ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2000, p. 3158–3162 Vol. 44, No. 11
0066-4804/00/$04.00+0

Nucleotide Sequence of the Chromosomal *ampC* Gene of *Enterobacter aerogenes*

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Received 18 January 2000/Returned for modification 18 May 2000/Accepted 2 August 2000

The AmpC β-lactamase gene and a small portion of the regulatory *ampR* sequence of *Enterobacter aerogenes* 97B were cloned and sequenced. The β-lactamase had an isoelectric point of 8 and conferred cephalosporin and cephamycin resistance on the host. The sequence of the cloned gene is most closely related to those of the *ampC* genes of *E. cloacae* and *C. freundii*.

Several members of the family *Enterobacteriaceae* are naturally resistant to cephalosporins due to the production of an inducible, chromosomally encoded cephalosporinase (7). In the uninduced state, transcription of the structural β-lactamase gene, *ampC*, is repressed by the product of the linked *ampR* gene (6, 12, 16). Chromosomal *ampC-ampR* systems have been described for *Citrobacter freundii* (11, 13), *Enterobacter cloacae* (4, 6), *Morganella morganii* (2, 16), and *Yersinia enterocolitica* (21). The *ampC* of *Escherichia coli* (8) lacks the regulatory gene, possibly as the result of a deletion (6).

Recently discovered plasmid-borne AmpC cephalosporinases such as MIR-1, ACT-1, and LAT-1 appear to be genetic descendants of these chromosomally encoded AmpC enzymes and have been implicated in the spread of the cephalosporin-resistant phenotype to other genera such as *Klebsiella* (3). In this paper, we describe a hitherto unknown chromosomal *ampC* from *E. aerogenes* that was cloned accidentally while searching for a potential plasmid-mediated cephalosporinase.

Enterobacter aerogenes isolates 97A and 97B, identified with the Vitek Identification System (bioMérieux Vitek, Inc., Hazelwood, Mo.), were obtained from the urine of a 30-year-old female patient receiving antibiotic therapy (ampicillin, cefazolin, and gentamicin) for recurring urinary tract infections. The specimen containing 97B was collected 17 h after collection of the one containing 97A. Standard disk diffusion tests (14) and Etests (AB Biodisk North America, Inc., Piscataway, N.J.) were used to determine antibiograms and MICs, respectively. Isolate 97B showed greater resistance than 97A to cefuroxime, cefotetan, cefazolin, ceftriaxone, ceftazidime, and ampicillin (AMP)-sulbactam (Table 1). Both isolates exhibited cefoxitin (FOX) resistance, with MICs of >256 μg/ml.

In a "shotgun" attempt to clone a possible plasmid-mediated cephamycinase, libraries of plasmid fragments from 97A and 97B were created by standard procedures (9, 19). Plasmid DNAs were combined with the vector pUC19, digested with P*st*I or *Kpn*I, and ligated. *E. coli* DH5α (Gibco-BRL, Gaithersburg, Md.) transformants were selected on agar containing 16 μg of FOX per ml or 50 μg of AMP per ml. This experiment produced only one recombinant, pACM200, from the 97B *Kpn*I-fragment library. The construct contained a 10-kb insert (Fig. 1).

Deletion subclones of pACM200 were selected on AMP agar and were subsequently tested for growth on FOX agar. FOX resistance was localized to the 2-kb *Sac*I-*Sal*I fragment in pACM204 (Fig. 1). Further subcloning of pACM204 by cutting the *Bam*HI or *Nru*I sites (pACM205, pACM206, and pACM207) eliminated FOX resistance (Table 1).

