ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2001, p. 2324–2330 Vol. 45, No. 8
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.8.2324–2330.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Molecular Characterization of Chromosomal Class C β-Lactamase and Its Regulatory Gene in *Ochrobactrum anthropi*

DAVID NADJAR,[1] ROGER LABIA,[2] CLAUDE CERCEAU,[2] CHANTAL BIZET,[3] ALAIN PHILIPPON,[4] AND GUILLAUME ARLET[1]*

Laboratoire de Bactériologie, Hôpital Tenon, UFR Saint-Antoine,[1] Collection de l'Institut Pasteur, Institut Pasteur,[3] and Service de Bactériologie, CHU Cochin,[4] Paris, and CNRS-UBO-MHN, Unité FRE 2125, Quimper,[2] France

Received 28 December 2000/Returned for modification 9 April 2001/Accepted 26 May 2001

***Ochrobactrum anthropi*, formerly known as CDC group Vd, is an oxidase-producing, gram-negative, obligately aerobic, non-lactose-fermenting bacillus of low virulence that occasionally causes human infections. It is highly resistant to all β-lactams except imipenem. A clinical isolate, SLO74, and six reference strains were tested. MICs of penicillins, aztreonam, and most cephalosporins tested, including cefotaxime and ceftazidime, were >128 μg/ml and of cefepime were 64 to >128 μg/ml. Clavulanic acid was ineffective and tazobactam had a weak effect in association with piperacillin. Two genes, *ampR* and *ampC*, were cloned by inserting restriction fragments of genomic DNA from the clinical strain *O. anthropi* SLO74 into pBK-CMV to give the recombinant plasmid pBK-OA1. The pattern of resistance to β-lactams of this clone was similar to that of the parental strain, except for its resistance to cefepime (MIC, 0.5 μg/ml). The deduced amino acid sequence of the AmpC β-lactamase (pI, 8.9) was only 41 to 52% identical to the sequence of other chromosomally encoded and plasmid-encoded class C β-lactamases. The kinetic properties of this β-lactamase were typical for this class of β-lactamases. Upstream from the *ampC* gene, the *ampR* gene encodes a protein with a sequence that is 46 to 62% identical to those of other AmpR proteins and with an amino-terminal DNA-binding domain typical of transcriptional activators of the Lys-R family. The deduced amino acid sequences of the *ampC* genes of the six reference strains were 96 to 99% identical to the sequence of the clinical strain. The β-lactamase characterized from strain SLO74 was named OCH-1 (gene, bla_{OCH-1}).**

The species of the genus *Ochrobactrum* form two groups: *Ochrobactrum anthropi* and *O. intermedium* (25). *O. anthropi*, formerly classified as CDC group Vd, is a nonfastidious, gram-negative bacillus that is strictly aerobic, oxidase positive, and motile (with peritrichous flagella), does not ferment lactose, and has strong urease activity (8, 16). *O. anthropi* is widespread and is distributed in water and hospital environments. In some cases, it has been isolated from water-based environments in hospitals (antiseptic solutions, dialysis fluids) (12). It has often been found on human clinical material: it often adheres to catheters, but pacemakers, intraocular lenses, and silicon tubing may also become infected (10, 18). Although only weakly virulent, *O. anthropi* causes hospital-acquired infections, often in immunocompromised hosts (7, 11, 14, 27). *O. anthropi* is usually resistant to β-lactams, such as broad-spectrum penicillins and oxyimino cephalosporins, except for cefepime in some cases and aztreonam (3). It is generally susceptible to carbapenems and aminoglycosides (19), trimethoprim-sulfamethoxazole (4), ciprofloxacin, and tetracyclines. The most effective antimicrobial agents for treating human infections are imipenem, trimethoprim-sulfamethoxazole, and ciprofloxacin (7), sometimes in conjunction with catheter removal (28). As this bacterium displays extensive resistance to β-lactams, we screened for and cloned a β-lactamase gene.

* Corresponding author. Mailing address: Service de Bactériologie, Hôpital Tenon, 4, rue de la Chine, 75970 Paris Cedex 20, France. Phone: 33 1 56 01 70 18. Fax: 33 1 56 01 61 08. E-mail: guillaume.arlet@tnn.ap-hop-paris.fr.

MATERIALS AND METHODS

Bacterial strains. Table 1 shows the bacterial strains used in this study. *O. anthropi* SLO74 was isolated in November 1991 at Saint-Louis Hospital (Paris, France) from a blood culture from a leukemic patient with catheter-related sepsis. Six reference strains of *O. anthropi* were obtained from the collection of the Pasteur Institute (Paris, France). *Escherichia coli* XL1-Blue (Stratagene, Amsterdam, The Netherlands) and *E. coli* HB101 (Bio-Rad, Marnes-La-Coquette, France) were used for cloning and subcloning experiments, respectively.

Antimicrobial agents and MIC determination. The antimicrobial agents used were standard laboratory powders. The antimicrobial agents used were as follows: amoxicillin, clavulanic acid, and ticarcillin (SmithKline Beecham, Nanterre, France); piperacillin and tazobactam (Wyeth-Lederle, Oullins, France); cephalothin and cefamandole (Eli-Lilly, Saint-Cloud, France); cefepime (Bristol-Myers Squibb, Nanterre, France); cefotaxime (Aventis, Paris, France); cefoxitin and imipenem (Merck Sharp & Dohme-Chibret, Paris, France); aztreonam (Sanofi, Paris, France); and ceftazidime (GlaxoWellcome, Marly-le-Roy, France).

