ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1999, p. 1350–1357 Vol. 43, No. 6
0066-4804/99/$04.00+0

Characterization and Nucleotide Sequence of a *Klebsiella oxytoca* Cryptic Plasmid Encoding a CMY-Type β-Lactamase: Confirmation that the Plasmid-Mediated Cephamycinase Originated from the *Citrobacter freundii* AmpC β-Lactamase

SHANG WEI WU,[1,2,3]* KATHRINE DORNBUSCH,[1] GÖRAN KRONVALL,[1] AND MARI NORGREN[3]

Department of Laboratory Medicine, Division of Clinical Microbiology, Karolinska Institute and Karolinska Hospital, 171 76 Stockholm,[1] and Department of Clinical Bacteriology, Umeå University, S-901 85 Umeå,[3] Sweden, and Laboratory of Microbiology, The Rockefeller University, New York, New York 10021[2]

Received 10 September 1998/Returned for modification 4 January 1999/Accepted 12 March 1999

Plasmid pTKH11, originally obtained by electroporation of a *Klebsiella oxytoca* plasmid preparation into *Escherichia coli* XAC, expressed a high level of an AmpC-like β-lactamase. The enzyme, designated CMY-5, conferred resistance to extended-spectrum β-lactams in *E. coli*; nevertheless, the phenotype was cryptic in the *K. oxytoca* donor. Determination of the complete nucleotide sequence of pTKH11 revealed that the 8,193-bp plasmid encoded seven open reading frames, including that for the CMY-5 β-lactamase (bla_{CMY-5}). The bla_{CMY-5} product was similar to the plasmidic CMY-2 β-lactamase of *K. pneumoniae* and the chromosomal AmpC of *Citrobacter freundii*, with 99.7 and 97.0% identities, respectively; there was a substitution of phenylalanine in CMY-5 for isoleucine 105 in CMY-2. bla_{CMY-5} was followed by the *Blc* and *SugE* genes of *C. freundii*, and this cluster exhibited a genetic organization identical to that of the *ampC* region on the chromosome of *C. freundii*; these results confirmed that *C. freundii* AmpC was the evolutionary origin of the plasmidic cephamycinases. In the *K. oxytoca* host, the copy number of pTKH11 was very low and the plasmid coexisted with plasmid pNBL63. Analysis of the replication regions of the two plasmids revealed 97% sequence similarity in the RNA I transcripts; this result implied that the two plasmids might be incompatible. Incompatibility of the two plasmids might explain the cryptic phenotype of bla_{CMY-5} in *K. oxytoca* through an exclusion effect on pTKH11 by resident plasmid pNBL63.

Extended-spectrum β-lactamases cause bacterial resistance to β-lactam antibiotics and contribute to therapeutic problems (16). Many of them are plasmid mediated and confer resistance to oxyimino-β-lactams, such as cefotaxime, ceftazidime, and the monobactam aztreonam (16). Plasmid-mediated β-lactamases are often expressed in large amounts and are encoded by transposons which can be easily transferred from one replicon to another (15). Chromosomally encoded β-lactamases are present in most gram-negative bacteria; these enzymes are often expressed at low levels and therefore may not contribute to clinical β-lactam resistance (22). Additionally, plasmid-mediated β-lactamases, such as MIR-1, CMY-1, and CMY-2, with high isoelectric points and cephalosporinase activity have been described (2, 4, 26). These findings have raised concern that chromosomal β-lactamases may cause serious therapeutic problems if their genes are translocated onto plasmids.

In previous studies (41, 42), a group of *Klebsiella oxytoca* isolates resistant to aztreonam (MIC, 32 μg/ml) and cefuroxime (MIC, 128 μg/ml) was characterized, and the resistance was found to be caused by an extended-spectrum β-lactamase with a pI of 5.25 and designated OXY-2a. The gene for this β-lactamase (bla_{OXY-2a}) was located on the chromosome of the *K. oxytoca* isolates (40). However, in an effort to transfer the β-lactam resistance along with plasmid DNA from the *K. oxytoca* isolates by electroporation into ampicillin-susceptible *Escherichia coli* XAC, transformants resistant to extended-spectrum β-lactams were obtained (42). The transformants produced a β-lactamase with a pI of 8.4 and designated CMY-5 and showed increased resistance to aztreonam, cefuroxime, cefotaxime, and ceftazidime (MIC, >32 μg/ml). The substrate and inhibition profiles of the CMY-5 β-lactamase were highly similar to those of the chromosomal AmpC enzyme in *E. coli* (42). However, the enzyme activity in the transformants was much higher than that in the susceptible recipient (42). The transformants harbored a 7.8-kb plasmid which differed from the plasmids seen in the donors and could be transferred into *E. coli* XAC or C600 recipients by the conventional transformation technique (42). Nevertheless, the gene for the CMY-5 β-lactamase (bla_{CMY-5}) was poorly expressed in *K. oxytoca*, as determined by the β-lactam resistance phenotype and enzymatic properties (42). Additionally, the extremely low frequency of transformation of pTKH11 by electroporation implied that pTKH11 existed in *K. oxytoca* at a low copy number.

In this study, the cryptic plasmid in *K. oxytoca* and the AmpC-like β-lactamase carried by this plasmid were further investigated.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. Bacteria were grown in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) or on Luria Bertani agar plates supplemented with appropriate antibiotics.

