# Genetic and Biochemical Characterization of FUS-1 (OXA-85), a Narrow-Spectrum Class D β-Lactamase from *Fusobacterium nucleatum* subsp. *polymorphum*

Christine Voha,[1] Jean-Denis Docquier,[2,3] Gian Maria Rossolini,[3] and Thierry Fosse[1]*

*Laboratoire de Bactériologie et Epidémiologie Moléculaire, Université de Nice, Centre Hospitalier Universitaire de Nice, F-06202 Nice, France[1]; Centre d'Ingénierie des Protéines et Laboratoire d'Enzymologie, Université de Liège, B-4000 Sart-Tilman, Belgium[2]; and Dipartimento di Biologia Molecolare, Laboratorio di Fisiologia e Biotecnologia dei Microrganismi, Università di Siena, I-53100 Siena, Italy[3]*

**Previous studies have reported β-lactamase-mediated penicillin resistance in *Fusobacterium nucleatum*, but no β-lactamase gene has yet been identified in this species. An *F. nucleatum* subsp. *polymorphum* strain resistant to penicillin and amoxicillin was isolated from a human periodontitis sample. DNA cloning and sequencing revealed a 765-bp open reading frame encoding a new class D β-lactamase named FUS-1 (OXA-85). A recombinant *Escherichia coli* strain carrying the $bla_{FUS-1}$ gene exhibited resistance to amoxicillin with a moderate decrease in the MICs with clavulanic acid. The $bla_{FUS-1}$ gene was found in two additional clonally unrelated *F. nucleatum* subsp. *polymorphum* isolates. It was located on the chromosome in a peculiar genetic environment where a gene encoding a putative transposase-like protein is found, suggesting a possible acquisition of this class D β-lactamase gene. The FUS-1 enzyme showed the closest ancestral relationship with OXA-63 from *Brachyspira pilosicoli* (53% identity) and with putative chromosomal β-lactamases of *Campylobacter* spp. (40 to 42% identity). FUS-1 presents all of the conserved structural motifs of class D β-lactamases. Kinetic analysis revealed that FUS-1 exhibits a narrow substrate profile, efficiently hydrolyzing benzylpenicillin and oxacillin. FUS-1 was poorly inactivated by clavulanate and NaCl. FUS-1 is the first example of a class D β-lactamase produced by a gram-negative, anaerobic, rod-shaped bacterium to be characterized.**

β-Lactam resistance in most gram-negative anaerobic bacteria is mediated by the production of class A extended-spectrum β-lactamases (group 2be, Bush). Such enzymes have been characterized in the genera *Bacteroides* (28, 30, 31, 33) and *Prevotella* (23) and in *Acidaminococcus fermentans*, a gram-negative coccus (11). In the case of oral cavity infections, the high prevalence of CfxA-like β-lactamase in *Prevotella* sp. and *Capnocytophaga* sp. strains accounted for most of the β-lactam resistance problem (9, 17). *Fusobacterium nucleatum* belongs to a heterogeneous group including five known subspecies and represents one of most prominent anaerobic gram-negative rods within the oral flora to be possibly involved in local or disseminated infections, alone or in mixed infections (6, 7, 8, 12, 15). In the past, it has been usually considered susceptible to penicillins (8). However, this view has been contradicted by a few studies which reported the production of a β-lactamase with penicillinase activity in *F. nucleatum*, although the nature of the β-lactamase-encoding gene(s) was not investigated (2, 15, 20, 29, 37). β-Lactamase-mediated penicillin resistance in *F. nucleatum* isolates from the oral cavity frequently occurs in childhood or in patients who have previously received antimicrobial therapy (25, 26, 38).

Class D β-lactamases (OXA-type enzymes) are characterized by a strong oxacillinase activity but widely differ in their genetic backgrounds and functional features (5). This fast-growing molecular class includes enzymes with narrow-to-broad substrate profiles (extended spectrum), some of which may exhibit carbapenemase activity (e.g., OXA-23, OXA-48), accounting for their increasing clinical relevance (24). Acquired enzymes are the most numerous and are commonly encoded by mobile genetic elements, being integron or plasmid borne, that promote their diffusion among hospital gram-negative pathogens. On the other hand, resident chromosome-encoded class D enzymes have been identified in several organisms that are members of the environmental microbiota (e.g., *Shewanella* spp.), some of which are also important opportunistic pathogens, such as *Aeromonas* spp. (OXA-12), *Pseudomonas aeruginosa* (OXA-50), and *Acinetobacter baumannii* (OXA-51).

Although class D enzymes have been reported in a wide variety of bacterial species, such β-lactamases have not yet been described in gram-negative anaerobic rods. This report describes the cloning and biochemical properties of a novel chromosome-encoded molecular class D β-lactamase, named FUS-1 (OXA-85), from a human periodontally pathogenic *F. nucleatum* clinical strain.

## MATERIALS AND METHODS

**Bacterial strains.** *F. nucleatum* subsp. *polymorphum* NI61 was isolated from a patient with periodontitis who received amoxicillin treatment. Two additional β-lactamase-positive *F. nucleatum* subsp. *polymorphum* strains, NI51 and NI487, were isolated from periodontal pockets of adult periodontitis outpatients from the Dental Department (Teaching University Hospital of Nice, France). The strains were identified with API rapid ID32A (bioMérieux, Marcy l'Etoile, France) and by 16S rRNA gene sequencing. *F. nucleatum* subsp. *nucleatum* ATCC 25586, *F. nucleatum* subsp. *animalis* ATCC 51191, *F. nucleatum* subsp. *fusiforme* ATCC 51190, and *F. nucleatum* subsp. *vincentii* ATCC 49256 were

* Corresponding author. Mailing address: Laboratoire de bactériologie, 151 route de St Antoine de Ginestière, Hôpital Archet, 06202 Nice Cédex 03, France. Phone: (33) 492036212. Fax: (33) 492036549. E-mail: fosse@unice.fr.