Probe 206 was made from the insert of pACM206 by using the Non-Radioactive Labeling and Detection Kit (Roche, Indianapolis, Ind.). Probe 206 failed to hybridize at 42°C with plasmid DNAs from either 97A or 97B (data not shown), even though the cloned fragment originated, presumably, in plasmid DNA from 97B. The probe was then applied to a Southern blot (19) of *Xba*I-digested total DNAs from a pulsed-field gel electrophoresis gel (20) (data not shown). The blot included DNAs from *E. aerogenes* strains ATCC 35028 and ATCC 35029 (American Type Culture Collection [ATCC], Rockville, Md.), isolates 97A and 97B, *E. coli* DH5α, and clinical isolates of *E. cloacae* and *Enterobacter agglomerans* (one isolate each). Probe 206 hybridized with a single large fragment (485 kb or larger) in each of three *E. aerogenes* lanes; in the fourth lane (with isolate ATCC 35028), the DNA was degraded to a smear that ran near the bottom of the gel, but the probe hybridized with the smeared DNA as well. The probe did not bind to the DNA of *E. coli* DH5α or *E. agglomerans*, and only a very faint band was present in the *E. cloacae* lane. These observations led to the conclusion that the cloned fragment had originated in residual chromosomal DNA in the plasmid preparation for 97B and serve as a reminder that the origin of any

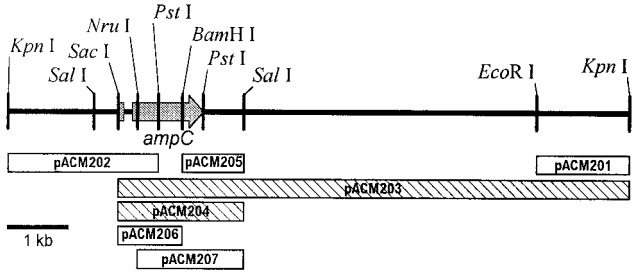

FIG. 1. Fragment of *E. aerogenes* chromosomal DNA cloned in recombinant pACM200. The fragments included in each subclone of pACM200 are indicated by boxes below the map; the hatched boxes indicate constructs that mediated FOX resistance. The coding sequence for *ampC* and the small portion of *ampR* that was sequenced are indicated by a gray arrow and a gray bar, respectively. Only relevant restriction sites are shown.

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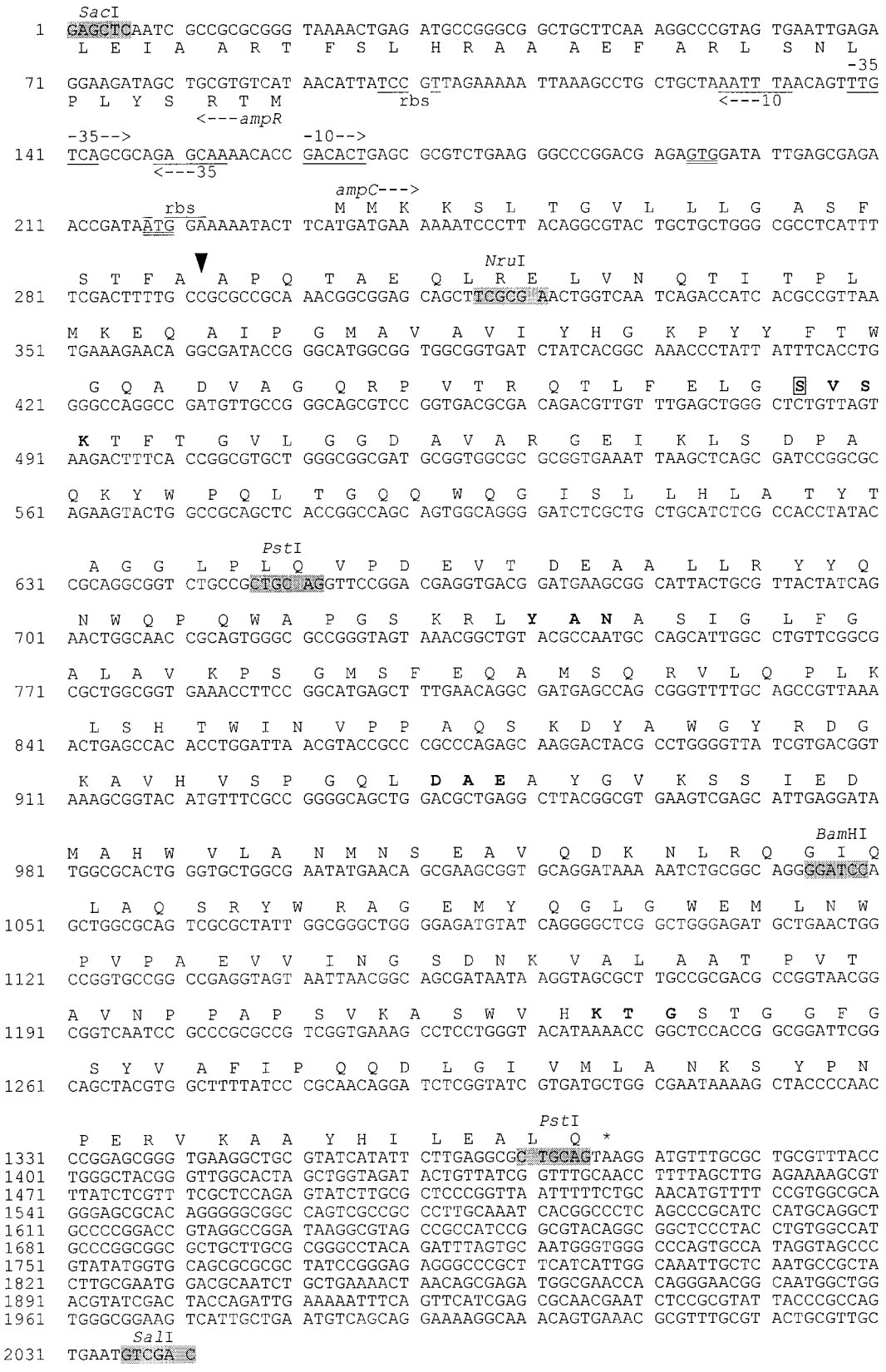