Recombinant DNA techniques. Plasmid DNA was prepared by use of Mini and Midi columns (Qiagen GmbH, Dusseldorf, Germany) as recommended by the manufacturer. Transformation of plasmid DNA was performed as described by Kushner (18). The antibiotics were used at 4 or 100 μg/ml for ampicillin, 8 μg/ml for aztreonam, and 100 μg/ml for cefotaxime and ceftazidime. Restriction enzymes, calf intestine alkaline phosphatase, and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.), and the conditions for enzy-

* Corresponding author. Mailing address: Box 152, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8278. Fax: (212) 327-8688. E-mail: wus@rockvax.rockefeller.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties and comments[a]	Source or reference(s)
Strains		
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 ϕ80 lacZΔM15*	Bethesda Research Laboratories
SN01	*ampA1*$^+$ *ampC*$^+$ *pyrB thr leu recA rpsL*	25
SN03	*ampA1 ampC8 pyrB thr leu recA rpsL*; spontanous SN01 mutant (mutations in structural and regulatory regions of *ampC*)	25
K-12 XAC	Na^r Ri^r	41
TKH11	*E. coli* XAC transformant from plasmid preparation of KH11	42
TNBL63	*E. coli* XAC transformant from plasmid preparation of NBL63	42
K. oxytoca		
KH11	Az^r Xm^r clinical isolate	41, 42
KH55	Az^r Xm^r clinical isolate	41, 42
KH78	Az^r Xm^r clinical isolate	41, 42
KH93	Az^r Xm^r clinical isolate	41, 42
NBL63	Az^r Xm^r clinical isolate	41, 42
Plasmids		
pGEM-3Z	Amp^r subcloning vector	Promega
pNU6	Amp^r harboring *ampC*	10
pKH11	Plasmid from *K. oxytoca* KH11 (subgroup 2)	41, 42
pNBL63	Plasmid from *K. oxytoca* NBL63 (subgroup 3)	41, 42
pTKH11	Plasmid from transformant TKH11	42
pTKH78	Plasmid from transformant TKH78	42
pTNBL63	Plasmid from transformant TNBL63	42
pSW-21	pGEM-3Z with 0.85-kb *Pst*I fragment of pTKH11	This study
pSW-22	pGEM-3Z with 2.74-kb *Pst*I-*Bgl*II fragment of pTKH11	This study
pSW-24	pGEM-3Z with 2.24-kb *Bgl*II fragment of pTKH11	This study
pSW-28	pGEM-3Z with 5.96-kb *Bgl*II fragment of pTKH11	This study
pSW-26	pGEM-3Z with 2.45-kb *Bgl*II fragment of pNBL63	This study

[a] Amp, ampicillin; Az, aztreonam; Na, nalidixic acid; Ri, rifampin; Xm, cefuroxime.

matic reactions were those suggested by the manufacturer. Analysis of plasmid DNA fragments was performed by electrophoresis in 0.7 to 1.5% agarose gels with TBE buffer (100 mM Tris base, 89 mM boric acid, 50 mM EDTA [pH 8.0]). As a size standard for the linear restriction fragments, a 1-kb DNA molecular weight marker (GIBCO BRL, Grand Island, N.Y.) was used.

Cloning of pTKH11 and the replication region of pNBL63. For the purpose of DNA sequencing the entire pTKH11, the plasmid was digested with *Bgl*II and with *Bgl*II plus *Pst*I, and the 0.85-kb *Pst*I, 2.74-kb *Pst*I-*Bgl*II, 2.24-kb *Bgl*II, and 5.96-kb *Bgl*II DNA fragments were separately ligated into plasmid vector pGEM-3Z to form recombinant plasmids pSW-21, pSW-22, pSW-24, and pSW-28, respectively (Table 1; see also Fig. 3). The 2.45-kb *Bgl*II fragment of plasmid pNBL63, which may include the replication region for this plasmid, was also ligated into pGEM-3Z to generate plasmid pSW-26.

DNA sequencing. The DNA sequence was determined by the dideoxy chain termination method (31) with an automated DNA sequencing system (model 377; PE/ABI, Foster City, Calif.). The template DNAs used for sequencing were plasmids pSW-21, pSW-22, pSW-24, pSW-26, and pSW-28; the primer walking was started with two vector-based primers specific for pGEM-3Z. Nucleotide and derived amino acid sequences were analyzed with the GCG program (Genetics Computer Group, Inc., Madison, Wis.) and DNA Star software (Lasergene, Madison, Wis.).

PCR. To detect the presence of plasmid pTKH11 in the *K. oxytoca* isolates, the following primer pair was used: primer S28R2M (5′-CAA TGT GTG AGA AGC AGT CT-3′; nucleotides [nt] 2186 to 2205 in the pTKH11 sequence) and primer S21R1 (5′-GTA CAT ATC GCC AAT ACG-3′; complementary to nt 3114 to 3231). This primer pair generates a 1,045-bp amplicon containing 188 bp upstream of bla_{CMY-5} and a 770-bp sequence 5′ of bla_{CMY-5}. A primer pair specific for the region and including a 298-bp sequence 3′ of bla_{CMY-5} and 231 nt downstream of bla_{CMY-5} was also used: primer S21F1 (5′-TTG GCG ATA TGT ACC AGG-3′; nt 3218 to 3235) and primer S22R1M (5′-TTC ATA CCA GGT TCC CAG-3′; complementary to nt 3730 to 3747). This primer pair produces a 529-bp PCR fragment.

Preparation of total DNA was previously described (39). The PCR mixture was prepared with a PCR reagent kit according to the standard method recommended by the manufacturer (Perkin-Elmer Cetus, Branchburg, N.J.). One to 4 μg of plasmid or total DNA from the *K. oxytoca* isolates and 40 pmol of each primer were included. PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Cetus) at the following temperature profiles: 94°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; 72°C for 5 min; and 4°C for the remainder of the reaction.

Southern blot analysis. Transfer of DNA to nylon filters was performed essentially as described by Southern (33). Labeling of DNA probes (as shown in Fig. 1) was performed with digoxigenin as described by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hybridization was performed at 70°C with buffers recommended in the instructions included in the digoxigenin kit from Boehringer.

Preparation of β-lactamase extracts and Western blotting. The preparation of crude β-lactamase extracts was previously described (41, 42). The protein content of crude enzyme preparations was determined by use of an AB Kemila-preparat protein assay (Bio-Rad, Sollentuna, Sweden) with lyophilized bovine serum albumin as a standard. Electrophoresis was performed with a 12% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (20). A low-molecular-weight protein standard from Pharmacia (Uppsala, Sweden) was run in parallel. Immunoblot transfer experiments were carried out essentially as described by Swanson et al. (34) with 1:1,000-diluted polyclonal antiserum raised against *E. coli* K-12 AmpC β-lactamase (5) as the primary antibody. Affinity-purified, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika Corp., West Chester, Pa.) was used as the secondary antibody.

