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ACI-1 from *Acidaminococcus fermentans*: Characterization of the First β-Lactamase in Anaerobic Cocci

J. C. GALÁN,[1] M. REIG,[1] A. NAVAS,[2] F. BAQUERO,[1] AND J. BLÁZQUEZ[1]*

Servicio de Microbiología, Hospital Ramón y Cajal, Instituto Nacional de Salud (INSALUD), 28034 Madrid,[1] and Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid,[2] Spain

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Acidaminococcus fermentans **belongs to the group of strictly anaerobic gram-negative cocci. All previously described *Acidaminococcus* strains are susceptible to β-lactam antibiotics. An *A. fermentans* strain (RYC-MR95) resistant to penicillin and expanded-spectrum cephalosporin (amoxicillin and cefotaxime MICs, 64 μg/ml) was isolated from a human perianal abscess. A fragment encoding a β-lactamase from genomic DNA was cloned in *Escherichia coli* K-12 strain HB101, and the recombinant strain expressed resistance to amoxicillin (MIC, 1,024 μg/ml) and cefotaxime (MIC, 4 μg/ml). Clavulanic acid decreased the MICs to 8 and 0.03 μg/ml, respectively. Analysis of the nucleotide sequence revealed a new class A β-lactamase, ACI-1. In accordance with its biochemical properties, we propose to assign ACI-1 to functional group 2be. The ACI-1 enzyme (estimated pI 4.3) had <50% amino acid identity with any other class A β-lactamases, the closest being ROB-1 from *Haemophilus influenzae* (44%). ACI-1 was closer to class A β-lactamases from some gram-positive organisms (41 to 44% amino acid identity with *Bacillus* β-lactamases) than to most class A enzymes from gram-negative organisms (TEM-1, 24.6%). The *aci1* gene had a G+C content of 42.1%, in contrast with 56% G+C content for genomic DNA from *A. fermentans*, thus suggesting that *aci1* may have been obtained by horizontal gene transfer.**

β-Lactamase-mediated resistance to β-lactams in anaerobic bacteria has been known since the early 1950s (14). During the last two decades, an increasing number of β-lactamases from anaerobes have been described, in particular among gram-negative rods (11, 20, 28, 33). β-Lactamases have been characterized for the genera *Bacteroides* (30, 34, 35, 38) and *Fusobacterium* (40). In *Prevotella* and *Porphyromonas*, as well as in *Bilophila* (12, 21, 29), the presence of β-lactamases is known only by positive nitrocefin reactions. Among gram-positive anaerobic bacteria, β-lactamases have been found in *Clostridium* (3, 17, 19). β-Lactamases from *Bacteroides* are cephalosporinases and/or penicillinases; all clostridial and fusobacterial β-lactamases are penicillinases. No β-lactamase has been described previously for anaerobic gram-negative cocci (including *Veillonella*, *Acidaminococcus*, and *Megasphaera*). Indeed, all strains described so far are susceptible to β-lactam antibiotics. In this work, cloning and sequencing of the *aci1* gene and molecular characterization of the new β-lactamase ACI-1 from a β-lactam-resistant *Acidaminococcus fermentans* clinical isolate are reported. To our knowledge, this is the first β-lactamase found in anaerobic gram-negative cocci.

MATERIALS AND METHODS

Strains, plasmids, culture conditions, and susceptibility testing procedures. The ampicillin-resistant *A. fermentans* strain RYC-MR95 was isolated from a perianal abscess sample from a diabetic male patient. Two susceptible *A. fermentans* isolates, RYC4093 and RYC4356, were isolated from clinical samples in the same year from different patients. These strains were grown anaerobically at 37°C in brucella agar supplemented with hemin and vitamin K_1 (Becton Dickinson, Meylan Cedex, France) and in prereduced brain heart infusion broth supplemented with yeast extract (Oxoid Ltd., Basingstoke, United Kingdom). Strains were identified based on conventional criteria, including detection by gas-liquid chromatography of the typical butyric acid accumulation (18). Antibiotic MICs were measured by the agar dilution method as recommended by the NCCLS (26), using Wilkins & Chalgren agar medium (Difco Laboratories, Detroit, Mich.) supplemented with 5% horse blood. Plates were incubated in an anaerobic chamber (Forma Scientific, Marietta, Ohio) at 37°C for 48 h.

Escherichia coli HB101 [F^- Δ(*gtp-proA*)*62 recA13 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *mtl-1 proA2 xyl-5 rpsL20*] (5) was the primary host used for cloning experiments. *E. coli* RYC1000 [F^- Δ(*argF-lac*)*U169 araD139 deoC1 flbB5301 pstF25 relA1 rpsL150* Δ*rib7 thiA gyrA recA56*] (15) was used in all subcloning experiments. *E. coli* JM109 [*endA1 hsdR17 gyrA96* Δ(*lac-proA*) *recAB1 relA supE44 thi* F′(*lac*I^q *lacZ*Δ*M15 proAB*$^+$ *tra*Δ*36*)] (42) was used for expression and biochemical characterization of the β-lactamase. Plasmid vectors used in this work were pBGS18$^-$ and pBGS19$^-$ (Kan^r) (39), pACYC184 (Chl^r Tet^r) (8), and pOGO-295 (Tet^r Amp^r) (41), as the expression vector.