MICs were determined with the standard agar dilution technique on Mueller-Hinton agar (Bio-Rad) with a multiple inoculator and an inoculum of 10^5 CFU per spot. All plates were incubated at 37°C for 18 h. The MICs of some β-lactams (amoxicillin, ticarcillin, and piperacillin) were determined alone or in combination with 2 μg of clavulanic acid per ml or 4 μg of tazobactam per ml.

Cloning experiments and recombinant plasmids. The chromosomal DNA of *O. anthropi* SLO74 was prepared as described by Grimont and Grimont (13). It was digested with *Eco*RI (Roche Biochemicals France S.A., Meylan, France) and ligated using T4 DNA ligase (Amersham Pharmacia Biotech, Saclay, France) into the *Eco*RI site of the pBK-CMV phagemid (Table 1) (Stratagene). Recombinant plasmids were introduced into *E. coli* XL1 by the standard $CaCl_2$ technique. Antibiotic-resistant colonies were selected on Drigaski agar (Bio-Rad) containing ceftazidime (2 μg/ml) and kanamycin (25 μg/ml) (Sigma, Saint-Quentin Falavier, France). Recombinant plasmid DNA was recovered using Qiagen columns (Qiagen, Courtaboeuf, France), and the size of the inserts was estimated by restriction enzyme digestion and electrophoresis in 1 to 3% agarose gels.

The recombinant plasmid pBK-OA1 was double digested with *Sac*II (Roche Biochemicals) and *Eco*RI, and the resulting fragment was ligated into the pBC SK$^+$ phagemid (Table 1) (Stratagene) digested with the same enzymes. Trans-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source
Strains		
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR47 supE44 relA1 lacI*^{q} ZDM15Tn*10* (Tet^r)	Stratagene
HB101	F$^-$ *mcrB mrr hsdS20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20* (Sm^r) *supE44*	Bio-Rad
O. anthropi		
SLO 74	Extended-spectrum cephalosporin resistance	Saint-Louis Hospital
CIP 82113		Collection of the Pasteur Institute
CIP 82115 T		
CIP 82116		
CIP 102332		
CIP 103949		
CIP 103952		
Plasmids		
pBK-CMV phagemid	Neo^r Kan^r	Stratagene
pBC SK$^+$ phagemid	Cam^r	Stratagene
pBK-OA1	4.7-kb DNA fragment from *O. anthropi* SLO74 that contained bla_{OCH-1} in the *Eco*RI site of pBK-CMV	This study
pSK$^+$-OA2	3-kb DNA fragment from the recombinant plasmid pBK-OA1 that contained bla_{OCH-1} in pBC SK$^+$ digested with *Sac*II and *Eco*RI	This study

formants were selected on the basis of resistance to amoxicillin (40 μg/ml) and chloramphenicol (25 μg/ml) (Sigma) using Drigalski agar plates.

Preparation of crude extracts of β-lactamase. The *O. anthropi* SLO74 strain and the six reference strains of *O. anthropi* (Table 1) were cultured overnight at 37°C in 100 ml of Trypticase soy broth. For cultures of the *E. coli* XL1(pBK-OA1) clone and the *E. coli* HB101(pSK$^+$-OA2) subclone, amoxicillin (40 μg/ml) and more kanamycin (25 μg/ml) or more chloramphenicol (25 μg/ml), respectively, were added to the medium to maintain selection pressure. Bacterial suspensions were pelleted (30 min at 5,800 × *g*), resuspended in 2 ml of 20 mM Tris buffer (pH 7.5), and disrupted by sonication (two times for 30 s each at 20 Hz) (Vibra Cell; Bioblock Scientific, Illkirch, France). The crude extracts were cleared by centrifugation at 48,000 × *g* for 30 min at 4°C.

IEF. All β-lactamase extracts were subjected to analytical isoelectric focusing (IEF) (1) on an ampholine polyacrylamide gel with a pH range of 3.5 to 10. The gel was put in a Multiphor apparatus (Amersham Pharmacia Biotech) for 18 h at 200 V, 15 mA, and 6 W. The focused β-lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Paris, France). The pI markers were those for the reference β-lactamases: for TEM-3 (pCFF04), pI is 6.3; for SHV-4 (pUD21), pI is 7.8; and for CMY-2 (pSenf), pI is 9.2.

β-Lactamase purification and kinetic measurements. Cells of the *E. coli* HB101 subclone containing the recombinant plasmid pSK$^+$-OA2 (Table 1) were obtained from 4-liter cultures in brain heart infusion broth (Difco) at 37°C. Cells were harvested by centrifugation at 5,800 × *g* for 30 min. The pellets (about 12 g [wet weight]) were washed by resuspending them in 24 ml of 0.15 M NaCl and centrifuging at 5,800 × *g* for 20 min. The supernatants were discarded and the pellets were resuspended under the same conditions, lysed by sonication (Branson Sonifier), and centrifuged at 5,800 × *g* for 1 h. The pellets were discarded. The crude extracts were cleared by centrifugation at 48,000 × *g* for 30 min at 4°C. Nucleic acids were precipitated by adding spermine (0.2 M) (Sigma) and collected by centrifugation (30,000 × *g* for 30 min at 4°C). The supernatant was dialyzed three times against 5 liters of distilled water and lyophilized. The enzyme was purified by chromatography on Bio-Rex 70 resin (weakly acidic cation exchanger) equilibrated with 10 mM Tris hydrochloride buffer, pH 7.0. The β-lactamase was eluted with a linear gradient of 0 to 0.6 M NaCl, and active fractions were pooled, desalted by three centrifugations and dilutions on an Ultrafree-20 centrifuge filter unit with a nominal molecular weight limit of 10,000 (Sigma), and used rapidly for kinetic studies. The kinetic constants k_{cat} and K_m for substrates were determined by a computerized microacidimetric assay at pH 7.0 and 37°C in 0.1 M NaCl as described by Labia et al. (20). One β-lactamase unit is defined as the amount of enzyme hydrolyzing 1 μmol of benzylpenicillin in 1 min at pH 7.0 and 37°C. In this test, the initial benzylpenicillin concentration is 500 μM.