TABLE 1. Primers used in this study

| Primer | Sequence[a] | Expected product size (bp) |
|---|---|---|
| Fus-1fwd | 5'-CGAATTCGATAACTCCTATTTTTTATTTTATAAC | 777 |
| Fus-1rev | 5'-GGGATCCTTATTATTTATGTTCTCGATTATTTC | |
| BlaAfwd | 5'-CGAATTCTTATTCAAGACTTTCCTCCATTTTG | 806 |
| BlaArev | 5'-GGGATCCGAAAAATATACAGAATGGAAGAAAG | |
| BlaAintF | 5'-GCCAAATAATACCTCCAT | 366 |
| BlaAintR | 5'-CTTATGATTATTGTAAGTG | |
| FUS-1-F | 5'-GC**CATATG**TTATTATTTATGTTCTCGAT | 778 |
| FUS-1-R | 5'-GC**GGATCC**TTATTTTATAACATTTATATTTTTG | |

[a] Added NdeI and BamHI linkers are in boldface in primers FUS-1-F and FUS-1-R, respectively.

purchased from the strain collection of the Pasteur Institute (Paris, France). *Escherichia coli* TOP10 (Invitrogen, Leek, The Netherlands) was used as the recipient for cloning experiments. *E. coli* BL21(DE3) (Stratagene Inc., La Jolla, Calif.) was used as the host for T7 promoter-based expression plasmids in overexpression experiments and large-scale enzyme production.

**Media, culture conditions, and determination of antimicrobial susceptibility.** *F. nucleatum* strains were grown anaerobically at 37°C in Schaedler broth (Bio-Rad, Marnes-la-Coquette, France). Antibiotic MICs were measured by the Etest method as recommended by the manufacturer (AB Biodisk, Solna, Sweden), with brucella agar medium (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% horse blood. Plates were incubated in an anaerobic workstation (Concept Plus, Leeds, United Kingdom) at 37°C for 48 h. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (32) for routine culture or Mueller-Hinton plates for antimicrobial susceptibility testing (Bio-Rad, Marnes-la-Coquette, France). Medium for autoinduction of T7 promoter-based expression systems, as described by Studier (34), was used with strain BL21(DE3) for recombinant enzyme production.

**Molecular methods.** Genomic DNA from *F. nucleatum* NI61 was prepared as previously described (23), partially digested with HindIII, and ligated to the HindIII site in plasmid pZErO-2 (Invitrogen, Leek, The Netherlands). The ligation mixture was transformed into *E. coli* TOP10, and transformants were successively selected on kanamycin (50 μg/ml) and ampicillin (50 μg/ml). The nucleotide sequence of the cloned DNA fragment was determined by primer walking (Eurogentec, Seraing, Belgium).

Plasmid DNA was isolated by alkaline lysis with the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) as previously described (32).

Detection of class A and class D β-lactamase genes in various *Fusobacterium* strains was achieved by PCR with oligonucleotides designed to amplify *bla*$_{FUS-1}$ and the class A β-lactamase gene (*blaA*) identified in *F. nucleatum* subsp. *nucleatum* ATCC 25586 (accession number AE009951) and *F. nucleatum* subsp. *polymorphum* ATCC 10953 (http://pedant.gsf.de) (Table 1). Amplification reactions were carried out in a thermal cycler (Perkin-Elmer 2400) with the following touchdown PCR program: initial denaturation at 94°C for 1 min, followed by 20 cycles of 45 s at 94°C, 45 s at 68°C (reduced by 0.4°C per cycle), and 45 s at 72°C, followed by 20 cycles of 45 s at 94°C, 45 s at 62°C, and 45 s at 72°C and then a final extension step at 72°C for 7 min.

Pulsed-field gel electrophoresis (PFGE) of undigested and SpeI-treated genomic DNA samples prepared from the various strains was performed as previously described, with the GenePath System (Bio-Rad, Hercules, Calif.) under the electrophoretic conditions recommended by the manufacturer (program 9) (17). Hybridization with *bla*$_{FUS-1}$ and *blaA* probes (791 and 806 bp, respectively) was carried out directly on dried gels (36). Briefly, the digoxigenin-labeled probes were obtained by PCR with the PCR DIG Probe Synthesis kit (Roche Applied Science, Mannheim, Germany) with the primers and amplification conditions described above. Prehybridization and hybridization were performed at 42°C with the DIG Easy Hib solution (Roche Applied Science) for 2 h and overnight, respectively. After hybridization, the gel was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate for 5 min at room temperature and then twice in 0.5× SSC containing 0.1% sodium dodecyl sulfate for 15 min at 68°C under constant agitation. Detection of hybridization signals was carried out with a DIG nucleic acid detection kit (Roche Applied Science).

The nucleotide sequence and the deduced amino acid sequence were analyzed by using the software at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The BLASTN and BLASTP programs were used to search for β-lactamases with homology to the *bla*$_{FUS-1}$ gene and FUS-1 β-lactamase sequences. Multiple-sequence alignment of the deduced peptide sequence was carried out at the University of Cambridge website with the program ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk/).

**Production and purification of FUS-1.** The *bla*$_{FUS-1}$ open reading frame was amplified by PCR with 50 pmol of primers FUS-1-F and FUS-1-R (Table 1), 3.5 U of the Expand High Fidelity DNA system (Roche Biochemicals, Mannheim, Germany) in the buffer system provided by the manufacturer, 200 μM deoxynucleoside triphosphates, and 20 ng of plasmid pZErO-2-FUS-1 as the template. The following cycling conditions were used: initial denaturation at 94°C for 3 min; denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min repeated for 30 cycles; and a final extension step at 72°C for 10 min. The PCR product was purified with the Wizard Gel and DNA purification kit and digested with restriction enzymes NdeI and BamHI, purified again, and ligated with vector pET-9a (Stratagene) digested with the same enzymes to yield plasmid pET-FUS-1. The cloned *bla*$_{FUS-1}$ gene was sequenced to rule out the presence of any PCR-generated mutations.