E. coli strains were grown in Luria-Bertani broth. MICs were determined by microdilution in Mueller-Hinton broth (Difco Laboratories) under aerobic conditions at 37°C for 24 h (27). When β-lactamase inhibitors were studied in combination with β-lactams, a fixed concentration of 2 μg/ml was used. Antibiotics and inhibitors were ampicillin, carbenicillin, and chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), kanamycin and tetracycline (Bio 101 Inc., Vista, Calif.), amoxicillin, ticarcillin, clavulanate, and cloxacillin (SmithKline Beecham Pharmaceuticals, Harlow, United Kingdom), cephalothin (Eli Lilly & Co., Indianapolis, Ind.), cephaloridine and cefotaxime (Hoechst-Roussel, Antony, France), ceftazidime (Glaxo-Wellcome, Verona, Italy), cefepime (Bristol-Myers Squibb, Wallingford, Conn.), cefoxitin and imipenem (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.), sulbactam (Pfizer, Groton, Conn.), and tazobactam (Lederle Wyeth, Pearl River, N.Y.).

DNA isolation and analysis. *A. fermentans* genomic DNA from the strains was prepared according to a previously described procedure (1). Plasmid DNA was obtained using Wizard Miniprep (Promega) and High Pure Plasmid (Roche Diagnostics). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc., and Roche Diagnostics, respectively. Transformation of plasmid DNA and Southern hybridization analysis were performed as recommended previously (37).

Cloning experiments and recombinant plasmid constructions. Genomic DNA from *A. fermentans* RYC-MR95 was digested with *Eco*RI and ligated to the *Eco*RI site in pBGS18$^-$. The ligation mixture was transformed into *E. coli* HB101, and transformants were selected on kanamycin (50 μg/ml) and ampicillin (30 μg/ml). Subcloning experiments were performed in pACYC184 and subsequently in pBGS19$^-$. pOGO-ACI was constructed by digesting plasmid pOGO295 with *Xba*I and *Bam*HI. The products of this digestion were separated by agarose gel electrophoresis. The *Xba*I-*Bam*HI fragment corresponding to the pOGO295 replicon (including the tetracycline resistance determinant) was recovered from the gel. The *aci1* gene was PCR amplified by using two primers (ACIFX and ACIRB) that were identical to ACIFE and ACIRH (see below) but contained *Xba*I and *Bam*HI sites instead of *Eco*RI and *Hin*dIII, respectively. The *aci1* amplicon was digested with *Xba*I and *Bam*HI and ligated to the *Xba*I-*Bam*HI-pOGO295 replicon to obtain the pOGO-ACI hybrid plasmid.

DNA sequencing. The nucleotide sequence was determined by the dideoxy polymerase chain termination method with a Sequenase, version 2.0, sequencing

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Ramón y Cajal, INSALUD, 28034 Madrid, Spain. Phone: 34-91-3368330. Fax: 34-91-3368809. E-mail: jblazquez@hrc.insalud.es.

kit (United States Biochemical Corp.) using an automated DNA sequencing system (model 377; PE/ABI, Foster City, Calif.). The nucleotide sequence and the deduced protein were analyzed by using the software at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). BLASTN and BLASTP programs were applied to search for β-lactamases with homology to the *aci1* gene and ACI-1 β-lactamase sequences. Multiple-sequence alignment of the deduced peptide sequence was carried out at the University of Cambridge website using the program ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk).

PCR conditions. Two oligonucleotide primers were synthesized to amplify only the β-lactamase gene: ACIFE (5′-GGGGAATTCAACAGATAGTAGGAGG T-3′) and ACIRH (5′-CGGCAAGCTTGATGCTATCAAGCCCCTT-3′), with *Eco*RI and *Hin*dIII restriction sites, respectively (underlined). Amplifications were carried out in a thermal cycler (Perkin-Elmer 2400) with the following conditions: 30 three-step cycles including denaturation at 94°C for 1 min, annealing at 47°C for 45 s, and extension at 72°C for 2 min. The amplicon was digested with *Eco*RI and *Hin*dIII and subsequently ligated to pBGS18$^-$ and transformed into *E. coli* RYC1000.

β-Lactamase preparation. The β-lactamase extract was prepared from 4 liters of an overnight culture of the JM109(pOGO-ACI-1) strain grown in Luria-Bertani broth with ampicillin (50 μg/ml) and tetracycline (20 μg/ml). Cells were harvested, washed twice with 50 mM phosphate buffer (pH 7.02), and resuspended in 4 ml of the same buffer. The suspension was sonicated for 10 min with a 2-s pulse (Sonicator Heat Systems-Ultrasonics, Inc.), debris was removed by centrifugation (10,000 × *g*, 30 min, 4°C), and the supernatant was assayed for β-lactamase activity with nitrocefin. The suspension was ultrafiltered with Centriplus-100 (Amicon, Inc., Beverly, Mass.). Fractions containing β-lactamase activity were pooled and further concentrated with Centriplus-10 (Amicon, Inc.). The final protein concentration was determined by the method of Bradford (6). The pI was determined by using a Pharmacia Phast system (LKB Biotechnology, Uppsala, Sweden) with LKB Ampholine polyacrylamide gel electrophoresis plates, and β-lactamase activity was detected by nitrocefin staining. Extracts of the β-lactamases TEM-1, TEM-3, TEM-15, TEM-24, SHV-1, SHV-3, and CblA from *Bacteroides uniformis*, ROB-1 from *Haemophilus influenzae*, and the β-lactamase from *Clostridium butyricum* were run in parallel. To study the intracellular location of the β-lactamase, cell fractionating procedures were applied (13).

