of *Staphylococcus aureus* (4) and from the group I beta-lactamase of *Bacillus cereus* (1). On the basis of the molecular weight of 26,000 the cysteine content corresponds well to two residues per enzyme molecule.

It is evident that this beta-lactamase from *F. nucleatum* does not resemble any other earlier described beta-lactamases. This new penicillinase from *F. nucleatum* may have important clinical implications since *F. nucleatum* is a common pathogen isolated from infections in the oropharynx such as peritonsillitis, Vincent's angina, and Ludwig's angina, for which penicillin is still the drug of choice (5). In treatment failures, the infection might be caused by beta-lactamase-producing *F. nucleatum*. This beta-lactamase may also inactivate the administered penicillin in the treatment of tonsillitis caused by group A streptococci, resulting in therapy failure or recurrence of the infection (19).

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