*E. coli* BL21(DE3) was transformed with plasmid pET-FUS-1 and grown in ZYP-0.8G (control) and ZYP-5052 (autoinducing) mediums (34). For optimization of production conditions, 1-ml aliquots of the culture were sampled at different times and centrifuged at 12,000 × *g* (10 min, 4°C). The bacterial cells were resuspended in 1 ml of 10 mM HEPES buffer (pH 7.0) (buffer H) and disrupted by sonication (five cycles of 20 s each at 45 W) with a B. Braun (Melsungen, Germany) Labsonic L sonicator. β-Lactamase activity in both culture supernatants and cell lysates was determined spectrophotometrically in buffer H with 1 mM ampicillin as the substrate. The β-lactamase was purified from 0.5 liter of culture grown overnight in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation, resuspended in 25 ml of 20 mM Tris-HCl buffer (pH 8.0), and disrupted with a One Shot cell disrupter at 23,000 lb/in$^2$ (Constant Systems Ltd., Daventry, United Kingdom). The preparation was centrifuged (15,000 × *g*, 1 h, 10°C) to remove cell debris, desalted with a HiPrep 26/10 desalting column, and loaded (flow rate, 2 ml/min) onto an XK 16/20 column packed with 30 ml of Q Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) and previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0) (buffer A). The enzyme was eluted with a linear gradient of NaCl in buffer A (0 to 400 mM in 400 ml). The purified enzyme preparation (1.1 ± 0.1 mg/ml) was stored at −20°C.

**Protein analysis techniques.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli with a 12% acrylamide concentration in the resolving gel. After electrophoresis, protein bands were stained with Coomassie brilliant blue. Analytical isoelectric focusing of the purified protein and zymographic detection of β-lactamase activity were carried out as previously described (21). The molecular mass of native FUS-1 was estimated by size exclusion chromatography with a Superdex 75 HR 10/30 column (Amersham Biosciences) as described previously (14). The column was calibrated with a mixture of bovine serum albumin (0.2 mg), ovalbumin (0.2 mg), chymotrypsinogen A (0.2 mg), and RNase A (0.5 mg). The protein concentration in solution was determined with a commercial kit (Bio-Rad protein assay; Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard. The molecular mass of the purified FUS-1 preparation was measured by electrospray mass spectrometry as described previously (14) with a Finnigan LTQ mass spectrometer equipped with an ion spray source (Thermo Electron Co., Schaumburg, Ill.). The data were analyzed with the software delivered with the instrument.

**Determination of kinetic parameters.** The steady-state kinetic parameters for β-lactam hydrolysis were measured in buffer H at 30°C as described previously (10, 14). Competition experiments with poor substrates were carried out with 1 mM oxacillin as the reporter substrate. The enzyme concentration in the assays ranged between 7 and 1,500 nM. Inhibition by clavulanic acid, tazobactam, and NaCl was investigated by incubating a 200 nM enzyme solution in the presence of several concentrations of the compound for 5 min at 30°C, and residual activity was measured with 1 mM oxacillin under the conditions described above.

**Nucleotide sequence accession number.** The nucleotide sequence of FUS-1 reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database and assigned accession number AY227054.

## RESULTS AND DISCUSSION

**Antibiotic susceptibility of the *F. nucleatum* isolate.** Isolate NI61 was isolated in 2001 from a gingival sample from a patient treated with amoxicillin for periodontitis and was identi-

TABLE 2. Susceptibilities of FUS-1-producing *F. nucleatum* subsp. *polymorphum* NI61 and *E. coli* NI533 carrying the cloned *bla*$_{FUS-1}$ gene to various β-lactam agents[a]

| Antibiotic(s) | MIC (μg/ml) for: | | | | |
| --- | --- | --- | --- | --- | --- |
| | *F. nucleatum* NI61 | *F. nucleatum* ATCC 25586 | *F. nucleatum* ATCC 49256 | *E. coli* NI533 | *E. coli* TOP10 |
| Penicillin G | >256 | 0.003 | 0.008 | | |
| Amoxicillin | 128 | <0.016 | 0.016 | >256 | 2 |
| Amoxicillin-clavulanic acid | 0.064 | <0.016 | <0.016 | 16 | 2 |
| Clavulanic acid | 4 | 4 | 2 | | |
| Oxacillin | >256 | 0.032 | <0.016 | | |
| Ticarcillin | 2 | 0.064 | 0.125 | >256 | 4 |
| Ticarcillin-clavulanic acid | <0.016 | <0.016 | <0.016 | 32 | 4 |
| Piperacillin | 32 | 0.032 | <0.016 | 8 | 1 |
| Piperacillin-tazobactam | <0.016 | <0.016 | <0.016 | 2 | 1 |
| Tazobactam | 4 | 8 | 2 | | |
| Cefoxitin | 0.25 | 0.125 | 0.06 | 4 | 4 |
| Cefuroxime | 0.047 | 0.047 | 0.25 | 4 | 4 |
| Cefotaxime | 0.094 | 0.047 | 0.25 | 0.064 | 0.064 |
| Cefepime | 1 | 0.75 | 1 | 0.032 | 0.032 |
| Ceftazidime | 2 | 2 | 2 | 0.25 | 0.25 |
| Imipenem | 0.016 | 0.016 | 0.016 | 0.25 | 0.25 |

[a] The susceptibilities of reference strains *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *vincentii* ATCC 49256 and of the *E. coli* strain used in cloning experiments are shown for comparison.

fied by biochemical methods and by 16S rRNA sequencing as *F. nucleatum* subsp. *polymorphum*. Although *F. nucleatum* reference strains appeared to be susceptible to all of the β-lactams tested, including oxacillin, *F. nucleatum* NI61 was resistant to penicillin, amoxicillin, piperacillin, and oxacillin while it remained susceptible to amoxicillin-clavulanate, whose MIC was 0.06 μg/ml (Table 2). Interestingly, two additional β-lactamase-positive *F. nucleatum* strains (NI51 and NI487) found in our collection and identified as *F. nucleatum* subsp. *polymorphum* presented the same resistance pattern.

**Cloning and sequencing of the class D β-lactamase gene.** A HindIII library of *F. nucleatum* NI61 genomic DNA was obtained in *E. coli* TOP10. Plasmid analysis of selected transformants able to grow in the presence of both kanamycin and ampicillin showed a cloned insert of approximately 2.5 kb. The plasmid insert found in *E. coli* clone NI533 was entirely sequenced and revealed a 765-bp open reading frame encoding a 254-amino-acid protein showing the highest similarity to class D β-lactamases and named FUS-1 (OXA-85). The G+C content of the *bla*$_{FUS-1}$ gene was 33%, which lies within the expected range of the G+C contents of *F. nucleatum* genes, suggesting that the *bla*$_{FUS-1}$ gene could be naturally occurring in some *F. nucleatum* subspecies.