Nucleotide sequence accession numbers. The nucleotide sequences described in this communication can be found in EMBL-GenBank under accession no. Y17716 for pTKH11 and Y17846 for the replication region of pNBL63.

RESULTS

Analysis of plasmid DNA with restriction endonucleases. To reveal the relationships among the plasmids in *K. oxytoca* donor strains (KH11 and NBL63) and the *E. coli* XAC transformants (42), restriction maps of the *K. oxytoca* plasmids and the plasmids from the *E. coli* transformants were constructed by single and double digestions with a series of restriction enzymes. As shown in Fig. 1, the restriction pattern of pKH11 was different from that of the *E. coli* transformant plasmid

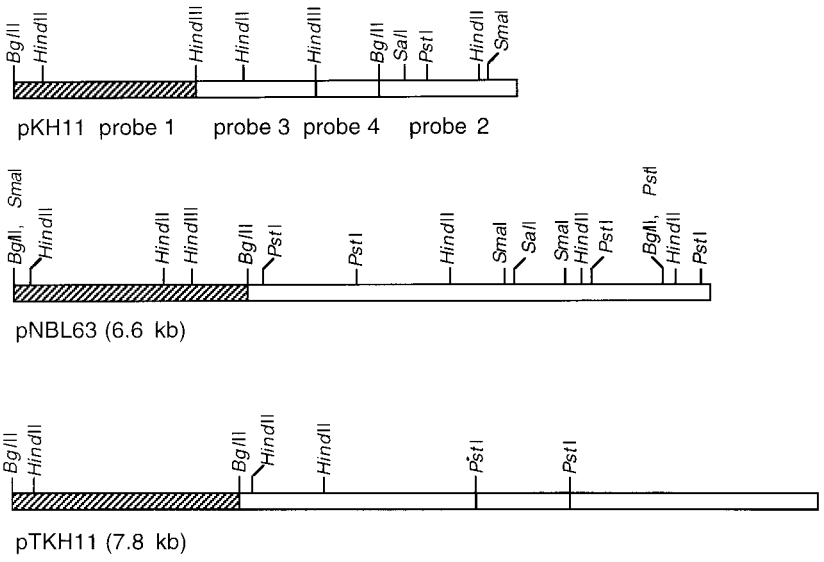

FIG. 1. Restriction maps of plasmids pKH11, pNBL63, and pTKH11. The restriction enzymes used in the construction of the maps are shown above the bars. The four fragments of pKH11 used as probes are indicated under the bars. The bars represent the DNA sequences of the plasmids. DNA fragments showing sequence similarities are indicated by hatched bars.

pTKH11 and that of pNBL63. However, the plasmid from each of the *E. coli* transformants had the same restriction map as pTKH11.

DNA hybridization. Southern blot analysis was performed to further examine the relationships among the plasmids. The 4.8-kb plasmid pKH11 was digested with *Bgl*II and *Hin*dIII, generating four DNA fragments: 1.75 kb by *Bgl*II-*Hin*dIII, 1.35 kb by *Bgl*II, 1.1 kb by *Hin*dIII, and 0.6 kb by *Bgl*II-*Hin*dIII (Fig. 1). Each of the fragments was labeled as a probe to hybridize with the DNA fragments produced by restriction digestion with *Bgl*II plus *Hin*dIII for pNBL63 and *Bgl*II plus *Pst*I for pTKH11. Under stringent conditions, the 1.75-kb *Bgl*II-*Hin*dIII fragment of pKH11 hybridized with the 2.4-kb *Bgl*II fragment of pNBL63 and the 2.2-kb *Bgl*II fragment of pTKH11 (Fig. 1). These homologous fragments may be the areas where the replication origins for the plasmids are located. The other probes from pKH11 failed to hybridize with any DNA fragment from either pTKH11 or pNBL63.

Phenotype analysis of plasmids in an *E. coli* AmpC$^-$ background. To distinguish enzyme activity encoded by the plasmid from that encoded by the chromosome, plasmid pTKH11 was transformed into both *E. coli* SN01, with a wild-type *ampC* gene, and its *ampA1 ampC8* mutant derivative, SN03 (25). Transformants were obtained from both strains selected on agar plates containing either aztreonam (8 μg/ml) or ceftazidime (100 μg/ml). Preparations of pKH11 and pNBL63 failed to transform *E. coli* SN01 and SN03 to β-lactam resistance.

Analysis of *ampC* β-lactamase expression. The expression of AmpC-like proteins was tested by Western blot analysis (Fig. 2). AmpC-like proteins were observed in crude β-lactamase preparations from *K. oxytoca* KH11, *E. coli* SN03/pTKH11, and *E. coli* SN03/pNU6. Depending on the dilution factor of the samples, the amount of the AmpC-like protein in *E. coli* SN03/pTKH11 was approximately 400-fold larger than that in *K. oxytoca* KH11 and 10-fold larger than that in *E. coli* SN03/pNU6 (10). No expression of the AmpC-like protein was seen in the *E. coli* SN03 recipient strain.

DNA sequence of pTKH11. The size of plasmid pTKH11 was determined to be 8,193 bp, and a *Ban*I restriction site was designated as the first nucleotide to linearize the circle sequence in order to facilitate description. The whole DNA sequence was analyzed for open reading frames (ORFs), and the amino acid sequences deduced from the ORFs were compared with the sequences of known polypeptides in both the Tblastn and the Blastp data banks. The regions containing no significant ORFs (more than 50 amino acid residues) were sent to the data banks for DNA sequence comparison. As shown in Fig. 3, the plasmid contained seven ORFs, five of which were transcribed in the same direction as the DNA sequence of pTKH11 and the other two of which were located on the opposite DNA strand. Each of the ORFs was preceded by putative Shine-Dalgarno and promoter consensus sequences, except for ORF1.