β-Lactamase activity. The activity of the β-lactamase preparation obtained from *E. coli* JM109 harboring the recombinant plasmid pOGO-ACI-1 was measured spectrophotometrically (Uvikon-940 spectrophotometer) against different β-lactam antibiotics. K_m and V_{max} values were obtained by double-reciprocal (Lineweaver-Burk) plots of the initial steady-state rates at different substrate concentrations. Inhibition studies were carried out by incubating the β-lactamase extract at various concentrations of inhibitors at 25°C for 10 min; then, 100 μM nitrocefin was added as the substrate. The IC_{50} was defined as the concentration of the inhibitor required to reach a 50% inhibition of the β-lactamase activity (4).

Comparison of β-lactamase sequences and phylogenetic analysis. Both the nucleotide sequence of the *aci1* gene and its deduced ACI-1 protein sequence from *A. fermentans* were compared with those of 35 other class A β-lactamases, including those from *Actinomadura* sp. strain R39, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, *Bacillus subtilis*, *Bacteroides fragilis*, *B. uniformis*, *Bacteroides vulgatus*, *Burkholderia cepacia*, *Citrobacter diversus*, *Enterobacter cloacae*, *E. coli* MEN-1, *E. coli* TEM-1, *H. influenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Nocardia farcinica*, *Nocardia lactamdurans*, *Pseudomonas aeruginosa* PSE-1, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptomyces badius*, *Streptomyces cacaoi*, *Streptomyces clavuligerus*, *Streptomyces fradiae*, *Serratia fonticola*, *Serratia marcescens*, and *Yersinia enterocolitica*. Sequences were classified by distance similarity and systematized by parsimony approach using the PAUP program (version 4; Sinauer Associates, Sunderland, Mass.). A matrix of pairwise mean distances (corrected for absolute distance for missing characters) among β-lactamases was computed and values were grouped in a dendrogram by the UPGMA linkage method. Parsimony analysis was carried out with the bootstrapping option for DNA sequences and the step matrix option for protein sequences. Amino acid sequences were analyzed in this way using a step matrix, which specifies the cost of changing from one amino acid to another, with the PROTPARS program of PHYLIP (version 3.4; J. Felsenstein, University of Washington, Seattle).

Nucleotide sequence accession number. The *aci1* nucleotide sequence is available at the EMBL database, with the accession number AJ007350.

RESULTS

Isolation of *A. fermentans* RYC-MR95. Strain RYC-MR95 was isolated in 1995 from a perianal abscess of a diabetic male patient who was admitted to the emergency room of the Hospital Ramón y Cajal (Madrid, Spain). Before admission, the patient was initially treated with a full course of amoxicillin and then, when the patient did not improve, a new treatment with ciprofloxacin was started, but signs and symptoms of infection continued. The isolate was obtained on brucella agar supplemented with hemin and vitamin K_1. Routine microdilution susceptibility testing revealed resistance to penicillin, amoxicillin, piperacillin, and tetracycline and susceptibility to amoxicillin-clavulanate, cefoxitin, and imipenem in addition to clindamycin, erythromycin, metronidazole, and chloramphenicol. A positive nitrocefin disk test revealed the production of a β-lactamase.

Cloning and characterization of the β-lactamase *aci1* gene. Genomic DNA from RYC-MR95 was digested with *Eco*RI and ligated to the *Eco*RI site in pBGS18$^-$. The ligation mixture was transformed into *E. coli* HB101, and transformants were selected on plates containing kanamycin (50 μg/ml) and ampicillin (30 μg/ml). The recombinant plasmid, pJC1, harbored an *Eco*RI fragment of 8.3 kb (Fig. 1). Plasmid DNA was not found in the wild-type RYC-MR95 strain when different plasmid extraction methods were used (such as alkaline lysis and the Nakamura technique [25]). The plasmid pJC1 was digested with *Hin*dIII and ligated to the *Hin*dIII site in pACYC184 and then transformed into *E. coli* RYC1000, using chloramphenicol (30 μg/ml) and ampicillin (50 μg/ml) as selectors for transformants. The new recombinant plasmid pJC3, harboring a *Hin*dIII fragment of 3.3 kb, was isolated. Finally, the plasmid pJC3 was digested with either *Pst*I or *Hin*dIII plus *Pst*I, ligated to pBGS19$^-$, and transformed into *E. coli* RYC1000. A recombinant plasmid, pJC4, harboring a *Pst*I fragment of 1.6 kb, provided no ampicillin resistance to the vector strain. However, the plasmid pJC5, which contained the 1.6-kb *Pst*I fragment plus an additional 0.5-kb *Hin*dIII-*Pst*I fragment, was able to confer ampicillin resistance. This 2.1-kb fragment was sequenced on both strands. Analysis of coding regions revealed an open reading frame (ORF) of 852 bp encoding a 284-amino-acid polypeptide (estimated size, 32 kDa). A BLAST search of the deduced polypeptide sequence against the GenBank database from the National Center for Biotechnology Information showed the presence of a single β-lactamase with homology to class A β-lactamases from gram-positive bacteria.