In *E. coli* NI533, the native *bla*$_{FUS-1}$ promoter region seems functional and good expression of the β-lactamase gene was observed. A specific activity of 4,800 ± 600 nmol/min/mg of protein (with 1 mM ampicillin as the substrate) was measured in the crude extract prepared from an early log phase culture which, on isoelectric focusing, revealed a single β-lactamase band with a pI of 5.3 ± 0.3. This strain presented a susceptibility profile similar to that of *F. nucleatum* NI61; the highest measured MICs were those of amoxicillin and ticarcillin, and there was a moderate decrease in the amoxicillin and ticarcillin MICs with clavulanic acid (16 and 32 μg/ml, respectively) (Table 2). The good activity of β-lactam–β-lactamase inhibitor combinations against *F. nucleatum* strains, including those producing FUS-1, could be due to the intrinsic activity of clavu-

lanic acid and tazobactam, with MICs ranging from 2 to 4 and from 2 to 8 μg/ml, respectively. These results were in agreement with previously published works (2, 20). Interestingly, a β-lactamase-positive *F. nucleatum* subsp. *polymorphum* strain was also found to be resistant to oxacillin (15) but this antibiotic was not tested in the other works (2, 20, 37).

**Genetic environment and distribution of the *bla*$_{FUS-1}$ gene.** Plasmid DNA could not be isolated from strain NI61 after repeated plasmid DNA extractions by various methods. After PFGE separation of undigested genomic DNA of strains NI61, NI51, and NI487, hybridization with a *bla*$_{FUS-1}$ probe yielded a single positive signal with the chromosomal DNA band, supporting a chromosomal location of the *bla*$_{FUS-1}$ gene in these three *F. nucleatum* isolates (Fig. 1 and data not shown). In addition, a PFGE analysis of the SpeI macrorestriction profiles carried out with these isolates revealed that they do not share any clonal relatedness (Fig. 1).

The region immediately downstream of the *bla*$_{FUS-1}$ gene exhibits strong homology with sequences found in the genomes of *F. nucleatum* subsp. *nucleatum* ATCC 25586 and *F. nucleatum* subsp. *polymorphum* ATCC 10953 (http://pedant.gsf.de) and includes an open reading frame (*orf1*) whose product is similar to transposases (Fig. 2). This putative transposase does not have insertion sequences or terminal repeats. This finding was in agreement with the genome sequences of *F. nucleatum* subsp. *nucleatum* and *F. nucleatum* subsp. *vincentii*, where transposases are often truncated and probably nonfunctional (18, 19). This lack of functional mobility might reflect an ancient chromosomal acquisition event of *bla*$_{FUS-1}$ in a subgroup of *F. nucleatum* species, as demonstrated for most oxacillinase genes (3).

PCRs specific for the amplification of the *bla*$_{FUS-1}$ were positive with strain NI61 and the two other β-lactam-resistant *F. nucleatum* subsp. *polymorphum* strains (NI51 and NI487) but were negative with the four reference *F. nucleatum* strains. In agreement with this finding, no oxacillinase gene was found in the sequenced genomes of *F. nucleatum* subsp. *nucleatum*
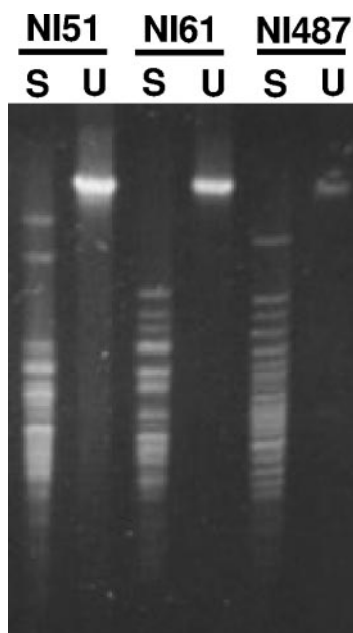
FIG. 1. PFGE analysis of genomic DNA samples isolated from FUS-1-producing *F. nucleatum* subsp. *polymorphum* strains NI51, NI61, and NI487 left undigested (U) or digested with restriction endonuclease SpeI (S).

and *F. nucleatum* subsp. *vincentii* (18, 19). However, a class A β-lactamase was reported in the genome of *F. nucleatum* subsp. *nucleatum* ATCC 25586 although this strain remains susceptible to β-lactams (Table 2). Similarly, two other open reading frames containing resistance genes (encoding 5-nitroimidazole resistance and chloramphenicol resistance) reported in the genome are apparently not expressed, possibly because of the lack of functional promoters.

A PCR carried out with external primers designed to amplify the whole class A β-lactamase gene (BlaAfwd and BlaArev) was positive with *F. nucleatum* subsp. *nucleatum* ATCC 25586

but failed to yield any amplification product with the other strains. However, a similar reaction carried out with internal primers (BlaAintF and BlaAintR) yielded amplification products with *F. nucleatum* subsp. *nucleatum* ATCC 25586, *F. nucleatum* subsp. *fusiforme* ATCC 51190, *F. nucleatum* subsp. *animalis* ATCC 51191, and isolates NI61 and NI487 but was negative with *F. nucleatum* subsp. *vincentii* ATCC 49256 and isolate NI51.

This finding was in agreement with the absence of a gene encoding a class A enzyme (*blaA*) in the released genome of *F. nucleatum* subsp. *vincentii* (19) and indicates an important heterogeneity in the sequence and distribution of this *blaA* gene among isolates of *F. nucleatum*, including those belonging to the same subspecies.

**Structural and functional properties of FUS-1.** $bla_{FUS-1}$ encodes a 254-amino-acid protein which exhibits all of the conserved structural motifs of class D β-lactamase ($S^{67}XFK$, $S^{115}XG$, and $K^{212}TG$, with the numbering of OXA-1 proposed by Sun et al. [35]), although these present infrequent amino acids, like a serine in position 58 (68 in the OXA-1 numbering), i.e., just after the active-site serine residue (a Ser residue only present in OXA-9 while all other OXA-type enzymes present a threonine) and a glutamine in position 107 (116 in the OXA-1 numbering; also present in a putative OXA-type β-lactamase found in the genome of *Campylobacter lari* RM2100 [GenBank accession no. ZP_00369367] but not in OXA-61 from the *C. jejuni* genome [1]) (Fig. 3). The highest homology was found with OXA-63 (53% amino acid identity; GenBank accession no. AY619003), described in a clinical strain of *Brachyspira pilosicoli*, a pathogenic intestinal anaerobic spirochete (4). Phylogenetic analysis (Fig. 4) showed that FUS-1 exhibits the closest ancestral relationship with putative enzymes found in the genomes of *Campylobacter* spp. and clusters with a group of chromosome-encoded enzymes including OXA-50 (40 to 42% identity on the basis of pairwise alignments) (13).