DNA sequencing, PCR amplification, and sequence analysis. The insert of the recombinant plasmid pBK-OA1 was sequenced using the method of Sanger et al. (30), with fluorescent dye-labeled dideoxynucleotides, thermal cycling with *Taq* polymerase (Amersham), and an ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). We studied the variability of the *ampC* genes of *O. anthropi* using two primers designed to amplify the entire coding region in the six reference strains: upper ochro (5′-AATTTTTCTAATGCCAAGTGCT-3′) and lower ochro (5′-GCCTATTGCTTGTTGTCGAG-3′) (see Fig. 2). The PCR products were sequenced and analyzed. The BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov) was used for database searches, and Clustal W (http://www.2.eib.ac.uk./clustalw) was used to align multiple protein sequences.

Nucleotide sequence accession numbers. The bla_{OCH} gene nucleotide sequence data appear in the EMBL nucleotide sequence database under accession no. AJ401618 for bla_{OCH-1}, AJ295340 for bla_{OCH-2}, AJ295341 for bla_{OCH-3}, AJ295342 for bla_{OCH-4}, AJ295343 for bla_{OCH-5}, AJ295344 for bla_{OCH-6}, and AJ294345 for bla_{OCH-7}.

RESULTS

Antimicrobial agent susceptibility. The MICs of β-lactams for all strains of *O. anthropi* showed that these strains were resistant to all β-lactams tested except imipenem (Table 2). Clavulanic acid was ineffective and tazobactam slightly reduced resistance to piperacillin. The *E. coli* XL1(pBK-OA1) clone and the *E. coli* HB101(pSK$^+$-OA2) subclone had similar resistance phenotypes: resistance to all penicillins (intermediate resistance to piperacillin in the clone harboring pBK-OA1), resistance to all cephalosporins except cefepime, intermediate susceptibility to aztreonam, and susceptibility to imipenem.

IEF. A band of β-lactamase activity was detected in each strain of *O. anthropi* in analytical IEF experiments. The corresponding pI was 8.9 for the parental strain *O. anthropi* SLO74. The pI values for the six reference strains of *O. anthropi* were from 8 to 9.2 (CIP 82113, pI = 9; CIP 83115T, pI = 8; CIP 83116, pI ≥ 9.2; CIP 102332, pI ≥ 9.2; CIP 103949, pI = 9.2; CIP 103952, pI = 8.5) (data not shown).

Cloning and sequence analysis of bla_{OCH-1} and subcloning. Total genomic DNA from *O. anthropi* SLO74 was digested with *Eco*RI and inserted into the *Eco*RI site of pBK-CMV. Four identical recombinant *E. coli* XL1 clones were obtained. One harboring pBK-OA1 (insert, 4.7 kb) was selected for further study. It produced a β-lactamase with a pI of 8.9. The entire 4,743-bp DNA insert was sequenced on both strands. We found that this insert contained five open reading frames (ORFs)

TABLE 2. MICs of β-lactams for *O. anthropi* SLO74, six reference strains of *O. anthropi*, the *E. coli* XL1(pBK-OA1) clone, the *E. coli* HB101(pSK⁺-OA2) subclone, and the strains *E. coli* XL1 and *E. coli* HB101

Strain	MIC (μg/ml) for the given strain[c]															
	AMX	AMC	TIC	TCC	PIP	TZP	CEF	FAM	CFM	FOX	CXM	CTX	FEP	CAZ	ATM	IPM
O. anthropi																
SLO74	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	128	≥128	≥128	≥128	≥128	≥128	0.5
CIP 82113	≥128	128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	128	≥128	≥128	64	≥128	≥128	1
CIP 82115 T	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	0.5
CIP 82116	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	1
CIP 102332	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	128	≥128	≥128	≥128	≥128	≥128	0.25
CIP 103949	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	1
CIP 103952	≥128	128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	64	≥128	≥128	64	≥128	≥128	0.25
E. coli																
XL1(pBK-OA1)[a]	≥128	≥128	≥128	≥128	16	8	≥128	≥128	≥128	128	≥128	32	0.5	128	8	0.12
HB101(pSK⁺-OA2)[b]	≥128	128	≥128	≥128	64	16	≥128	≥128	≥128	64	≥128	64	0.5	≥128	16	0.25
XL1	4	1	0.5	0.12	1	0.25	2	0.5	0.25	2	0.5	0.06	0.06	0.12	0.06	0.5
HB101	4	1	0.5	0.12	0.5	0.25	4	0.5	0.25	2	0.5	0.06	0.06	0.12	0.06	0.5

[a] This strain, harboring the recombinant multicopy plasmid pBK-OA1, produced OCH-1 β-lactamase (in the presence of the regulator gene).

[b] This strain, harboring the recombinant multicopy plasmid pSK⁺-OA2, produced OCH-1 β-lactamase (in the absence of the regulator gene).

[c] AMX, amoxicillin; AMC, AMX + clavulanic acid (2 μg/ml), TIC, ticarcillin; TCC, TIC + clavulanic acid; PIP, piperacillin; TZP, PIP + tazobactam (4 μg/ml); CEF, cephalothin; FAM, cefamandole; CFM, cefixime; FOX, cefoxitin; CXM, cefuroxime; CTX, cefotaxime; FEP, cefepime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem.