The nucleotide sequence of this ORF is shown below in Fig. 3. The G+C content of this sequence was 42%, quite dissimilar to the 56% overall G+C content of *A. fermentans* chromosomal DNA (36). A possible Shine-Dalgarno ribosome-binding site (AGGAGG) was identified 5 bp prior to the start codon.

To isolate the putative gene, two primers, ACIFE and ACIRH, were designed and synthesized to amplify the ORF, including its putative Shine-Dalgarno site. The amplification product, 0.8 kb, was cloned into pBGS18$^-$, yielding a plasmid, pJC10, which conferred resistance to ampicillin in *E. coli* RYC1000. In summary, the β-lactamase found in the resistant *A. fermentans* clinical isolate corresponded to a new member of the class A β-lactamases that we propose to name ACI-1 (for "*Acidaminococcus*").

Antimicrobial susceptibility pattern. The MICs of different β-lactam antibiotics for the ACI-1-positive and -negative *A. fermentans* strains, as well as for the *E. coli* strains harboring (or not) the recombinant plasmids, are shown in Table 1. The results showed that organisms harboring this new class A β-lactamase displayed resistance to penicillins (penicillin, amoxicillin, and ticarcillin) and expanded-spectrum cephalosporins (cefotaxime and ceftazidime). The cefotaxime MIC was 128-fold higher for the original resistant strain than for the susceptible *Acidaminococcus* strains. A similar decrease in susceptibility occurred in the *E. coli* strains harboring the recombinant ACI-1-encoding plasmids, compared with the strains harboring no plasmids or plasmids encoding a truncated enzyme. The presence of clavulanic acid (2 μg/ml) strongly reduced the MICs of both penicillins and cephalosporins. The presence of

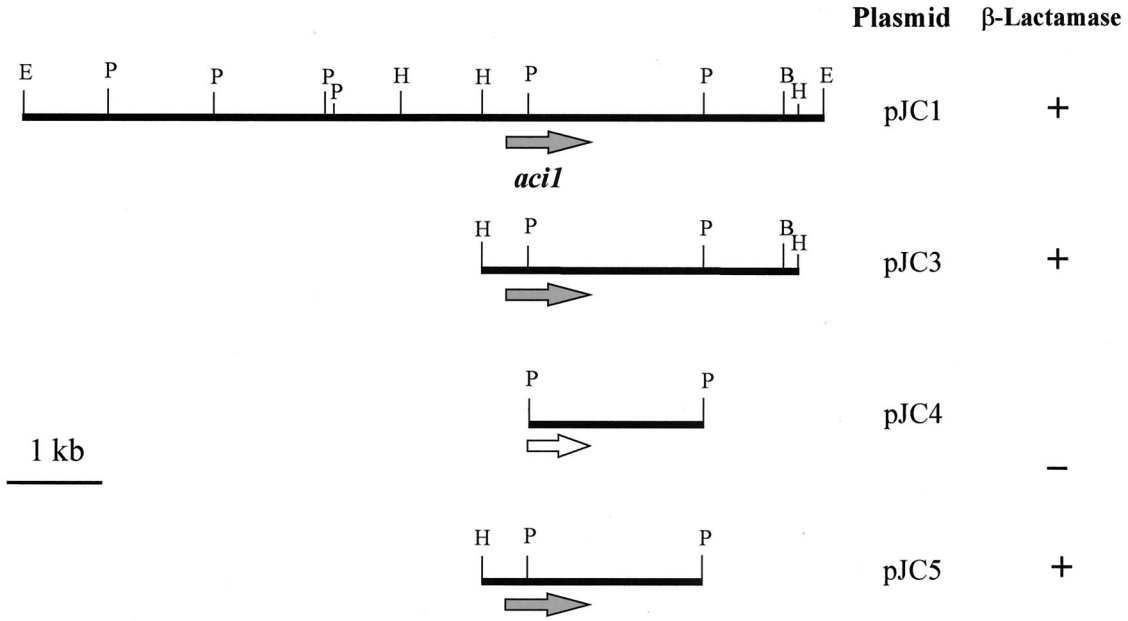

FIG. 1. Physical map of plasmid pJC1 and subcloning strategy. The solid arrow represents the *aci1* β-lactamase gene, and the open arrow represents the truncated gene. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I. The enzymatic activity was detected by nitrocefin reaction.

ACI-1 in *E. coli* strains slightly increased the MICs of cefepime but not of imipenem or cefoxitin.

Absence of *aci1* in ampicillin-susceptible *A. fermentans* strains. To verify that *aci1* was not present in ampicillin-susceptible *A. fermentans* strains, we developed the Southern blot hybridization shown in Fig. 2. The 8.3-kb *Eco*RI chromosomal fragment present in plasmid pJC1 was labeled and used as a probe against genomic DNAs from *A. fermentans* RYC-MR95 and RYC4093 that were previously digested with *Eco*RI or *Bam*HI. The results suggest that *A. fermentans* RYC-MR95 harbors only one copy of the β-lactamase gene and flanking regions into genomic DNA, while the susceptible strain RYC4093 did not show any hybridization signal. Similarly, the *aci1* gene was not detected by PCR in another susceptible *A. fermentans* RYC4356 clinical isolate (data not shown).