FIG. 2. Nucleotide sequence of the insert of pACM204 and deduced amino acid sequences for two ORFs corresponding to *ampR* and *ampC*. The standard single-letter code for amino acids is used; the stop codon is marked with an asterisk. Putative ribosomal binding sites (rbs), promoters (−10, −35), and selected restriction sites are indicated. Two in-frame alternate start codons for *ampC* are double underlined. The putative active-site serine is boxed; amino acids in boldface are characteristic motifs of serine β-lactamases. The predicted cleavage point for the signal peptide is marked with an arrowhead.

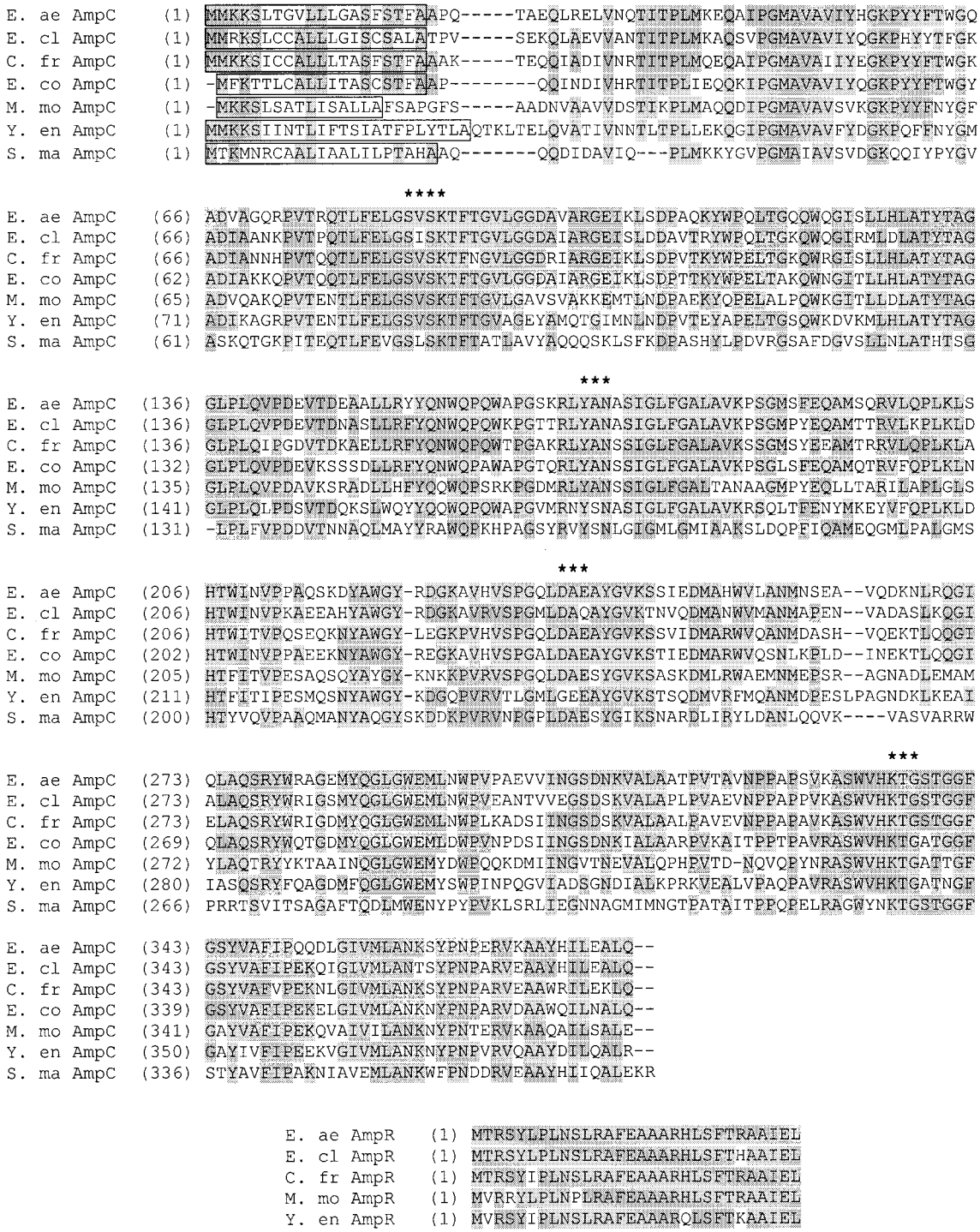

FIG. 3. Alignment of deduced amino acid sequences for seven chromosomal AmpC β-lactamases and the first 30 amino acids of five regulatory AmpR proteins (Table 2). The AmpC signal peptides (predicted or experimentally determined by each author) are boxed, and motifs involved in the formation of the active site are marked with asterisks. Amino acids conserved in more than half the sequences are shaded. E. ae, *E. aerogenes*; E. cl, *E. cloacae*; C. fr, *C. freundii*; M. mo, *M. morganii*; Y. en, *Y. enterocolitica*; S. ma, *Serratia marcescens*.

cloned fragment must be confirmed by hybridization with donor DNA.

A collection of 43 *E. aerogenes* strains dating back to the 1980s was screened by in situ colony hybridization (19) with probe 206 (data not shown). All *E. aerogenes* colonies were positive. Other enteric organisms were negative (*Klebsiella oxytoca*, *E. coli*, *C. freundii*, *E. agglomerans*) or only weakly positive (*E. cloacae*). These results indicate that the cloned gene is specific to *E. aerogenes*.