(Fig. 1). Two ORFs (ORF4 and ORF5) showed no sequence identity with DNA sequences in databases in BLASTN searches. ORF1 was 98% identical to a gene encoding a 25-kDa outer membrane protein in *Brucella abortus* (9). ORF3 was 1,169 bp long and encoded a 390-amino-acid sequence. This ORF was preceded by putative −35 (TTGTCG) and −10 (GTATAT) promoter regions and a putative ATG initiation codon at position 1070 (Fig. 2). The consensus sites, SVSK and KTG, characteristic of serine β-lactamases were detected in the deduced amino acid sequence of the protein (17). The structural element characteristic of class C β-lactamases, YSN (24), was also detected (Fig. 3). The deduced amino acid sequence (OCH-1) was 42 to 52% identical to those of other chromosomally encoded and plasmid-encoded class C β-lactamases (Fig. 4). Immediately upstream from the $bla_{\text{OCH-1}}$ gene was ORF2, an 867-bp ORF containing an *ampR* gene. This *ampR* gene had an overlapping and divergently oriented promoter (2): the sequences of boxes −35 and −10 were AACGCG and GCAATA, and a Lys-R motif (CTTTTTAAACC) (15, 21) was found (Fig. 2). The deduced amino acid sequence of the AmpR protein showed this protein to have a helix-turn-helix domain at the N terminus (15, 21). The AmpR protein of *O. anthropi* was 46 to 62% identical to other AmpR proteins (Fig. 5).

pBK-OA1 was double digested with *Sac*II and *Eco*RI, and the resulting fragment was inserted into pBC SK⁺. The *E. coli* HB101 subclone harbored a recombinant plasmid, pSK⁺-OA2. DNA sequencing showed this plasmid to contain an insert of 3,141 bp. The *ampC* gene (ORF3) and a truncated *ampR* gene (ORF2) were identified in this insert (Fig. 1). This subclone was used for kinetic measurements for the OCH-1 β-lactamase.

Biochemical properties of OCH-1. The β-lactamase of *O. anthropi* produced by the *E. coli* HB101(pSK⁺-OA2) subclone was overproduced, and crude extracts were found to have a specific activity of about 1,000 mU per mg. The enzyme was purified and the final preparation of the enzyme was >90% pure, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was similar to results published for other class C β-lactamases (5). The isoelectric point of the enzyme, determined by analytical IEF (1), was 8.9. This enzyme had a low k_{cat} for penicillins, a high k_{cat} for cephalothin and cefaloridine, and similar k_{cat} values for oxyimino cephalosporins (Table 3), but the K_m measured for cefotaxime was low (9 μM), whereas those measured for cefepime, cefpirome, and ceftazidime were high. Aztreonam was a poor substrate, with no detectable hydrolysis and a high K_i (data not shown). For cephamycins (cefoxitin and cefotetan), no hydrolysis was detected, but the K_is were very low (excellent affinity) (Table 3).

Diversity of the *O. anthropi* AmpC β-lactamase. An amplification product of about 1.3 kb was obtained for the six reference strains of *O. anthropi*. Sequencing of these products showed the *ampC* gene to be present in all six. Amino acid