Since a rather large amount of recombinant enzyme was produced by *E. coli* BL21(DE3)/pET-FUS-1 (>50 mg of en-
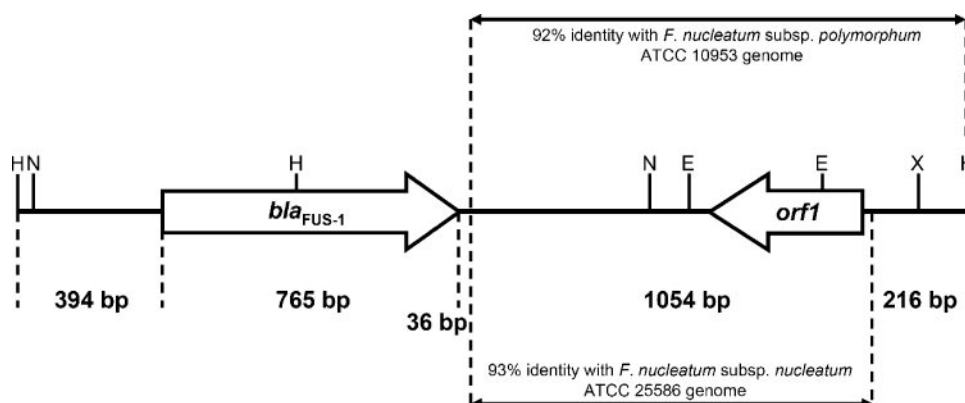


FIG. 2. Schematic representation of the 2.5-kb insert from *E. coli* NI533 showing the organization of the $bla_{FUS-1}$ locus in *F. nucleatum* subsp. *polymorphum* NI61. Restriction sites for EcoRV (E), HindIII (H), NdeI (N), and XbaI (X) are shown. The 394-bp region directly upstream of the $bla_{FUS-1}$ gene did not exhibit any significant similarity to other DNA sequences. A sequence similar to the 1,054-bp region including the *orf1* gene was found in the genome of *F. nucleatum* subsp. *nucleatum* ATCC 25586 (identity, 93%), while a larger region (1,270 bp) was found in the *F. nucleatum* subsp. *polymorphum* ATCC 10953 genome (identity, 92%). *orf1*, shown as starting from the initiator codon found in this sequence, might be longer if an alternative initiator codon were downstream of the HindIII site used for cloning (the reading frame is uninterrupted from this site to the stop codon).
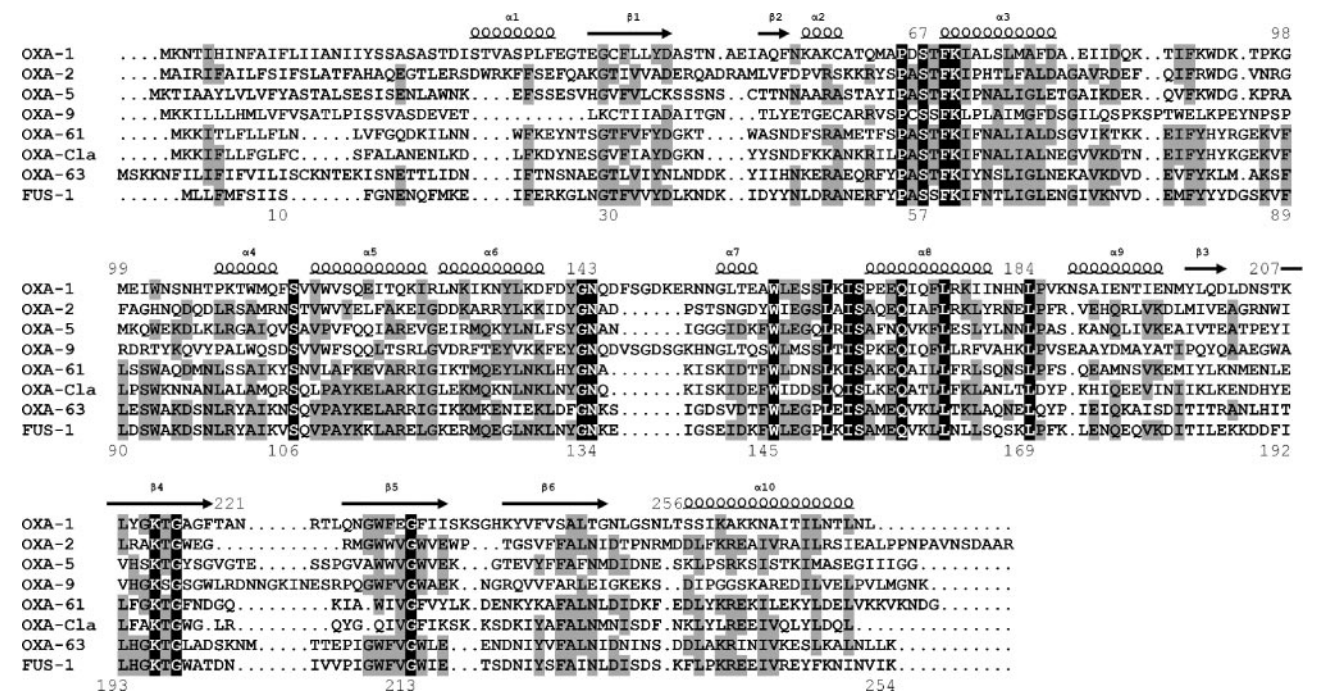
FIG. 3. Amino acid sequence alignment of FUS-1 with other class D enzymes. The structural elements of OXA-1 (α, alpha helices; β, beta strands) are shown above the sequence (35). The numbering above the OXA-1 sequence derives from a structure-based sequence alignment as proposed by Sun et al. (35), while the numbering of FUS-1 is indicated below its sequence.
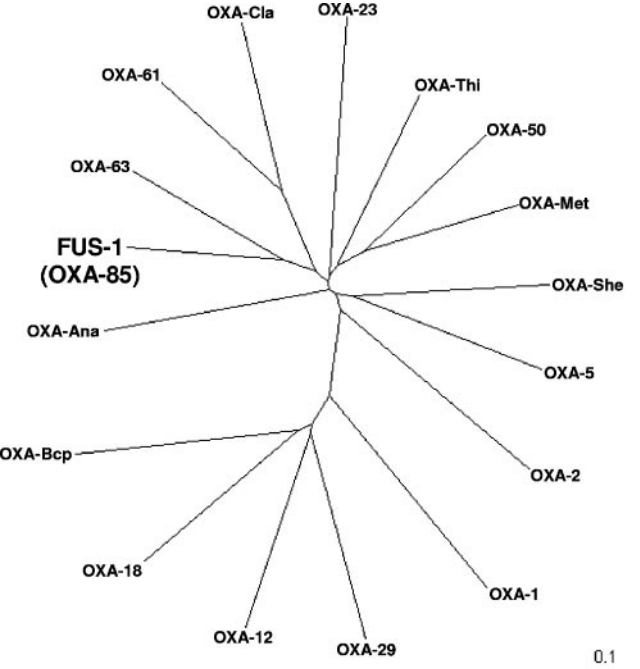