The isoelectric point of the cloned β-lactamase was determined by focusing, as described previously (22), crude protein extracts prepared with the B-PER II Bacterial Protein Extrac-

TABLE 1. β-Lactam disk diffusion zone sizes for *E. aerogenes* isolates, the cloning host, and recombinants clones

Antibiotic	Zone size (mm)				
	E. aerogenes isolate 97A	*E. aerogenes* isolate 97B	*E. coli* DH5α	*E. coli* DH5α (pACM204)	*E. coli* DH5α (pACM206)
AMP	6	6	19	6	6
AMP-Sulbactam	15	6	24	6	6
Pipericillin	22	22	30	6	6
Pipericillin-tazobactam	21	21	29	17	17
Cefazolin	7	6	26	6	12
Cefuroxime	16	6	22	8	20
FOX	6	6	25	10	25
Cefotetan	22	6	32	22	31
Ceftriaxone	24	20	31	24	31
Ceftazidime	24	19	30	25	30

TABLE 2. Sequences discussed in the text and included in alignment figures

Species and strain	Abbreviation in Fig. 3 to 5	Nucleotide sequence accession no(s).	Reference
Enterobacter aerogenes 97B	E. ae	AF211348	This work
Enterobacter cloacae P99	E. cl	X07247	4 (*ampC*)
Enterobacter cloacae MHN1		X04730	6 (*ampR*, i-cis[a])
Escherichia coli K-12	E. co	J01611	8
Citrobacter freundii OS60	C. fr	X03866, M27222	11 (*ampC*) 13 (*ampR*, i-cis)
Morganella morganii SLM01	M. mo	Y10283	2
Serratia marcescens SR50	S. ma	X52964	15
Yersinia enterocolitica IP97	Y. en	X63149	21

[a] i-cis, intercistronic region.

tion Reagent (Pierce, Rockford, Ill.). A sonicated preparation of *K. oxytoca* β-lactamases (22) provided reference enzymes with known isoelectric points. Focused β-lactamases (data not shown) were detected with the chromogenic cephalosporin pyridinium-2-azo-*p*-dimethylaniline chromophore (Calbiochem, La Jolla, Calif.) (10). The cloned β-lactamase had an isoelectric point of approximately 8 and comigrated with the single β-lactamase produced by each of four *E. aerogenes* strains (ATCC and clinical strains), including the DNA donor 97B. Since there is no evidence that 97B produced any β-lactamase other than the one that was cloned, we ruled out a novel plasmidic β-lactamase gene as the source of increased cephalosporin resistance.

The nucleotide sequence of the insert of pACM204 was determined as described previously (17). The sequence was analyzed with Vector NTI Suite software (InforMax, North Bethesda, Md.) and compared to sequences in the GenBank database through BLAST (1). The insert of pACM204 (Fig. 2) has an open reading frame (ORF) that encodes 381 amino acids. The coding sequence has a 73% identity to the *ampC* of *E. cloacae* P99, a 72% identity to the *ampC* of *C. freundii* OS60, and a 70% identity to the *ampC* of *E. coli* K-12 (Table 2). The *ampC* ORF is preceded by an incomplete ORF (30 codons) on the complementary strand that corresponds to the beginning of the *ampR* regulatory genes of *E. cloacae* and *C. freundii*. Putative promoters and ribosomal binding sites of the intercistronic region are indicated. There are two possible alternate start codons preceding and in frame with the indicated start of the *ampC* coding sequence. On the basis of a comparison with other *ampC* sequences, the indicated start codon is predicted to be the actual start codon. There is no evidence of a transcript terminator sequence following *ampC*. The nucleotide sequence following *ampC* has no significant similarity to any other nucleotide sequences in the GenBank database; translations of short ORFs in this region have no protein homologs.

The deduced amino acid sequence has characteristics typical of AmpC enzymes from other enteric organisms. The first 20 amino acids fit predictions for a putative signal peptide (23). The predicted mature peptide of 361 amino acids has a calculated molecular mass and isoelectric point of 39.5 kDa and 7.99, respectively. The mature peptide includes four "signature" motifs [SXXK, Y(A or S)N, D/E, and KTG] that are involved in the formation of the active site in the "active-site serine" enzymes (5). The putative active-site serine occurs at amino acid 64 in the mature peptide sequence.

The alignments of the deduced amino acid sequences for seven AmpC sequences and five partial AmpR sequences (AmpR is absent in *E. coli* and unknown in *S. marcescens*) are shown in Fig. 3. The dendrogram (Fig. 4) depicts the relationship between the AmpC sequences calculated by the neighbor joining method (18).