The 188-amino-acid product of ORF1 showed no similarities

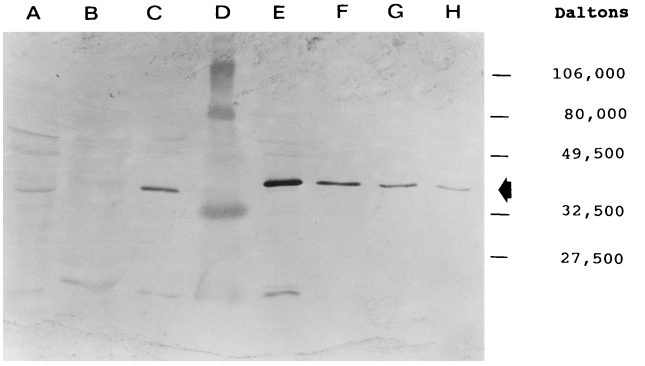

FIG. 2. Western blot of crude β-lactamase from an *E. coli* recipient, a *K. oxytoca* donor, and their transformants with AmpC β-lactamase-specific antisera. The AmpC β-lactamase-specific band is indicated by an arrow. Lane A, 50 μg of crude β-lactamase from *K. oxytoca* KH11; lane B, 50 μg of crude β-lactamase from *E. coli* SN03; lane C, 5 μg of crude β-lactamase from *E. coli* SN03/pNU6; lane D, molecular weight standards; lane E, 5 μg of crude β-lactamase from *E. coli* SN03/pTKH11; lane F, 0.5 μg of crude β-lactamase from *E. coli* SN03/pTKH11; lane G, 0.25 μg of crude β-lactamase from *E. coli* SN03/pTKH11; lane H, 0.125 μg of crude β-lactamase from *E. coli* SN03/pTKH11.

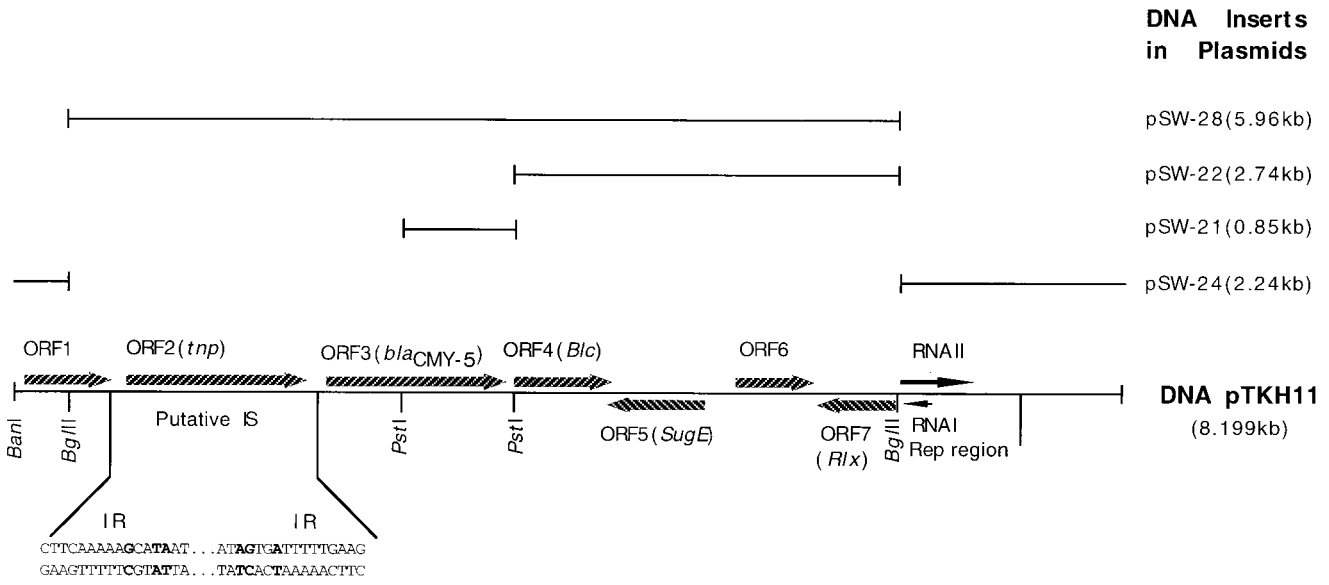

FIG. 3. Genetic organization of the genes identified on plasmid pTKH11. The direction of gene transcription is shown by the arrows. The DNA inserts in various recombinant plasmids are represented by lines of different lengths, and the significant restriction sites are indicated. The inverted repeat (IR) in the putative insertion element (IS) is shown, and mismatched nucleotides are indicated by bold letters.

with any protein in the data banks. Located 97 bp downstream of ORF1, ORF2, which encoded a polypeptide of 420 amino acid residues, showed 12% identity and 38% similarity to the transposase of IS*1247* (accession no. X84038) from *Xanthobacter autotrophicus* (37). An inverted repeat of 16 bp was identified at 151 nt upstream (nt 628 to 644) and 163 nt downstream (complementary to nt 2220 to 2235) of ORF2 (Fig. 3). The inverted repeat was imperfect, with three nucleotide mismatches. No direct repeat which might serve as the target site for an insertion element could be found at the junction regions of the inverted repeat.

The 381-amino-acid product of ORF3, which followed ORF2 at an interval of 317 nt, showed 99.7 and 97% identities to the CMY-2 β-lactamase (1) and AmpC of *Citrobacter freundii* (13), respectively. Thus, ORF3 was identified as bla_{CMY-5}. The only difference noted in the two plasmidic enzymes was the substitution of Leu-105 in CMY-2 with Phe-105 in CMY-5 (Fig. 4). Analysis of the bla_{CMY-5} flanking region, including 466 upstream and 124 downstream nt, revealed that the DNA sequence of the bla_{CMY-5} region was similar to that of CMY-2 by over 99%, and the same consensus sequences were shared by both β-lactamases.