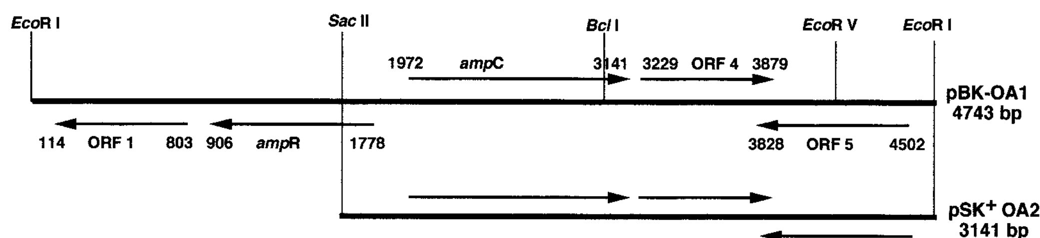

FIG. 1. Schematic restriction endonuclease map of the recombinant plasmids pBK-OA1 and pSK⁺-OA2. The genes *ampR* and *ampC* and the other ORFs are indicated.

```
   1 tcagttgttcgcggcatctgtcagccagtcgcgaaatgcggccatcgcttgcgtttccgtccgcgactgtagccttgtca 80
  81 gccagtaactgcccatcgtcaccgtcgtctcgaacggctggacaatggtttccgacagcaattgccgggagaacatcagt 160
 161 ggcggggcaagcgcaatgcccgctccctgcatggcggcttccatcatcgcgagcgacgaatcaaacatgatgcttcgcgg 240
 241 caggggcatgtcgcccgtgacgcctgctgccacaaaccactgcgtccattcatcggcacgataggagcgcagcaaagtat 320
 321 gtcgtgcgagatcgaccggtgttcgcaattcgcgcgccagttccgggatgcaaagtggcgacaggggcgcttccaataat 400
 401 cgggttgcgtcgatgccgtgccaggcgccgctcccgaaacggatcgcgtaatccagtccctccgccgcaatatccacccg 480
 481 gttgttgttggtcgaaagccgcaggtcgatgaacgggtagcgattctgaaaatcgcgcaaacgtggcagtagccagccga 560
 561 cggccaaggtgccgacagcacccacacgcaagatttcccgataatgccccgcttcaaaacgctctagcgttccggcaatc 640
 641 ctgtcgaatgcgtcctttaatgtgggaagcagcgtttctccttcgctcgtcagcatcagcccgcggggcaggcgctcgaa 720
 721 caatgtcacgttcagacgggtttcgaggctcttgacctgatggctgacagctgcctgcgtgacgcaaagttcaatcgcgg 800
                                                                              ampR
 801 ccttcgtaaagctcagatggcgcgccgatgcctcgaaggcgcgcagggaattgagcgggagatgcggtcgaaccatgttg 880
                                   -10       -35        -35        -10
 881 ccccaaatttttctaatgccaagtgctagataacgtcgtttgtcgcgccgcgcaggcattgtatataagctgggcctga 960
      Lys-R Motif
 961 aggcatatatcccgcaaagcgggcttcaatataatggaccgcgcaataccggctatgtcttgagaaagatgcagcggaa 1040
                                    ampC
1041 cgcataatcatgaggtttatttatctgtcatgagaaatctacgacacttttgatcggtttcctcaccactgccgctatt 1120
1121 atcccgaatagcggcgcgctggctgcgagcaaggtgaatgatggcgacttgcgccgtattgtcgatgaaacggtgcgccc 1200
1201 gctcatggccgagcagaaaatccccggcatggcggttgccataaccatcgacggcaagagccacttcttcggttatggtg 1280
1281 tggcatcgaaagaaagcgggcaaaaagtcactgaagacacgattttcgagatcggttcggtcagcaagaccttcactgca 1360
1361 atgcttggcggttacgggctggcgacaggcgcgttctccctgtccgatcccgcgaccaaatgggctcctgaactggcagg 1440
1441 cagcagcttcgacaagatcaccatgcgtgatcttgggacctacacgccgggcggattgcccctccagtttcccgatgctg 1520
1521 tcaccgatgacagttcgatgctggcatatttcaagaaatggaagccggactatccggcagggacgcagcgtcgctattcg 1600
1601 aatcccagcatcggcctgttcggctatctggcggcacgaagcatggacaagccgttcgacgttttgatggagcaaaagct 1680
1681 tctgcctgcattcggcctgaagaacaccttcatcaatgtgccggcaagccagatgaagaactacgcctacggctattcca 1760
1761 aagccaacaagccgatccgggtatcgggcggggcgctggatgcacaagcctatggcatcaagaccaccgcgcttgatctt 1840
1841 gcccgcttcgtcgaactgaacatcgacagctcatctctggagcctgatttccagaaagccgtcgccgcaacgcataccgg 1920
1921 ttactaccatgtcggagcgaacaatcagggacttggctgggagttctacaactatccgactgcgctcaagacgcttcttg 2000
2001 agggcaactcgtcggacatggcgctgaagtcgcacaaaatcgagaaattcgatacacctcgccaaccgtcagctgatgtg 2080
2081 ctgatcaataagacaggctcaaccaacggctttggcgcttatgcggcctttattcctgcgaagaagaccggaattgttct 2160
2161 gcttgccaaccggaattatccgatcgatgagcgcgtaaaggctgcctatcggatattgcaggcgctcgacaacaagcaat 2240
2241 aggc                                                                              2244
```

FIG. 2. Nucleotide sequence of the 2,244-bp fragment of pBK-OA1 containing the *ampC* and *ampR* coding regions. The putative promoter sequences are indicated as −35 and −10 regions (boxed). The start codons and the Lys-R motif are indicated by arrows, and the stop codons are underlined. The PCR primers upper ochro and lower ochro are in italics.

sequence analysis showed few differences (Fig. 3). The percentage of identity was between 96 and 99%.

DISCUSSION

O. anthropi is a gram-negative, mobile, aerobic, oxidase-positive, rod-shaped bacterium that often infects immunocompromised hosts (11, 14). The most frequent infection due to *O. anthropi* is central venous catheter-related bacteremia (10, 18). *O. anthropi* is generally resistant to all β-lactams except imipenem (4). The clinical strain SLO74 has this restoring resistance phenotype. The antimicrobial agent susceptibility of this strain and of the six reference strains confirmed this resistance to all β-lactams except imipenem. β-Lactamase inhibitors were inactive (clavulanic acid) or had only weak activity (tazobactam) in restoring susceptibility to penicillins. Among the gram-negative bacteria, *O. anthropi* is the most resistant to β-lactams (e.g., *Stenotrophomonas maltophilia*, which in addition is resistant to carbapenems) (31).

We investigated the cause of this high level of resistance to β-lactams by searching for β-lactamase production. All strains studied had a β-lactamase with a pI in the alkaline range (8 to

FIG. 3. Multiple alignment of AmpC amino acid sequences deduced from the sequence of the *ampC* gene present in *O. anthropi* SLO74 and in the six reference strains of *O. anthropi*. The specific SVSK and KTG boxes of the serine-active β-lactamases and the KTG box specific for class C β-lactamases are boxed. Identical amino acids are indicated by dashes.

>9.2). The pattern of susceptibility, inactivity of inhibitors, and alkaline pI suggested the presence of a class C β-lactamase (6). Using total DNA digested with *Eco*RI, we obtained four identical recombinant clones. The *E. coli* XL1 clone harboring the recombinant plasmid pBK-OA1 was resistant to all β-lactams (intermediate resistance to piperacillin and aztreonam) except cefepime and imipenem. This profile differed from that of the parental strain, *O. anthropi* SLO74, essentially in the level of resistance to cefepime. Cefepime is known to be stable and to be unaffected by class C β-lactamases (29). Therefore, the resistance to cefepime observed in the parental strain and in the six reference strains may be due to an impermeability mechanism (26). Analysis of the DNA sequence of the 4,743-bp insert of pBK-OA1 showed the presence of a gene with less than 50% identity to genes encoding class C β-lactamases. The deduced amino acid sequence contained two sites characteristic of active-site serine β-lactamases (SXXK and KXG) and a site characteristic of class C β-lactamases (YXN). The protein, OCH-1, was 42 to 52% identical to various different chromosomally encoded and plasmid-encoded class C β-lactamases.