Figure 5 shows the alignment of the *ampR-ampC* intercistronic regions for six enteric organisms. Promoters, the *ampC* ribosomal binding site, and the AmpR binding region (determined experimentally for *C. freundii* by Lindquist et al. [13]) occur in highly conserved areas of the sequence. The putative *ampR* ribosomal binding site is less well conserved, which might reflect the observed weak expression of this gene (6, 13). Because of the close relationship between the *E. aerogenes* sequence and others of its type, the intercistronic region is assumed to function in a manner similar to those of *C. freundii* (13) and *E. cloacae* (6).

Although cloned unintentionally, the *E. aerogenes ampC* sequence adds another branch to the phylogenetic tree of species-specific chromosomally encoded enzymes. As new sequences are added, a better understanding of evolutionary relationships among species is achieved.

Nucleotide sequence accession number. The nucleotide se-

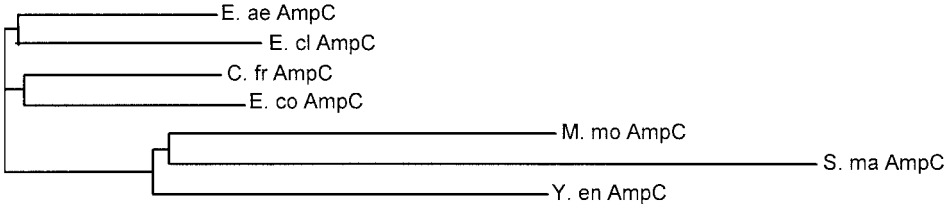

FIG. 4. A dendrogram calculated by the neighbor joining method (18) shows the relatedness of the AmpC amino acid sequences; branch lengths are proportional to the number of amino acid changes between sequences. E. co, *E. coli*; see the legend to Fig. 3 for definitions of the other abbreviations.

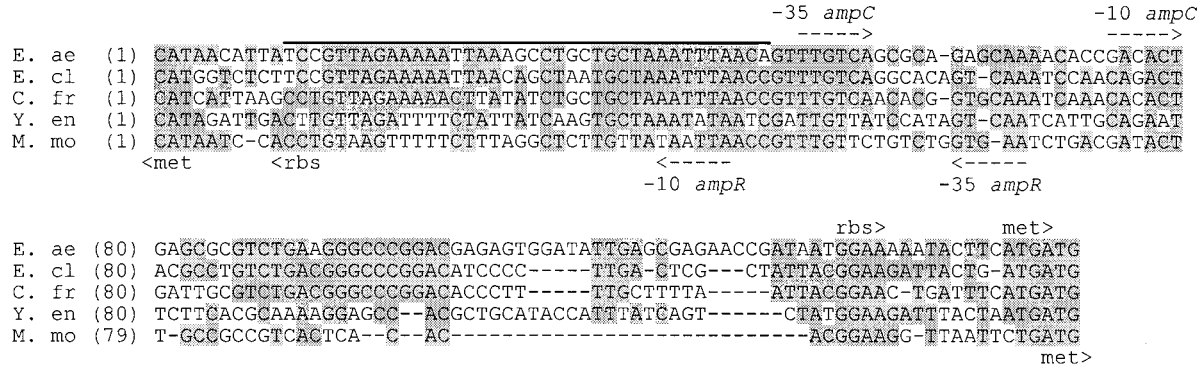

FIG. 5. Alignment of the intercistronic regions of five *ampR-ampC* nucleotide sequences (Table 2). Nucleotides conserved in more than half the sequences are shaded. Putative start codons (met), promoters (−10, −35), and ribosomal binding sites (rbs) are indicated. The overlined sequence corresponds to the 38-bp AmpR binding region experimentally determined for *C. freundii* (13). See the legend to Fig. 3 for definitions of organism abbreviations.

quence of the insert of pACM204 has been deposited in GenBank under accession number AF211348.

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