ORF4 was located 94 bp downstream of bla_{CMY-5} and its 177-amino-acid peptide product exhibited 98% identity with the outer membrane lipoprotein encoded by gene *Blc* from *C. freundii* (7). ORF5, which encoded a product of 105 amino acids, was located on the complementary strand of the pTKH11 sequence, and the stop codon of this ORF overlapped that of ORF4 by 2 nt. The deduced amino acid sequence of the product of ORF5 was identical to that of the SugE protein of *C. freundii* (unpublished data; accession no. U21727). ORF6, which encodes a putative protein with unknown function, was transcribed in the same direction and was located between ORF5 and ORF7. At 56 bp downstream of ORF6, ORF7 was located in the same orientation as ORF5 and encoded a putative protein with 70% identity and 77% similarity to the relaxation protein (accession no. X01654) of *Salmonella typhimurium* (6).

Within the 1.8-kb DNA sequence at the 3′ region (nt 6254 to

```
      1                                                  50
CMY-5 MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAV
CMY-2 MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAV
BLACF MMKKSICCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAV

     51                                                 100
CMY-5 AVIYQGKPYYFTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIAR
CMY-2 AVIYQGKPYYFTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIAR
BLACF AIIYEGKPYYFTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIAR
       *  *
    101                                                 150
CMY-5 GEIKFSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAA
CMY-2 GEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAA
BLACF GEIKLSDPVTKYWPELTGKQWRGISLLHLATYTAGGLPLQIPDDITDKAA
         *                 *  *                       **
    151                                                 200
CMY-5 LLHFYQNWQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ
CMY-2 LLHFYQNWQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ
BLACF LLRFYQNWQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ

    201                                                 250
CMY-5 PLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEAYGVKSSVIDM
CMY-2 PLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEAYGVKSSVIDM
BLACF PLKLAHTWITVPQSEQKNYAWGYREGKPVHVSPGQLDAEAYGVKSSVIDM
          *  *
    251                                                 300
CMY-5 ARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
CMY-2 ARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
BLACF ARWVQANMDASHVQEKTLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKA
                          *
    301                                                 350
CMY-5 DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVP
CMY-2 DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVP
BLACF DSIINGSDSKVALAALPAVEVNPPVPAVKASWVHKTGSTGGFGSYVAFVP
                          *
    351         381
CMY-5 EKNLGIVMLANKSYPNPVRVEAAWRILEKLQ
CMY-2 EKNLGIVMLANKSYPNPVRVEAAWRILEKLQ
BLACF EKNLGIVMLANKSYPNPVRVEAAWRILEKLQ
```

FIG. 4. Multiple amino acid sequence alignments of the CMY-5 β-lactamase with *K. pneumoniae* CMY-2 and *C. freundii* AmpC (BLACF) β-lactamases. The amino acid stretches in bold letters are the active site (SVSK), the conserved triad (KTG), and the class C-typical motif (YXN). Amino acid substitutions are indicated with asterisks, and Phe-105 in CMY-5 and Leu-105 in CMY-2 are shown in bold letters.

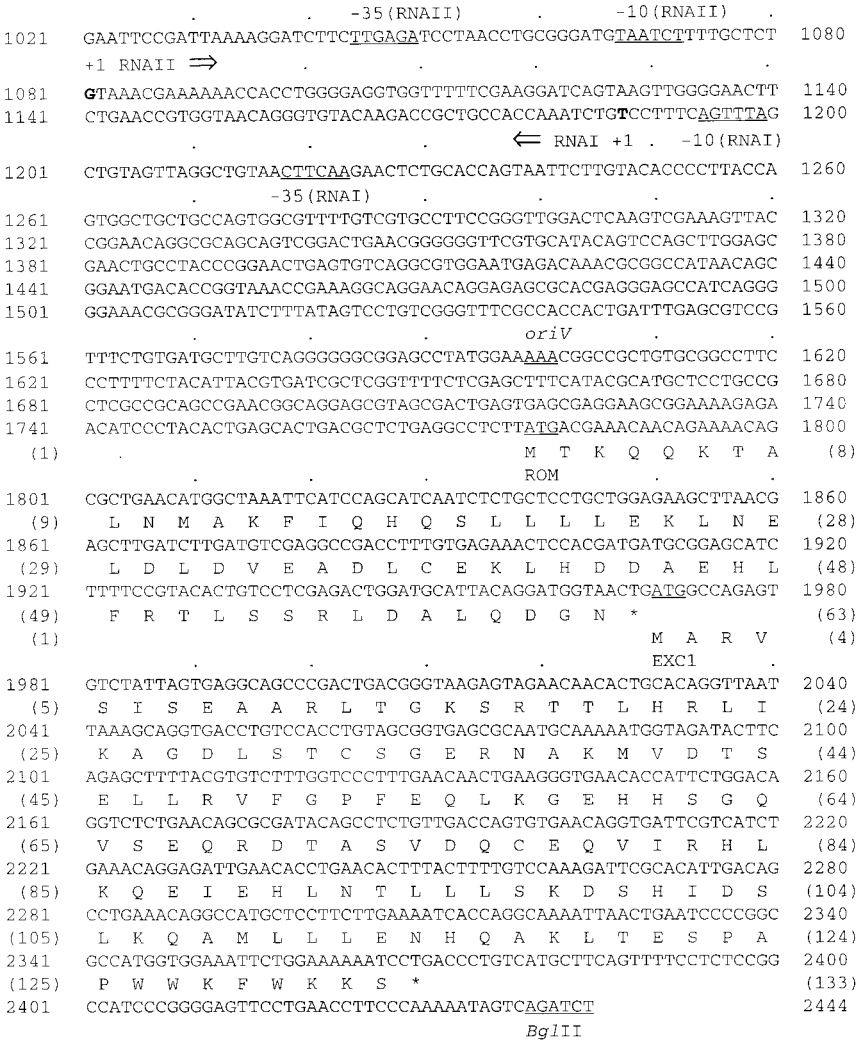

FIG. 5. Nucleotide sequence of the 1,424-bp replication region of pNBL63. The sequence includes the 1,424-bp 3′ part of the 2,444-bp *Bgl*II fragment of pNBL63. The consensus sequences are underlined and labeled. The putative transcription start sites are indicated by bold letters. The putative start codons are underlined, the stop codons are designated with asterisks, and the gene designations are given below the deduced amino acids, which are specified by standard one-letter abbreviations.