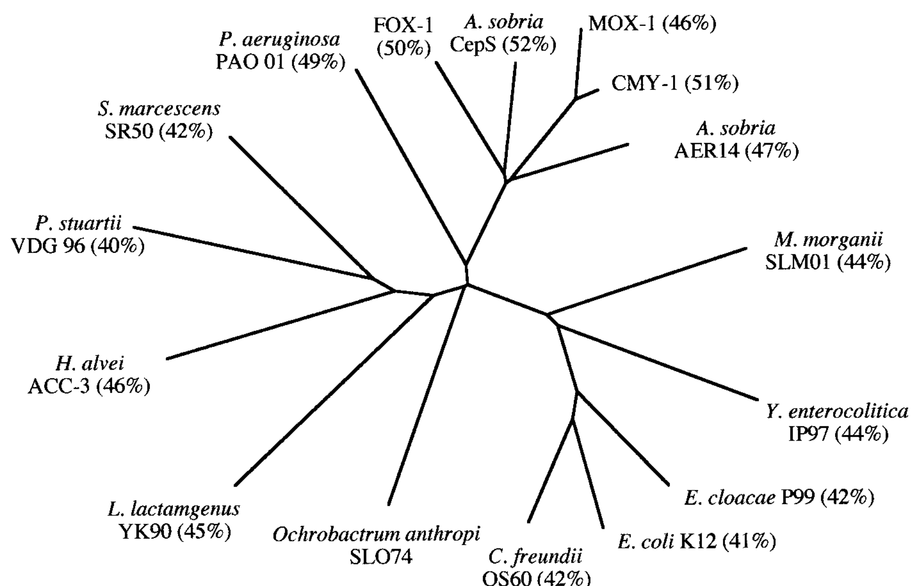

FIG. 4. Schematic dendrogram obtained for 16 representative chromosomally encoded and plasmid-encoded class C β-lactamases. Percentages in brackets are percent identities between the indicated amino acid sequence and that of OCH-1.

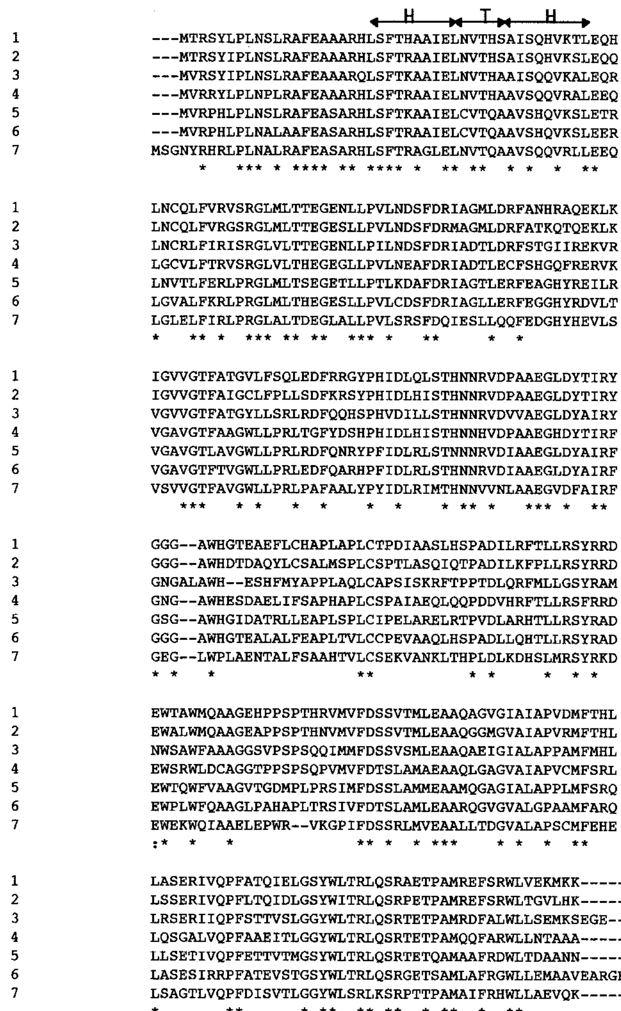

FIG. 5. Multiple alignment of deduced amino acid sequences of AmpR, which regulates cephalosporinase genes. The origins of the AmpR sequences are as follows: 1, *E. cloacae* MHN-1; 2, *Citrobacter freundii* OS 60; 3, *Yersinia enterocolitica* IP97; 4, *Morganella morganii* GUI-1; 5, *O. anthropi* SLO74; 6, *P. aeruginosa* PAO01; 7, *Providencia stuartii* VDG 96. Identical amino acids are indicated by an asterisk. The predicted helix-turn-helix motif (HTH) of the Lys-R family is indicated by arrows.

The highest level of sequence identity to OCH-1 was recorded with a class C β-lactamase called CepS (*Aeromonas sobria*) (32), with 52% identity. It is a new class C β-lactamase, very distantly related to other known class C enzymes.