Structural characteristics of ACI-1. Within the deduced protein sequence, all characteristic motifs of penicillin-binding proteins were found. A *bla* active-site (STHK) tetrad was detected at positions 65 to 68 (positions 70 to 73 of TEM-1 β-lactamase in the numbering scheme of Ambler [2]). An SDN motif at positions 123 to 125 (positions 130 to 132 of TEM-1) and a KSG motif at positions 226 to 228 (positions 234 to 236 of TEM-1) were also found. In addition, a specific feature of class A β-lactamases was found: the Ω loop region (EPELN) at positions 159 to 163 (Fig. 3).

Amino acid analysis showed 41 to 44% identity with class A

TABLE 1. MICs of β-lactams for *A. fermentans* RYC-MR95, *E. coli* harboring recombinant plasmids which produce β-lactamase ACI-1, and reference strains[a]

Antibiotic	MIC (μg/ml)					
	RYC-MR95	RYC4093	RYC4356	RYC1000/(pJC10)	RYC1000/(pJC4)	RYC1000
Penicillin	128	≤0.5	≤0.5			
Amoxicillin	64	1	≤0.5	1,024	8	8
Amoxicillin-Cla	≤0.03	≤0.03	≤0.03	8	4	4
Ticarcillin	256	1	≤0.5	≥1,024	4	4
Ticarcillin-Cla	0.25	0.5	≤0.03	16	4	4
Cephalothin	4	≤0.5	≤0.5	16	2	2
Cephalothin-Cla	2	0.5	0.25	4	2	2
Ceftazidime	32	2	2	16	0.12	0.12
Ceftazidime-Cla	2	2	0.5	0.5	0.12	0.12
Cefotaxime	64	0.5	0.5	4	0.03	0.03
Cefotaxime-Cla	0.25	0.5	0.25	0.03	0.03	0.03
Cefepime				1	0.015	0.03
Cefepime-Cla					0.015	0.03
Cefoxitin	4	1	1	1	0.5	0.5
Imipenem	0.5	0.5	0.5	0.12	0.06	0.06

[a] RYC-MR95 is wild-type *A. fermentans* producing ACI-1. RYC4093 and RYC4356 are β-lactam-susceptible *A. fermentans* strains. RYC1000 harboring recombinant plasmid pJC10 produced the ACI-1 β-lactamase. RYC1000 harboring recombinant plasmid pJC4 contained a truncated *aci1* gene. Cla, clavulanic acid at a fixed concentration of 2 μg/ml.

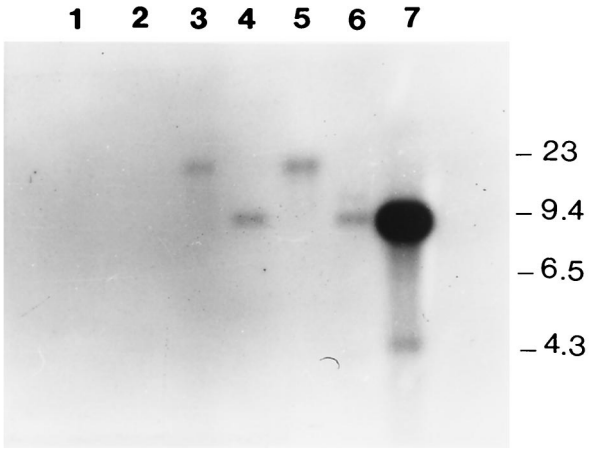

FIG. 2. Southern hybridization. The *A. fermentans* 8.3-kb *Eco*RI DNA fragment was labeled and used as a probe for Southern blot analysis. Lane 1, RYC4093 genomic DNA digested with *Bam*HI; lane 2, RYC4093 genomic DNA digested with *Eco*RI; lane 3, RYC-MR95 genomic DNA digested with *Bam*HI; lane 4, RYC-MR95 genomic DNA digested with *Eco*RI; lanes 5 and 6, same as lanes 3 and 4, respectively, with slightly higher concentrations of DNA; lane 7, plasmid pJC1 digested with *Eco*RI. Molecular sizes are shown, in kilobases.

penicillinases from some gram-positive bacteria, particularly with those of *B. licheniformis* (43.3%), *B. amyloliquefaciens* (44%), *B. cereus* (40.8%), and *S. aureus* (43%) and with ROB-1 from *H. influenzae* (44%). A lower homology with common class A β-lactamases from gram-negative bacteria (24.6% with TEM-1 and 28.2% with SHV-1) was found. The sequence of the ACI-1 enzyme showed higher homology with non-TEM and non-SHV extended-spectrum β-lactamases, such as CTX-M-3 from *Salmonella enterica* serovar Typhimurium (35.2%) (16).