8199) of pTKH11, no significant ORFs could be identified. By DNA sequence comparison, it was shown that the 1- to 1.5-kb region 5′ of ORF7, coordinating nt 6371 to 7370 or nt 7870 (Fig. 3), was significantly homologous to replication regions of various plasmids (9, 23, 24, 28). The highest similarity was shown by the replicon of pJHCMW1, which was first identified in a pathogenic multiresistant *K. pneumoniae* strain isolated from the cerebrospinal fluid of a neonate with meningitis (38). These sequence similarities indicated that the 733-bp sequence from nt 6371 to nt 7105 might be the replicon of pTKH11. The RNA transcripts RNA I and RNA II, which serve as functional elements for plasmid replication (27), were identified (Fig. 3). RNA I and RNA II of pTKH11 were 70% and 81% similar, respectively, to the corresponding transcripts from pJHCMW1 (9).

DNA sequence of the replication region of plasmid pNBL63. The 1,423-bp 3′ part of the 2,444-bp *Bgl*II fragment of pNBL63 was highly similar to the replication regions of several RNA priming plasmids (8, 9, 24). By DNA comparison with these well-characterized plasmids, the replication region of pNBL63 was identified (Fig. 5). The region can be divided into three functionally different parts. The first part, from nt 1037 to nt 1731, was the DNA sequence containing genes for RNA I and RNA II, which showed 84% similarity with the corresponding region in pJHCMW1. The 103-bp RNA I and the 521-bp RNA II transcripts of pNBL63 exhibited 71 and 87% similarities to those of pJHCMW1, respectively. The promoters for RNA I and RNA II were completely conserved in the two plasmids. A consensus sequence for the replication origin (*oriV*) of ColE1 (36) was also found at an appropriate location (nt 1599 to 1601). In the second part, the DNA sequence encoding a peptide of 63 amino acids was observed 178 bp downstream of *oriV* and showed 65% identity and 76% similarity to the genetic product of *Rom* (17). In the third part, the DNA sequence encoding a putative protein of 133 amino acid residues was identified downstream of that encoding the Rom homolog and was 29% identical and 44% similar in the amino terminus to entry exclusion protein 1 (Exc1) produced by the ColE1 plasmid (8).

Similarity between the replication regions of pNBL63 and pTKH11. The regions containing genes for RNA I and RNA II, which were 745 bp for pNBL63 (nt 1035 to 1780) and 784 bp

FIG. 6. Bestfit comparison of the pNBL63 and pTKH11 RNA Is and RNA IIs. Identical nucleotides are indicated by vertical bars, and gaps are shown by periods.

for pTKH11 (nt 6370 to 7154), were 83% similar between the two plasmids. The RNA I transcript of pNBL63 was highly similar to that of pTKH11, with an identity of 97% (Fig. 6a). The RNA II transcripts of the two plasmids were estimated to be 521 nt for pNBL63 and 560 nt for pTKH11, with an identity of 90% (Fig. 6b). Nevertheless, no *Rom*- and *Exc*1-like genes were identified in the region downstream of the RNA I-RNA II region of pTKH11.

Detection of plasmid pTKH11 in DNA preparations from *K. oxytoca* isolates by PCR amplification. To confirm the presence of pTKH11 in the *K. oxytoca* isolates, a plasmid preparation from strain NBL63 and total DNA samples from strains KH11, KH55, KH78, and KH93 were used as templates for the detection of pTKH11. PCR products of 1.05 and 0.5 kb were obtained with primer pairs S28R2M-S21R1 and S21F1-S22R1M, respectively. Nevertheless, template DNAs had to be used in relatively large amounts (2 to 4 μg/100-μl reaction). The PCR products were confirmed to be the DNA fragments of the bla_{CMY-5} region in pTKH11 by DNA sequencing.

DISCUSSION

In a previous study, a plasmid having a low copy number and a cryptic phenotype with respect to β-lactam resistance in clinical isolates of *K. oxytoca* was found to express an AmpC-like β-lactamase in *E. coli* XAC electrotransformants (42). In this investigation, the plasmid, designated pTKH11, was identified as being harbored by each of the electrotransformants and as existing in most of the *K. oxytoca* donors. Each of the *K. oxytoca* isolates also contained an additional plasmid, such as pNBL63, which could be readily extracted by a routine procedure. pTKH11 was obtained only through electroporation of the plasmid into *E. coli* (42), and the detection of pTKH11 in *K. oxytoca* by PCR required a large amount of template DNA. These results suggested that pNBL63 was predominantly replicated, while pTKH11 obviously existed in very few copies in the *K. oxytoca* isolates. A similar finding was recently reported by other investigators (21).

Plasmid pTKH11 was introduced into the *ampC* β-lactamase null mutant SN03 (25), and the resultant transformant showed resistance to aztreonam and ceftazidime and hyperproduction of an AmpC-like protein, designated CMY-5 (Fig. 2). The level of production of the CMY-5 β-lactamase was low in *K. oxytoca* KH11. These data strongly support the notions that CMY-5 was encoded by pTKH11 and that the plasmid indeed existed in the *K. oxytoca* isolates. Moreover, the low level of expression of CMY-5 was consistent with the extremely low copy number of plasmid pTKH11 in *K. oxytoca*.

A comparison of amino acid sequences showed that CMY-5 differed from CMY-2 by only one residue and showed 97% identity with *C. freundii* AmpC (Fig. 4). The high degree of amino acid identity conferred the same catalytic activities on the enzymes (1, 42) and implied a close relationship among the three β-lactamases examined here.