FIG. 4. Unrooted tree showing the relationships among OXA-type enzymes displaying the greatest homology to FUS-1 (OXA-63 and OXA-61) and with representative members of each phylogenetic subgroup, including some homologues detected in the genomes of various bacterial species (OXA-Ana from *Anabaena* sp. strain PCC 7120 [accession no. NP_486520], OXA-Bcp from *Burkholderia* sp. strain 383 [*B. cepacia* complex; accession no. ABB12960], OXA-Cla from *Campylobacter lari* RM2100 [accession no. ZP_00369367], OXA-Met from *Methylobacillus flagellatus* KT [accession no. ZP_00564423], OXA-She from *Shewanella algae* N511 [accession no. AAL47570], and OXA-Thi from *Thiomicrospira denitrificans* ATCC 33889 [accession no. YP_393464]).

zyme per liter of culture in ZYP-5052 medium was obtained in the cell lysates after 20 h of growth), a preparation of the FUS-1 β-lactamase of satisfactory purity (>98%, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [data not shown]) could be obtained after a single anion-exchange chromatographic step. In analytic isoelectric focusing, the purified enzyme preparation showed a single β-lactamase band with a pI of $5.3 \pm 0.3$, similar to that observed with an *E. coli* NI533 crude extract. The molecular mass of the native protein, estimated by size exclusion chromatography, was 30 kDa, indicating that the enzyme was monomeric under our conditions. Electrospray mass spectrometry confirmed the purity of the enzyme preparation obtained from *E. coli* and revealed two protein species (present in roughly equivalent concentrations) of $28,908 \pm 2$ and $29,054 \pm 2$ Da, corresponding to the cleavage of the N-terminal extremity after residue Phe6 (theoretical mass, 28,908.11 Da) or Met5 (theoretical mass, 29,055.28 Da), respectively.

In kinetic assays, FUS-1-catalyzed reactions did not follow biphasic kinetics with the substrates tested, as frequently observed for other class D enzymes. The enzyme exhibited a narrow substrate profile, efficiently hydrolyzing penicillins, cephalothin, CENTA, and to a lesser extent cefuroxime (Table 3). Oxacillin was a good substrate, with a high turnover rate ($k_{cat}$, 780 s$^{-1}$) and consequently a high catalytic efficiency ($k_{cat}/K_m$, $2.1 \times 10^7$ M$^{-1}$ s$^{-1}$), being hydrolyzed 30-fold more efficiently than ampicillin. Ticarcillin and cefuroxime were weakly recognized by the enzyme ($K_m$, >250 μM), acting as poor substrates, while expanded- and broad-spectrum cephalosporins were not hydrolyzed by FUS-1 (Table 3).

In comparison with OXA-2, another narrow-spectrum en-

TABLE 3. Kinetic parameters of FUS-1 for the hydrolysis of various β-lactam substrates in comparison with OXA-2 (22)

| Substrate | $k_{cat}$ (S$^{-1}$) | | $K_m$ (μM) | | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | | Relative $k_{cat}/K_m$ | |
|---|---|---|---|---|---|---|---|---|
| | FUS-1 | OXA-2 | FUS-1 | OXA-2 | FUS-1 | OXA-2 | FUS-1 | OXA-2 |
| Ampicillin | 45 | 110 | 76 | 25 | $5.7 \times 10^5$ | $4.4 \times 10^6$ | 0.7 | 20 |
| Benzylpenicillin | 830 | 90 | 10 | 4 | $8.3 \times 10^7$ | $2.2 \times 10^7$ | 100 | 100 |
| Ticarcillin | >10 | NA$^a$ | >250 | | $4.0 \times 10^4$ | | 0.04 | |
| Oxacillin | 780 | 900 | 36 | 360 | $2.1 \times 10^7$ | $2.5 \times 10^6$ | 25 | 11 |
| Cephalothin | 4.1 | 3.4 | 47 | 7 | $8.7 \times 10^4$ | $4.9 \times 10^5$ | 0.1 | 2 |
| Cefuroxime | >1.8 | NA | >400 | | $4.5 \times 10^3$ | | 0.005 | |
| Cefotaxime | NH$^b$ | NA | | | | | | |
| Ceftazidime | NH | 0.02 | | 12 | | $1.7 \times 10^3$ | | 0.00007 |
| Cefepime | NH | NA | | | | | | |
| CENTA | 0.42 | NA | 23 | | $1.8 \times 10^4$ | | 0.02 | |

$^a$ NA, data not available.
$^b$ NH, no hydrolysis detected (see text for details).

zyme (22), FUS-1 exhibited differences in kinetic parameters with some substrates that account for variations in the spectrum of activity, i.e., better activity on oxacillin but lower activity on ampicillin. With oxacillin, a lower $K_m$ value was measured with FUS-1, resulting in $k_{cat}/K_m$ ratios 10-fold higher than those measured with OXA-2. By contrast, activity on ampicillin was lower with FUS-1 because of both a higher $K_m$ value and lower $k_{cat}$ values (Table 3). Although ceftazidime was a bad substrate for OXA-2, no hydrolysis of that compound could be detected with FUS-1 (at enzyme concentrations as high as 1.5 μM).