Biochemical properties of ACI-1 β-lactamase. The isoelectric point of ACI-1 was studied on preparations of the wild-type RYC-MR95 and recombinant RYC1000(pJC10) strains. In both cases, two bands were observed when the polyacrylamide gel was stained with nitrocefin. The main band showed a pI of 4.3, and a second band appeared around pI 6.7. When cell fractionating procedures were applied, the pI 4.3 band was found only in the periplasmic extract. Conversely, in the cytoplasmic extract only the pI 6.7 band was detected. The ACI-1 β-lactamase from *E. coli* strain JM109(pOGO-ACI-1) was partially purified. ACI-1 is a broad-spectrum β-lactamase which hydrolyzes both penicillins (penicillin, carbenicillin, ticarcillin, and cloxacillin) and cephalosporins (cephaloridine and cefotaxime). The highest hydrolysis rate corresponded to penicillin, but cefotaxime was also efficiently hydrolyzed, better than cephaloridine. The β-lactamase affinity for cefotaxime was the highest among the tested compounds (Table 2). The clavulanate IC_{50} for ACI-1 was 0.018 μM, lower than that for TEM-1 (0.08 μM). The IC_{50} obtained for sulbactam was 0.008 μM (TEM-1, 8 μM), and that for tazobactam was 0.007 μM (TEM-1, 0.16 μM). The ACI-1 hydrolytic effect was not inhibited by EDTA. These results suggest that the ACI-1 enzyme is a β-lactamase that may belong in the group 2be β-lactamases of the Bush classification (7).

ACI-1 sequence comparison with other class A β-lactamases. The alignment of amino acid sequences generated 408 protein positions that served to compare ACI-1 with other class A β-lactamases. Although alignment of the nucleotide sequence was also done, scarce resolution in any tree was obtained by using such sequences. No significant cophenetic correlation between sets of data was detected. Therefore,

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attgtatgaagtgatgtattagtaatggcaataagagtgctaattgctgatgatcacgct   60
                                                      RBS
ctattgagccaatacgacccgttgaatttggcgggtcgttcaacagatagtaggaggtta  120

ttATGAAGAAATTTTGTTTTTTGTTTTTGATAATCTGTGGCTTGATGGTTTTCTGCCTTC  180
   M  K  K  F  C  F  L  F  L  I  I  C  G  L  M  V  F  C  L

AGGATTGTCAAGCGCGGCAGAAATTAAATCTTGCTGATCTGGAAAATAAATATAACGCCG  240
Q  D  C  Q  A  R  Q  K  L  N  L  A  D  L  E  N  K  Y  N  A

TGATTGGTGTTTACGCCGTTGACATGGAGAATGGAAAAAAAATTTGCTACAAACCTGATA  300
V  I  G  V  Y  A  V  D  M  E  N  G  K  K  I  C  Y  K  P  D

CGCGTTTTTCCTACTGCTCGACACACAAAGTTTTTACGGCTGCAGAATTGCTAAGACAAA  360
T  R  F  S  Y  C  S  T  H  K  V  F  T  A  A  E  L  L  R  Q

AAAATACCTCCGATTTGAATGAAATTCGTAAGTTTTCGGCGGAAGATATTTTGTCCTACG  420
K  N  T  S  D  L  N  E  I  R  K  F  S  A  E  D  I  L  S  Y

CGCCAATCACCAAAGACCATGTTGCTGATGGCATGACGCTGGCGGAAATTTGTTCGGCAT  480
A  P  I  T  K  D  H  V  A  D  G  M  T  L  A  E  I  C  S  A

CGCTCAGGTGGAGTGACAACACGGCGGCAAATTTAATTTTGCAGGAGATCGGCGGCGTGG  540
S  L  R  W  S  D  N  T  A  A  N  L  I  L  Q  E  I  G  G  V

AAAATTTCAAGGTGGCACTTAAAAATATTGGCGACAAAACTACCAAACCTGCGCGAAATG  600
E  N  F  K  V  A  L  K  N  I  G  D  K  T  T  K  P  A  R  N

AACCTGAACTTAATCTTTTCAATCCAAAAGATAATCGTGATACTAGCACGCCGAGACAGA  660
E  P  E  L  N  L  F  N  P  K  D  N  R  D  T  S  T  P  R  Q

TGGTAAAAAATTTGCAAGTCTATATATTCGGCGATATTTTGAGCGACGACAAGAAAAAAC  720
M  V  K  N  L  Q  V  Y  I  F  G  D  I  L  S  D  D  K  K  K

TGCTGATTGATTGGATGAGCGACAATTCCATAACCGACACGCTTATCAAGGCAGAAACTC  780
L  L  I  D  W  M  S  D  N  S  I  T  D  T  L  I  K  A  E  T

CGCAAGGTTGGAAAGTTATCGACAAGAGCGGTTCAGGCGATTATGGGGCGCGGAATGATA  840
P  Q  G  W  K  V  I  D  K  S  G  S  G  D  Y  G  A  R  N  D

TTGCCGTGATTTATCCGCCCAATCGCAAACCCATTGTCATGGCGATAATGTCGCGCCGCA  900
I  A  V  I  Y  P  P  N  R  K  P  I  V  M  A  I  M  S  R  R

CGGAAAAAAATGCAAAATCTGACGACGCTATGATTGCGGAGGCGGCAAAACGAATTTTTG  960
T  E  K  N  A  K  S  D  D  A  M  I  A  E  A  A  K  R  I  F