The β-lactamases CMY-2, LAT-1, and BIL-1 are members of the plasmidic cephamycinases (CMYs) of amber class C

β-lactamases (1, 11) and are believed to have a very close evolutionary relationship with *C. freundii* AmpC because of their high homologies (>94%) (1). Furthermore, a plasmid carrying a CMY-2 β-lactamase gene was characterized in *C. freundii*, and the plasmid was transferable to *K. pneumoniae* and *E. coli* (3). In this investigation, it was shown that bla_{CMY-5} was followed by *Blc* and *SugE* (Fig. 3); bla_{CMY-5} was 98% similar to *C. freundii Blc* and 100% identical to *C. freundii SugE*. Assembly of the DNA sequences for the *ampC* region of *C. freundii* GC3 (13) and the *Blc* and *SugE* regions of *C. freundii* (accession no. U21727) revealed that the genetic organization of these genes was identical to that found in pTKH11. The high degree of similarity in both the gene products and the genetic organization provided evidence that the chromosomally encoded AmpC of *C. freundii* is the origin of the plasmid-mediated CMYs. Thus, *C. freundii* AmpC should be regarded as the evolutionary precursor of CMYs rather than a homologous enzyme in the same classification group.

The *Blc* product may provide a starvation response function (7). The product of *SugE* is involved in nitrogen fixation in bacterial cells (12). Therefore, neither *Blc* nor *SugE* is related to the expression of the *ampC* gene. The insertion sequence-like element observed upstream of bla_{CMY-5} might be responsible for the translocation of the β-lactamase. ORF7 of pTKH11 specified a protein similar to an *S. typhimurium* relaxation protein which is involved in the formation of the relaxation complex during the mobilization of plasmid pSC101 (6). Accordingly, the product of ORF7 may also play a role in the spread of pTKH11.

bla_{CMY-5} was preceded by an intact promoter sequence; hence, the CMY-5 β-lactamase could be highly expressed in *E. coli* XAC and *E. coli* SN03. Thus, it was surprising that the bla_{CMY-5} gene product failed to confer resistance in the *K. oxytoca* isolates. Analysis of the replication regions for pNBL63 and pTKH11 showed that they both belonged to the RNA priming category of plasmids (29, 30, 32). In these plasmids, the replication process is regulated by the frequency of RNA I priming (14). The sequence homology between RNA Is also determines plasmid incompatibility through the cross suppression of replication by RNA I (19, 35). The extremely low copy number of pTKH11 in *K. oxytoca* might be due to incompatibility between pNBL63 and pTKH11, since a high degree of sequence similarity in the RNA I transcripts (97%) was noted. pNBL63 may have originated from *K. oxytoca* based on the fact that its RNA II transcript showed a high degree of similarity to that of pJHCMW1 of *K. pneumoniae*; pTKH11, on the other hand, may have been transferred from *C. freundii* into *K. oxytoca*. Thus, pTKH11 could not be stably maintained in the *K. oxytoca* host.

In conclusion, *K. oxytoca* plasmid pTKH11 was characterized as harboring a β-lactamase from the CMY family. CMY-5 was cryptic in the *K. oxytoca* clinical isolates but was highly expressed in *E. coli*. The cryptic phenotype was probably caused by the presence of an incompatible plasmid in the *K. oxytoca* host cell. The CMY β-lactamases were demonstrated to have originated from the chromosomal *ampC* gene of *C. freundii* based on the identical genetic organizations seen in the bla_{CMY-5} and *C. freundii ampC* gene regions. These results present direct evidence for the translocation of a β-lactamase-associated gene region from the chromosome to a plasmid.

ACKNOWLEDGMENTS

M. Norgren was supported by the Medical Research Council (grant 08675) and the medical faculty at Umeå University. S. W. Wu was partially supported by the Kempe Foundation.