TABLE 3. Kinetic parameters of β-lactamase OCH-1 for various substrates

β-Lactam	k_{cat} (s^{-1})[a]	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Benzylpenicillin	20[b]	2.0	10.0
Amoxicillin	2.0	1.0	2.0
Cephalothin	480	260	1.84
Cephaloridine	400	415	0.96
Cefepime	2.8	760	0.004
Cefpirome	3.2	800	0.004
Cefotaxime	3.6	9.0	0.4
Ceftazidime	4.0	124	0.032
Cefoxitin	—[c]	0.1	NA[d]
Cefotetan	—[c]	0.1	NA[d]

[a] For compounds with a k_{cat} lower than 10 s^{-1}, K_i values were determined instead of K_m values, using cephalothin as the substrate.
[b] Standard deviations for k_{cat} values were 15%, and standard deviations were about 20% for K_m and K_i values. Each determination was made at least in triplicate.
[c] k_{cat} of <0.05 s^{-1}.
[d] NA, not applicable.

The kinetic parameters of the purified OCH-1 β-lactamase from *O. anthropi* are typical of class C β-lactamases such as that produced by *Enterobacter cloacae* P99 (23). OCH-1 conferred resistance to all β-lactams except cefepime and imipenem. The level of resistance to extended-spectrum cephalosporins is high and comparable to that observed in *Enterobacteriaceae* overproducing the chromosomal class C β-lactamase, and it was therefore not possible to observe the inducible effect of cefoxitin or imipenem by standard diffusion (2).

Immediately upstream from the *ampC* gene was a gene in the opposite orientation that was 45 to 60% identical to known *ampR* genes. A helix-turn-helix motif was detected at the N-terminal end of the deduced amino acid sequence, as observed in other AmpR proteins and typical of transcriptional activators of the the Lys-R family (15, 21).

Analysis of the intercistronic region showed the presence of a Lys-R motif. The DNA sequence of this motif displayed a higher level of identity to the corresponding region in *Pseudomonas aeruginosa* PAO01 (22) than in *Enterobacteriaceae* (Fig. 6). This region is very long (193 bp) and is longer than the intercistronic region present in *P. aeruginosa* PAO01 (22). The putative promoters of the *ampC* and *ampR* genes overlapped and were divergently oriented, as previously described for the *ampC-ampR* regulatory system (23).

Comparison of the seven deduced AmpC amino acid sequences from the parental strain, SLO74, and the six reference strains of *O. anthropi* showed that there were seven different β-lactamases, with amino acid sequences that were 96 to 99%

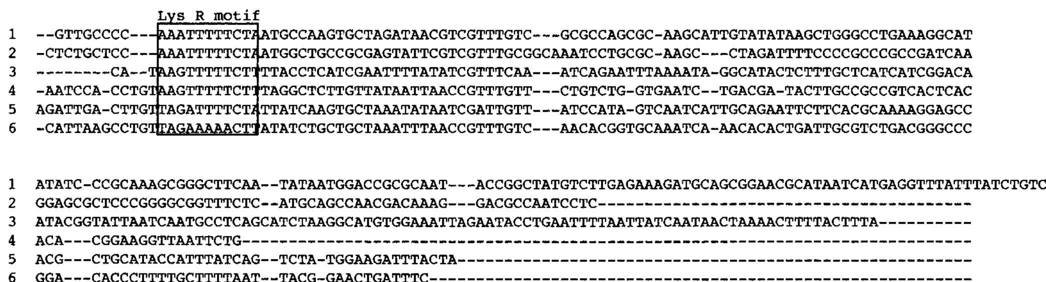

FIG. 6. Alignment of the *ampC-ampR* intercistronic region from the β-lactamase of the following strains: 1, *O. anthropi* SLO74; 2, *P. aeruginosa* PAO01; 3, *P. stuartii* VDG 96; 4, *M. morganii* SLM 01; 5, *Y. enterocolitica* IP 97; 6, *C. freundii* OS 60. The Lys-R motif is boxed.

identical. We obtained seven different pIs, confirming this observation. These seven β-lactamases are very distantly related to the other group of class C β-lactamases. The β-lactamase present in strain SLO74 was named OCH-1, and the others present in the six reference strains of *O. anthropi* were named OCH-2 (CIP 82113), OCH-3 (CIP 82115T), OCH-4 (CIP 82116), OCH-5 (CIP 102332), OCH-6 (CIP 103949), and OCH-7 (CIP 103952).

ACKNOWLEDGMENTS

This work was financed by grants from the Ministère de l'Education Nationale, de la Recherche et de la Technologie (Réseau β-lactamase), Paris, from the UFR Saint-Antoine, Paris, and from the Institut Beecham, La Défense, France.