ATAATTTAGTATTTTAAaggggcttgatagcatcggtattgccgatgggcagagagggcc 1020
D  N  L  V  F  -

atcacgcaaaaatatttcgccaaacatttatatctcactcaaacttcgcacacctgaaac 1080

ccttactattatatcatattc                                        2001
```

FIG. 3. Nucleotide sequence of the *A. fermentans aci1* β-lactamase gene and its flanking regions. The predicted amino acid sequence is shown below the nucleotide sequence. The putative initiation codon and conserved motifs are shown in bold. The pair of arrows indicates the putative transcription terminator. The predicted Shine-Dalgarno sequence is also shown as RBS.

the tree in Fig. 4 only expresses relationships among protein sequences, because of its higher resolution and consistency. This figure suggests evolutionary relationships based on parsimony criteria in which the outgroup was considered paraphyletic. The tree shows that only the β-lactamase BRO-1 from *M. catarrhalis* could be considered apart. The ACI-1 enzyme from *A. fermentans* was consistently found in or near the basal node of the main phylogenetic class A β-lactamase group. That was coincident to distance analysis criteria (tree not

TABLE 2. Hydrolytic activity of ACI-1 enzyme against different β-lactam antibiotics

β-Lactam agent	K_m (μM)	Relative V_{max}[a]	Relative V_{max}/K_m
Penicillin	3 ± 0.4	100	100
Ticarcillin	13.3 ± 3	27 ± 1	6.7
Carbenicillin	116 ± 26	30 ± 2	6.5
Cloxacillin	29 ± 9	33 ± 4	2.7
Cephaloridine	6 ± 0.6	64 ± 8	36
Cefotaxime	2.1 ± 0.2	26 ± 2	42.2
Imipenem	747 ± 65	0.4 ± 0.1	3

[a] Penicillin was a substrate reference, set at 100. Data are the means and standard deviations from three independent experiments.

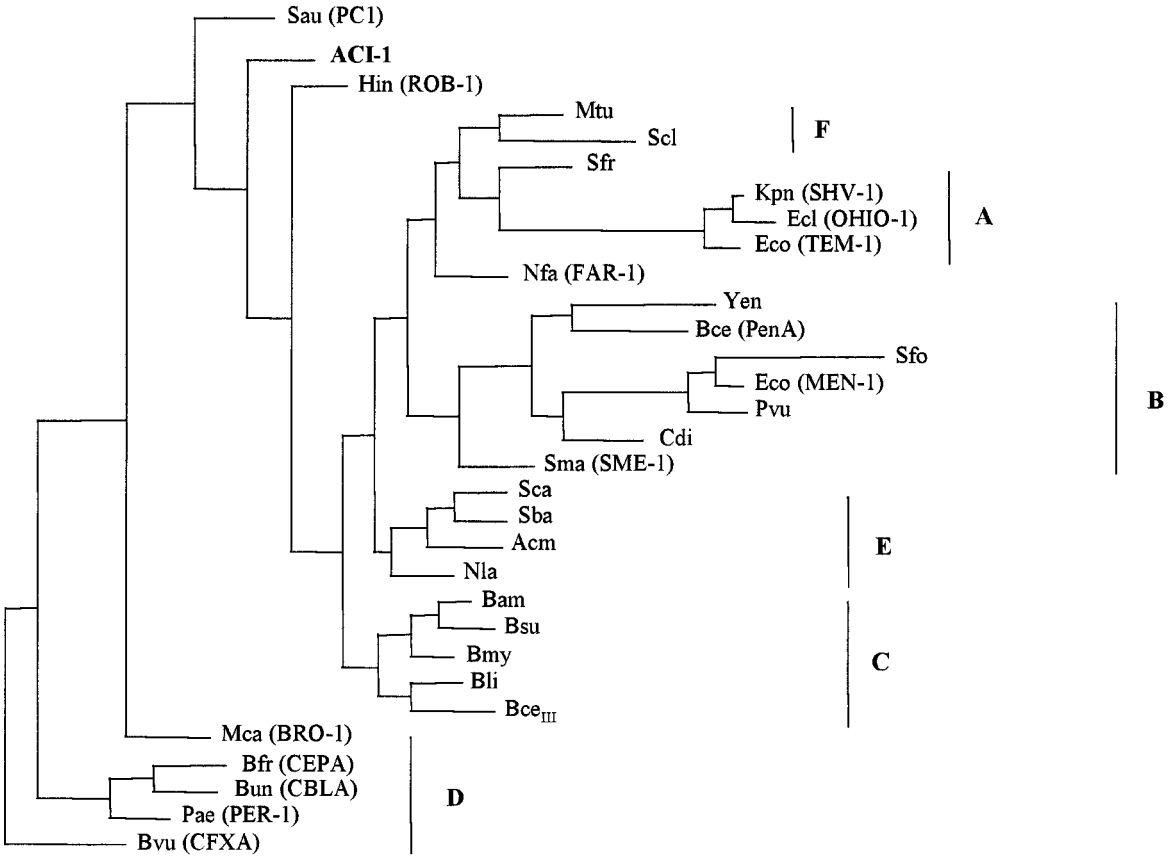

FIG. 4. Phylogenetic trees obtained for 31 class A β-lactamases, according to the parsimony criteria. Abbreviations: Sau, *S. aureus* PC1 (P00807); Hin, *H. influenzae* ROB-1 (P33949); Mtu, *M. tuberculosis* (Q10670); Scl, *S. clavuligerus* (Z54190); Nfa, *N. farcinica* FAR-1 (AF024601); Acm, *Actinomadura* sp. strain R39 (X53650); Sca, *S. cacaoi* (P14560); Sba, *S. badius* (P35391); Nla, *N. lactamdurans* (Q06316); Bam, *B. amyloliquefaciens* (Q44674); Bsu, *B. subtilis* (P39824); Bmy, *B. mycoides* (P28018); Bli, *B. licheniformis* (P00808); BceIII, *B. cereus* β-lactamase type III (P06548); Yen, *Y. enterocolitica* (Q01166); Bce, *B. cepacia* (U85041); Sfo, *S. fonticola* (P80545); Eco (MEN-1), *E. coli* (P28585); Cdi, *C. diversus* (P22390); Pvu, *P. vulgaris* (P52664); Sma, *S. marcescens* Sme-1 (P52682); Sfr, *S. fradiae* (P35392); Kpn, *K. pneumoniae* SHV-1 (P23982); Ecl, *E. cloacae* (OHIO-1) (P18251); Eco (TEM-1), *E. coli* (P00810); Mca, *M. catarrhalis* (BRO-1) (Q59514); Bvu, *B. vulgatus* (CfxA) (P30899); Bfr, *B. fragilis* (CepA) (L13472); Bun, *B. uniformis* (CblA) (P30898); Pae, *P. aeruginosa* (PER-1) (P37321). The codes in parentheses correspond to listed accession numbers (see reference 23 and the European Bioinformatics Institute website [http://www.ebi.ac.uk]).

shown). A similar situation was found for ROB-1 from *H. influenzae*, with both constituting the possible origin of independent monophyletic lines.

DISCUSSION