REFERENCES

1. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, and H. Giamarellou.** 1996. Characterization of the plasmidic beta-lactamase CMY-2, which is responsible for cephamycin resistance. Antimicrob. Agents Chemother. **40:**221–224.
2. **Bauernfeind, A., and G. Horl.** 1987. Novel R-factor born beta-lactamase of *Escherichia coli* conferring resistance to cephalosporins. Infection **15:**257–259.
3. **Bauernfeind, A., O. Ang, C. Bal, and R. Jungwirth.** 1994. Plasmidic cephamycinase gene in *Citrobacter freundii* transferable to *Klebsiella pneumoniae* and *Escherichia coli*. Presented at the Scientific Meeting of the European Society of Chemotherapy, abstr. OC2. Coimbra, Portugal.
4. **Bauernfeind, A., S. Schweighart, K. Dornbusch, and H. Giamarellou.** 1990. A transferable cephamycinase (CMY-ase) in *Klebsiella pneumoniae* (*K. pn.*), abstr. 190, p. 118. *In* Program and abstracts of the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
5. **Bergstrom, S., and S. Normark.** 1979. Beta-lactam resistance in clinical isolates of *Escherichia coli* caused by elevated production of the *ampC*-mediated chromosomal beta-lactamase. Antimicrob. Agents Chemother. **16:**427–433.
6. **Bernardi, A., and F. Bernardi.** 1984. Complete sequence of pSC101. Nucleic Acids Res. **12:**9415–9426.
7. **Bishop, R. E., S. S. Penfold, L. S. Frost, J. V. Holtje, and J. H. Weiner.** 1995. Stationary phase expression of a novel *Escherichia coli* outer membrane lipoprotein and its relationship with mammalian apolipoprotein D. Implications for the origin of lipocalins. J. Biol. Chem. **270:**23097–23103.
8. **Chan, P. T., H. Ohmori, J. Tomizawa, and J. Lebowitz.** 1985. Nucleotide sequence and gene organization of ColE1 DNA. J. Biol. Chem. **260:**8925–8935.
9. **Dery, K. J., R. Chavideh, V. Waters, R. Chamorro, L. S. Tolmasky, and M. E. Tolmasky.** 1997. Characterization of the replication and mobilization regions of the multiresistance *Klebsiella pneumoniae* plasmid pJHCMW1. Plasmid **38:**97–105.
10. **Edlund, T., T. Grundstrom, and S. Normark.** 1979. Isolation and characterization of DNA repetitions carrying the chromosomal beta-lactamase of *Escherichia coli* K-12. Mol. Gen. Genet. **173:**115–125.
11. **Fosberry, A. P., D. J. Payne, E. J. Lawlor, and J. E. Hodgson.** 1994. Cloning and sequencing analysis of bla_{BIL-1}, a plasmid-mediated class C β-lactamase gene in *Escherichia coli* BS. Antimicrob. Agents Chemother. **38:**1182–1185.
12. **Greener, T., D. Govezensky, and A. Zamir.** 1993. A novel multicopy suppressor of a groEL mutation includes two nested open reading frames transcribed from different promoters. EMBO J. **12:**889–896.
13. **Haruta, S., K. Taniguchi, M. Nukaga, and T. Sawai.** 1996. Unpublished data.
14. **Itoh, T., and J. Tomizawa.** 1980. Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. Proc. Natl. Acad. Sci. USA **77:**2450–2454.
15. **Jacoby, G. A.** 1994. Extrachromosomal resistance in Gram-negative organisms: the evolution of β-lactamase. Trends Microbiol. **2:**357–360.
16. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
17. **Keenleyside, W. J., and C. Whitfield.** 1995. Lateral transfer of *rfb* genes: a mobilizable ColE1-type plasmid carries the rfbO:54 (O:54 antigen biosynthesis) gene cluster from *Salmonella enterica* serovar Borreze. J. Bacteriol. **177:**5247–5253.
18. **Kushner, S. R.** 1987. An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids, p. 17. *In* H. B. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland Publishing Co., Amsterdam, The Netherlands.
19. **Lacatena, R. M., and G. Cesareni.** 1981. Base pairing of RNAI with its complementary sequence in the primer precursor inhibits ColE1 replication. Nature **294:**623–626.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Liu, Y., B. L. Mee, and L. Mulgrave.** 1997. Identification of clinical isolates of indole-positive *Klebsiella* spp., including *Klebsiella planticola*, and a genetic and molecular analysis of their beta-lactamases. J. Clin. Microbiol. **35:**2365–2369.
22. **Matthew, M., and A. M. Harris.** 1976. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. J. Gen. Microbiol. **94:**55–67.
23. **Mikiewicz, D., B. Wrobel, G. Wegrzyn, and A. Plucienniczak.** 1997. Isolation and characterization of a ColE1-like plasmid from *Enterobacter agglomerans* with a novel variant of rom gene. Plasmid **38:**210–219.
24. **Nomura, N., and Y. Murooka.** 1994. Characterization and sequencing of the region required for replication of a non-selftransmissible plasmid pEC3 isolated from Erwinia carotovora subsp. carotovora. J. Ferment. Bioeng. **78:**250–254.
25. **Normark, S., and L. G. Burman.** 1977. Resistance of *Escherichia coli* to penicillins: fine mapping and dominance of chromosomal β-lactamase mutations. J. Bacteriol. **132:**1–7.
26. **Papanicolaou, G. A., A. A. Medeiros, and G. A. Jacoby.** 1990. Novel plasmid-

mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and α-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34:**2200–2209.
27. **Polisky, B.** 1988. ColE1 replication control circuitry: sense from antisense. Cell **55:**929–932.
28. **Rose, R. E.** 1988. The nucleotide sequence of pACYC177. Nucleic Acids Res. **16:**356.
29. **Sakakibara, Y., and J. I. Tomizawa.** 1974. Replication of colicin E1 plasmid DNA in cell extracts. Proc. Natl. Acad. Sci. USA **71:**802–806.
30. **Sakakibara, Y., and J. I. Tomizawa.** 1974. Replication of colicin E1 plasmid DNA in cell extracts. II. Selective synthesis of early replicative intermediates. Proc. Natl. Acad. Sci. USA **71:**1403–1407.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Selzer, G., T. Som, T. Itoh, and J. Tomizawa.** 1983. The origin of replication of plasmid p15A and comparative studies on the nucleotide sequences around the origin of related plasmids. Cell **32:**119–129.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
34. **Swanson, J., L. W. Mayer, and M. R. Tam.** 1982. Antigenicity of *Neisseria gonorrhoeae* outer membrane protein(s) III detected by immunoprecipitation and Western blot transfer with a monoclonal antibody. Infect. Immun. **38:**668–672.
35. **Tomizawa, J., and T. Itoh.** 1981. Plasmid ColE1 incompatibility determined by interaction of RNAI with primer transcripts. Proc. Natl. Acad. Sci. USA **78:**6096–6100.
36. **Tomizawa, J.-I., H. Ohmori, and R. E. Bird.** 1977. Origin of replication of colicin E1 plasmid DNA. Proc. Natl. Acad. Sci. USA **74:**1865–1869.
37. **Van Der Ploeg, J., M. Willemsen, G. Van Hall, and D. B. Janssen.** 1995. Adaption of *Xanthobacter autotrophicus* GJ10 to bromoacetate due to activation and mobilization of the haloacetate dehalogenase gene by insertion element IS1247. J. Bacteriol. **177:**1348–1356.
38. **Woloj, M., M. E. Tolmasky, M. Roberts, and J. H. Crosa.** 1986. Plasmid-encoded amikacin resistance in multiresistant strains of *Klebsiella pneumoniae* isolated from neonates with meningitis. Antimicrob. Agents Chemother. **29:**315–319.
39. **Wu, S., H. de Lencastre, and A. Tomasz.** 1998. Genetic organization of the *mecA* region in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus sciuri*. J. Bacteriol. **180:**236–242.
40. **Wu, S. W., K. Dornbusch, and G. Kronvall.** Genetic characterization of resistance to extended-spectrum beta-lactams in *Klebsiella oxytoca* septicemia isolates recovered from hospitals in the Stockholm area. Antimicrob. Agents Chemother., in press.
41. **Wu, S. W., K. Dornbusch, E. Goransson, U. Ransjo, and G. Kronvall.** 1991. Characterization of *Klebsiella oxytoca* septicaemia isolates resistant to aztreonam and cefuroxime. J. Antimicrob. Chemother. **28:**389–397.
42. **Wu, S. W., K. Dornbusch, M. Norgren, and G. Kronvall.** 1992. Extended spectrum beta-lactamase from *Klebsiella oxytoca*, not belonging to the TEM or SHV family. J. Antimicrob. Chemother. **30:**3–16.