REFERENCES

1. **Barthélémy, M., M. Guionie, and R. Labia.** 1978. β-Lactamases: determination of their isoelectric points. Antimicrob. Agents Chemother. **13:**695–698.
2. **Bennett, P. M., and I. Chopra.** 1993. Molecular basis of β-lactamase induction in bacteria. Antimicrob. Agents Chemother. **37:**153–158.
3. **Bizet, C., and J. Bizet.** 1995. Sensibilité comparée de *Ochrobactrum anthropi, Agrobacterium tumefaciens, Alcaligenes faecalis, Alcaligenes denitrificans* subsp. *denitrificans, Alcaligenes denitrificans* subsp. *xylosoxydans* et *Bordetella bronchiseptica vis-à-vis* de 35 antibiotiques dont 17 β-lactamamines. Path. Biol. **43:**258–263.
4. **Brivet, F., M. Guibert, M. Kiredjian, and J. Dormont.** 1993. Necrotizing fasciitis, bacteremia, and multiorgan failure caused by *Ochrobactrum anthropi*. Clin. Infect. Dis. **17:**516–518.
5. **Bulychev, A., and S. Mobashery.** 1999. Class C β-lactamases operate at the diffusion limit for turnover of their preferred cephalosporin substrates. Antimicrob. Agents Chemother. **43:**1743–1746.
6. **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.
7. **Cieslak, T. J., M. L. Robb, C. J. Drabick, and G. W. Fischer.** 1992. Catheter-associated sepsis caused by *Ochrobactrum anthropi:* report of a case and review of related nonfermentative bacteria. Clin. Infect. Dis. **14:**902–907.
8. **Déliere, E., H. Vu-Thien, V. Levy, S. Barquins, L. Schlegel, and A. Bouvet.** 2000. Epidemiological investigation of *Ochrobactrum anthropi* strains isolated from a haematology unit. J. Hosp. Infect. **44:**173–178.
9. **de Wergifosse, P.** 1995. Cloning and nucleotide sequence of the gene coding for the 25-kilodalton outer membrane protein of *Brucella abortus*. J. Bacteriol. **177:**1911–1914.
10. **Earhart, K. C., K. Boyce, W. D. Bone, and M. R. Wallace.** 1997. *Ochrobactrum anthropi* infection of retained pacemaker leads. Clin. Infect. Dis. **24:** 281–282.
11. **Gill, M. V., H. Ly, M. Mueenuddin, P. E. Schoch, and B. A. Cunha.** 1997. Intravenous line infection due to *Ochrobactrum anthropi* (CDC group Vd) in a normal host. Heart Lung **26:**335–336.
12. **Grandsden, W. R., and S. J. Eykyn.** 1992. Seven cases of bacteremia due to *Ochrobactrum anthropi*. Clin. Infect. Dis. **15:**1068–1069.
13. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal nucleic acid gene restriction as a potential taxonomic tool. Ann. Microbiol. **137B:**165–175.
14. **Haditsch, M., L. Binger, G. Tschurtschenthaler, R. Watschinger, G. Zauner, and H. Mittermayer.** 1994. Bacteremia caused by *Ochrobactrum anthropi* in an immunocompromised child. Infection **22:**291–292.
15. **Henikoff, S., G. W. Haughn, D. L. Wulff, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
16. **Holmes, B., M. Popoff, M. Kiredjian, and K. Kersters.** 1988. *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. Int. J. Syst. Bacteriol. **38:**406–416.
17. **Joris, B., J. M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. M. Frere, J. A. Kelly, J. C. Boyington, P. C. Moews, and J. R. Knox.** 1988. The active-site-serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. Eur. J. Biochem. **250:**313–324.
18. **Kern, W. V., M. Oethinger, A. Kaufhold, E. Rozdzinski, and R. Marre.** 1993. *Ochrobactrum anthropi* bacteremia: report of four cases and short review. Infection **21:**306–310.
19. **Klein, J. D., and S. C. Eppes.** 1993. *Ochrobactrum anthropi* bacteremia in a child. Del. Med. J. **65:**493–495.
20. **Labia, R., J. Andrillon, and F. Le Goffic.** 1973. Computerized microacidimetric determination of β-lactamase Michaelis Menten constants. FEBS Lett. **33:**42–44.
21. **Lindberg, F., L. Westman, and S. Normark.** 1987. Regulatory components in *Citrobacter freundii amp*C β-lactamase induction. Proc. Natl. Acad. Sci. USA **82:**4620–4624.
22. **Lodge, J. M., S. D. Minchin, L. J. Piddock, and J. W. Busby.** 1990. Cloning and sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal β-lactamase. Biochem. J. **272:** 627–631.
23. **Matagne, A., A. Dubus, M. Galleni, and J. M. Frere.** 1999. The β-lactamase cycle: a tale of selective pressure and bacterial ingenuity. Nat. Prod. Rep. **16:** 1–19.
24. **Matsumura, N., S. Minami, and S. Mitsuhashi.** 1998. Sequences of homologous β-lactamases from clinical isolates of *Serratia marcescens* with different substrate specificities. Antimicrob. Agents Chemother. **42:**176–179.
25. **Peltroche-Llacsahuanga, H., V. Brandenburg, J. Riehl, and G. Hasse.** 2000. *Ochrobactrum anthropi* peritonitis in a CAPD patient. J. Infect. **40:**299–301.
26. **Piddock, L. J., and E. A. Traynor.** 1991. β-Lactamase expression and outer membrane protein changes in cefpirome-resistant and ceftazidime-resistant gram-negative bacteria. J. Antimicrob. Chemother. **28:**209–219.
27. **Roberto, M., N. Anna, F. Morena, C. Leonardo, T. Marina, and C. Francesco.** 1999. *Ochrobactrum anthropi* as an agent of nosocomial septicemia in the setting of AIDS. Clin. Infect. Dis. **28:**692–694.
28. **Saavedra, J., C. Garrido, D. Folgueira, M. J. Torres, and J. T. Ramos.** 1999. *Ochrobactrum anthropi* bacteremia associated with a catheter in an immunocompromised child and review of the pediatric literature. Pediatr. Infect. Dis. J. **18:**586–660.
29. **Sanders, C. C.** 1993. Cefepime: the next generation? Clin. Infect. Dis. **17:** 369–379.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Spencer, R. C.** 1995. The emergence of epidemic, multiple-antibiotic-resistant *Stenotrophomonas* (*Xanthomonas*) *maltophilia* and *Burkholderia* (*Pseudomonas*) *cepacia*. J. Hosp. Infect. **30**(Suppl.)**:**453–464.
32. **Walsh, T. R., J. Hall, A. P. MacGowan, and P. M. Bennett.** 1995. Sequence analysis of two chromosomally mediated inducible β-lactamases from *Aeromonas sobria*, strain 163a, one a class D penicillinase, the other an AmpC cephalosporinase. J. Antimicrob. Chemother. **36:**41–52.