As already observed for other class D β-lactamases, the enzyme was poorly inhibited by clavulanate (50% inhibitory concentration [IC$_{50}$], 500 ± 70 μM), while tazobactam was a better inhibitor (IC$_{50}$, 2.5 ± 0.5 μM). These data are in good agreement with the susceptibility profile of *E. coli* NI533, a clone carrying the cloned $bla_{FUS-1}$ gene, that was resistant to most penicillins, whose antimicrobial activity was not fully restored by clavulanic acid (Table 2).

Interestingly, addition of rather high NaCl concentrations had a poor effect on the enzyme activity (IC$_{50}$, >1 M), as also observed for OXA-29 (10). This feature represents an important difference from other OXA-type enzymes, which are usually more sensitive to chloride ions (IC$_{50}$ = 250 mM) (14, 24, 27).

**Concluding remarks.** *F. nucleatum* represents a heterogeneous group including at least five known subspecies whose identification by means of phenotypic tests might be difficult. β-Lactamase-negative strains are usually found to be susceptible to oxacillin, and resistance to this β-lactam could be an additional phenotypic marker for the detection of β-lactamase production (15). β-Lactamase production by *F. nucleatum* strains was most frequently reported in the subspecies *polymorphum* or *nucleatum* but more rarely in the subspecies *vincentii* (19). To date, the $bla_{FUS-1}$ gene has only been detected in isolates of *F. nucleatum* subsp. *polymorphum*, as identified by sequencing of the 16S rRNA gene. The chromosomal location of this gene, its peculiar genetic environment, and the absence of clonal relatedness between the FUS-1-producing strains might suggest ancient acquisition of $bla_{FUS-1}$. The study of the distribution of a β-lactamase gene(s) in a larger set of phylogenetically representative isolates in relation to subspecies and pathogenic characteristics would surely yield valuable data to address these issues.

From the clinical standpoint, differences in pathogenic potential among *F. nucleatum* subspecies have been reported, such as the ability to attach to and invade human gingival epithelial cells, as well as to stimulate the production of proinflammatory interleukin-8 (16). Penicillin resistance due to β-lactamase production by oral *F. nucleatum* occurs frequently in childhood (20, 25, 26), where β-lactams are among the antimicrobials most commonly used in bacterial pediatric infections.

This work underlines an important diversity among *F. nucleatum* strains for the presence of β-lactamase genes and shows that some isolates might produce a functional class D enzyme. FUS-1 is the first oxacillinase described in gram-negative anaerobic rods. It presents a particular narrow-spectrum substrate profile with predominant oxacillinase and penicillinase activity. A previous report described a β-lactamase with strong penicillinase activity from an *F. nucleatum* strain (37). Although both enzymes present similar isoelectric pHs and overall compatible kinetic properties, they exhibit significant differences in amino acid composition, indicating that the strain investigated by Tunér et al. may produce a different enzyme. It will be important to clarify the contribution of FUS-1 (and eventually the class A β-lactamase) to the resistance to β-lactams exhibited by clinical isolates of *Fusobacterium* spp. that might represent a serious therapeutic problem.

### REFERENCES

1. **Alfredson, D. A., and V. Korolik.** 2005. Isolation and expression of a novel molecular class D β-lactamase, OXA-61, from *Campylobacter jejuni*. Antimicrob. Agents Chemother. **49:**2515–2518.
2. **Appelbaum, P. C., S. K. Spangler, and M. R. Jacobs.** 1990. β-Lactamase production and susceptibilities to amoxicillin, amoxicillin-clavulanate, ticarcillin, ticarcillin clavulanate, cefoxitin, imipenem, and metronidazole of 320 non-*Bacteroides fragilis Bacteroides* isolates and 129 fusobacteria from 28 U.S. centers. Antimicrob. Agents Chemother. **34:**1546–1550.
3. **Barlow, M., and B. G. Hall.** 2002. Phylogenetic analysis shows that the OXA β-lactamase genes have been on plasmids for millions of years. J. Mol. Evol. **55:**314–321.
4. **Brooke, C. J., D. J. Hampson, and T. V. Riley.** 2003. In vitro antimicrobial

susceptibility of *Brachyspira pilosicoli* isolates from humans. Antimicrob. Agents Chemother. **47:**2354–2357.

5. **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.

6. **Candoni, A., C. Filì, R. Trevisan, F. Silvestri, and R. Fanin.** 2003. *Fusobacterium nucleatum*: a rare cause of bacteremia in neutropenic patients with leukemia and lymphoma. Clin. Microbiol. Infect. **9:**1112–1115.

7. **Dzink, J. L., M. T. Sheenan, and S. S. Socransky.** 1990. Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov.; *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov.; and *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. Int. J. Syst. Bacteriol. **40:**74–78.

8. **Finegold, S. M., and H. M. Wexler.** 1988. Therapeutic implications of bacteriologic findings in mixed aerobic-anaerobic infections. Antimicrob. Agents Chemother. **32:**611–616.

9. **Fosse, T., I. Madinier, L. Hannoun, C. Giraud-Morin, C. Hitzig, Y. Charbit, and S. Ourang.** 2002. High prevalence of *cfxA* β-lactamase in aminopenicillin-resistant *Prevotella* strains isolated from periodontal pockets. Oral Microbiol. Immunol. **17:**85–88.

10. **Franceschini, N., L. Boschi, S. Pollini, R. Herman, M. Perilli, M. Galleni, J. M. Frere, G. Amicosante, and G. M. Rossolini.** 2001. Characterization of OXA-29 from *Legionella* (*Fluoribacter*) *gormanii*: molecular class D β-lactamase with unusual properties. Antimicrob. Agents Chemother. **45:**3509–3516.

11. **Galán, J. C., M. Reig, A. Navas, F. Baquero, and J. Blázquez.** 2000. ACI-1 from *Acidaminococcus fermentans*: characterization of the first β-lactamase in anaerobic cocci. Antimicrob. Agents Chemother. **44:**3144–3149.

12. **Gharbia, S. E., and H. N. Shah.** 1992. *Fusobacterium nucleatum* subsp. *fusiforme* subsp. nov. and *Fusobacterium nucleatum* subsp. *animalis* subsp. nov. as additional subspecies within *Fusobacterium nucleatum*. Int. J. Syst. Bacteriol. **42:**296–298.

13. **Girlich, D., T. Naas, and P. Nordmann.** 2004. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **48:**2043–2048.