The antibiotic susceptibility of the different gram-negative anaerobic cocci isolated from humans remains largely unknown. The isolation of such microorganisms from clinical samples is relatively infrequent, and among them, *Acidaminococcus* has been very rarely reported. Most general studies on anaerobes do not distinguish *Acidaminococcus* from *Veillonella* and *Megasphaera*; others deal only with strains of veterinary origin (32). Penicillin resistance in *Veillonella* has previously been reported by our group, but the strains were in all cases β-lactamase negative (M. Reig, N. Mir, and F. Baquero, Letter, Antimicrob. Agents Chemother. **41:**1210, 1997). To date, *Acidaminococcus* has been considered fully susceptible to β-lactam antibiotics. β-Lactamases are the main mechanism of resistance in anaerobic gram-negative rods (33), but these enzymes had never been found among gram-negative anaerobic cocci. In this work, the presence of a novel class A β-lactamase (ACI-1) was detected in an *A. fermentans* clinical strain resistant to penicillins and cephalosporins. The nucleotide sequence of ACI-1 revealed a closer relationship with class A β-lactamases from some gram-positive bacteria than with many enzymes from gram-negative bacteria. The ACI-1 enzyme shares all characteristics common with those of class A β-lactamases. The highest homology was found with β-lactamases from *Bacillus* and with ROB-1 from *H. influenzae*. The phylogenetic analysis of sequences strongly suggests that ACI-1, like ROB-1, is located in an independent monophyletic line, very near the basal node that constitutes the common root of most class A β-lactamases.

What is the origin of the *aci1* β-lactamase gene found in the chromosome of *A. fermentans*? No plasmids were found in the resistant isolate. The G+C content of 42% for the *aci1* structural gene was very dissimilar to that of *A. fermentans* chromosomal DNA (56%). The flanking regions of the *aci1* gene have a 41.8% G+C content upstream and a 52.4% G+C content downstream. Moreover, the 8.3-kb fragment containing the *aci1* gene was not detected in two β-lactam-susceptible *A. fermentans* strains. Altogether, these data strongly suggest that the β-lactamase gene could be part of a transposable element, as has also been proposed for the origin of the ROB-1-encoding gene in *Pasteurella* (22). The higher similarity of ACI-1 was found with class A β-lactamases from some gram-positive organisms. Members of the family *Veillonellaceae*, which includes *Veillonella*, *A. fermentans*, and *Megasphaera elsdenii*, are anaerobic gram-negative cocci but may stain weakly as gram positive. On the other hand, the 16S rRNA gene sequences of

these three genera have allowed their classification within cluster IX (*Sporomusa* subbranch) of the low-G+C-content *Bacillus*/*Clostridium* gram-positive subphylum (10). The taxonomic position of *Acidaminococcus* may explain the presence of a β-lactamase similar to those of gram-positive bacteria. The consequences of a broad-spectrum β-lactamase in *Acidaminococcus* are difficult to evaluate. These organisms are part of the resident microbiota of the gastrointestinal tract in humans and animals, although their prevalence and density are low compared with those of *B. vulgatus* or *Fusobacterium prausnitzii* (24). Even though *Acidaminococcus* is rarely involved in clinical infections, it has been isolated from abdominal and pulmonary abscesses (9) and in bacteremia (31), always as part of a mixed anaerobic flora. The results from this study suggest that *A. fermentans* may have an indirect effect on human health and may contribute to the origin or spreading of resistance genes encoding both penicillin- and cefotaxime-hydrolyzing β-lactamases in one of the most complex microbial ecosystems known.

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