14. **Giuliani, F., J. D. Docquier, M. L. Riccio, L. Pagani, and G. M. Rossolini.** 2005. OXA-46, a new class D β-lactamase of narrow substrate specificity encoded by a *bla*$_{VIM-1}$-containing integron from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **49:**1973–1980.

15. **Goldstein, E. J., P. H. Summanen, D. M. Citron, M. H. Rosove, and S. M. Finegold.** 1995. Fatal sepsis due to a β-lactamase-producing strain of *Fusobacterium nucleatum* subspecies *polymorphum*. Clin. Infect. Dis. **20:**797–800.

16. **Han, Y. W., W. Shi, G. T. Huang, S. Kinder Haake, N. H. Park, H. Kuramitsu, and R. J. Genco.** 2000. Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. Infect. Immun. **68:**3140–3146.

17. **Handal, T., C. Giraud-Morin, D. A. Caugant, I. Madinier, I. Olsen, and T. Fosse.** 2005. Identification of chromosome- and plasmid-encoded β-lactamases in *Capnocytophaga* spp. Antimicrob. Agents Chemother. **49:**3940–3943.

18. **Kapatral, V., I. Anderson, N. Ivanova, G. Reznik, T. Los, A. Lykidis, A. Bhattacharyya, A. Bartman, W. Gardner, G. Grechkin, L. Zhu, O. Vasieva, L. Chu, Y. Kogan, O. Chaga, E. Goltsman, A. Bernal, N. Larsen, M. D'Souza, T. Walunas, G. Pusch, R. Haselkorn, M. Fonstein, N. Kyrpides, and R. Overbeek.** 2002. Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. J. Bacteriol. **184:**2005–2018.

19. **Kapatral, V., N. Ivanova, I. Anderson, G. Reznik, A. Bhattacharyya, W. L. Gardner, N. Mikhailova, A. Lapidus, N. Larsen, M. D'Souza, T. Walunas, R. Haselkorn, R. Overbeek, and N. Kyrpides.** 2003. Genome analysis of *F.*

*nucleatum* subsp. *vincentii* and its comparison with the genome of *F. nucleatum* ATCC 25586. Genome Res. **13:**1180–1189.

20. **Könönen, E., A. Kanervo, K. Salimen, and H. Jousimies-Somer.** 1999. β-Lactamase production and antimicrobial susceptibility of oral heterogeneous *Fusobacterium nucleatum* population in young children. Antimicrob. Agents Chemother. **43:**1270–1273.

21. **Lauretti, L., M. L. Riccio, A. Mazzariol, G. Cornaglia, G. Amicosante, R. Fontana, and G. M. Rossolini.** 1999. Cloning and characterization of *bla*$_{VIM}$, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.

22. **Ledent, P., and J. M. Frère.** 1993. Substrate-induced inactivation of the OXA2 β-lactamase. Biochem. J. **295:**871–878.

23. **Madinier, I., T. Fosse, J. Giudicelli, and R. Labia.** 2001. Cloning and biochemical characterization of a class A β-lactamase from *Prevotella intermedia*. Antimicrob. Agents Chemother. **45:**2386–2389.

24. **Naas, T., and P. Nordmann.** 1999. OXA-type β-lactamases. Curr. Pharm. Des. **5:**865–879.

25. **Nyfors, S., E. Könönen, A. Takala, and H. Jousimies-Somer.** 1999. β-Lactamase production by oral anaerobic gram-negative species in infants in relation to previous antimicrobial therapy. Antimicrob. Agents Chemother. **43:**1591–1594.

26. **Nyfors, S., E. Kononen, R. Syrjanen, E. Komulainen, and H. Jousimies-Somer.** 2003. Emergence of penicillin resistance among *Fusobacterium nucleatum* populations of commensal oral flora during early childhood. J. Antimicrob. Chemother. **51:**107–112.

27. **Paetzel, M., F. Danel, L. De Castro, S. C. Mosimann, M. G. Page, and N. C. Strynadka.** 2000. Crystal structure of the class D β-lactamase OXA-10. Nat. Struct. Biol. **7:**918–925.

28. **Parker, A. C., and C. J. Smith.** 1993. Genetic and biochemical analysis of a novel Ambler class A β-lactamase responsible for cefoxitin resistance in *Bacteroides* species. Antimicrob. Agents Chemother. **37:**1028–1036.

29. **Paula, M. O., J. E. Gaetti-Jardim, and M. J. Avila-Campos.** 2003. Plasmid profile in oral *Fusobacterium nucleatum* from humans and *Cebus apella* monkeys. Rev. Inst. Med. Trop. Sao Paulo. **45:**5–9.

30. **Rasmussen, B. A., Y. Gluzman, and F. P. Tally.** 1990. Cloning and sequencing of class B β-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **34:**1590–1592.

31. **Rogers, M. B., A. C. Parker, and C. J. Smith.** 1993. Cloning and characterization of the endogenous cephalosporinase gene, *cepA*, from *Bacteroides fragilis* reveals a new subgroup of Ambler class A β-lactamases. Antimicrob. Agents Chemother. **37:**2391–2400.

32. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

33. **Smith, C. J., T. K. Bennett, and A. C. Parker.** 1994. Molecular and genetic analysis of the *Bacteroides uniformis* cephalosporinase gene, *cblA*, encoding the species-specific β-lactamase. Antimicrob. Agents Chemother. **38:**1711–1715.

34. **Studier, F. W.** 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. **41:**207–234.

35. **Sun, T., M. Nukaga, K. Mayama, E. H. Braswell, and J. R. Knox.** 2003. Comparison of β-lactamases of classes A and D: 1.5-Å crystallographic structure of the class D OXA-1 oxacillinase. Protein Sci. **12:**82–91.

36. **Tsao, S. G., C. F. Brunk, and R. E. Pearlman.** 1983. Hybridization of nucleic acids directly in agarose gels. Anal. Biochem. **131:**365–372.

37. **Tunér, K., L. Lindqvist, and C. E. Nord.** 1985. Purification and properties of a novel β-lactamase from *Fusobacterium nucleatum*. Antimicrob. Agents Chemother. **27:**943–947.

38. **Tunér, K., and C. E. Nord.** 1986. Emergence of β-lactamase producing anaerobic bacteria in the tonsils during penicillin treatment. Eur. J. Clin. Microbiol. **4:**399–404.