

Cloning, Sequence Analyses, Expression, and Distribution of *ampC-ampR* from *Morganella morganii* Clinical Isolates

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Shotgun cloning experiments with restriction enzyme-digested genomic DNA from *Morganella morganii* 1, which expresses high levels of cephalosporinase, into the pBKCMV cloning vector gave a recombinant plasmid, pPON-1, which encoded four entire genes: *ampC*, *ampR*, an *hybF* family gene, and *orf-1* of unknown function. The deduced AmpC β -lactamase of pI 7.6 shared structural and functional homologies with AmpC from *Citrobacter freundii*, *Escherichia coli*, *Yersinia enterocolitica*, *Enterobacter cloacae*, and *Serratia marcescens*. The overlapping promoter organization of *ampC* and *ampR*, although much shorter in *M. morganii* than in the other enterobacterial species, suggested similar AmpR regulatory properties. The MICs of β -lactams for *E. coli* MC4100 (*ampC* mutant) harboring recombinant plasmid pACYC184 containing either *ampC* and *ampR* (pAC-1) or *ampC* (pAC-2) and induction experiments showed that the *ampC* gene of *M. morganii* 1 was repressed in the presence of *ampR* and was activated when a β -lactam inducer was added. Moreover, transformation of *M. morganii* 1 or of *E. coli* JRG582 (Δ *ampDE*) harboring *ampC* and *ampR* with a recombinant plasmid containing *ampD* from *E. cloacae* resulted in a decrease in the β -lactam MICs and an inducible phenotype for *M. morganii* 1, thus underlining the role of an AmpD-like protein in the regulation of the *M. morganii* cephalosporinase. Fifteen other *M. morganii* clinical isolates with phenotypes of either low-level inducible cephalosporinase expression or high-level constitutive cephalosporinase expression harbored the same *ampC-ampR* organization, with the *hybF* and *orf-1* genes surrounding them; the organization of these genes thus differed from those of *ampC-ampR* genes in *C. freundii* and *E. cloacae*, which are located downstream from the fumarate operon. Finally, an identical AmpC β -lactamase (DHA-1) was recently identified as being plasmid encoded in *Salmonella enteritidis*, and this is confirmatory evidence of a chromosomal origin of the plasmid-mediated cephalosporinases.

Species of the family *Enterobacteriaceae* including *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Serratia marcescens*, and *Yersinia enterocolitica* are naturally resistant to aminopenicillins and the early cephalosporins. This resistance phenotype is mediated by chromosomally encoded β -lactamases (AmpC) belonging to class C enzymes, also commonly named cephalosporinases (2, 5, 6). The inducible biosynthesis of these cephalosporinases has been reported phenotypically for these bacterial species. In *C. freundii* and *E. cloacae*, an *ampR* gene is located upstream of *ampC* and is divergently transcribed compared to *ampC*. Its deduced protein, a transcriptional regulator of the LysR family, acts as a repressor in the basal level of AmpC biosynthesis and favors its biosynthesis upon induction by several β -lactams (5). In these enterobacterial species, mutations in the promoter or the structural gene of *ampD* result in constitutive overproduction of cephalosporinase and explain the acquired resistance to expanded-spectrum cephalosporins (5, 11, 12, 17).

The mechanism of cephalosporinase expression has been studied in detail for *E. cloacae* and was found to be related to peptidoglycan components (13). Briefly, during normal growth in the absence of β -lactam as an inducer, the AmpR regulator is maintained in an inactive form by a peptidoglycan precursor, uridine pyrophosphoryl-*N*-acetyl muramyl-L-alanyl-D-glutamyl-

meso-diaminopimelic acid-D-alanyl-D-alanine (UDP-MurNac-pentapeptide). This negative effect occurs by direct binding of UDP-MurNac-pentapeptide to the regulator. In this inactive form, AmpR binds to its operator site between the *ampC* and *ampR* structural genes, leading to repression of *ampC* expression. This inactivation of AmpR can be relieved by both knockout mutations in the *ampD* gene or the presence of β -lactams. Inactivation of *ampD* which encodes a cytosolic amidase specific for the recycling of mucopeptides results in an increase in the concentration of its substrate, the 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid (anh-MurNac-tripeptide). The higher concentration of this mucopeptide inside the cell is sufficient to displace the UDP-MurNac-pentapeptide from its AmpR binding site, thereby reactivating AmpR. In *ampD*⁺ cells, addition of β -lactams results in increased biosynthesis of cell wall degradation fragments and in a higher intracellular level of anh-MurNac-tripeptide by titrating the available AmpD activity. More recently, other mucopeptides have been recognized as a signal for β -lactamase induction, such as anh-MurNac-pentapeptide (31).

As for other cephalosporinase-producing enterobacterial species, *M. morganii* strains may be grouped into two β -lactamase expression phenotypes, i.e., oxyimino-cephalosporin sensitive, with low-level and inducible cephalosporinase production on the one hand, and oxyimino-cephalosporin resistant, with high-level and constitutive cephalosporinase production on the other hand (32). While this work was in progress, the sequence of the cephalosporinase gene from an *M. morganii* isolate, strain SLM01, was reported along with the se-

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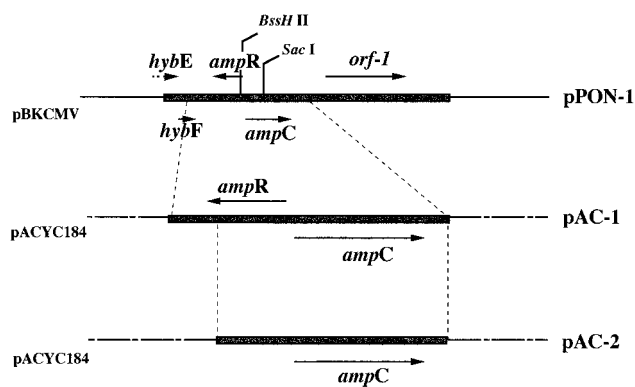


FIG. 1. Restriction endonuclease maps of the inserts from recombinant plasmid pPON-1, which codes for the *ampR* and *ampC* genes from *M. morgani* 1, and of pAC-1 (*ampR*, *ampC*) and pAC-2 (*ampC*) pACYC184 derivatives. The thick lines represent the cloned inserts from *M. morgani* 1, the thin lines indicate vector pBKCVMV, and the dotted lines represent vector pACYC184. The five ORFs found in the 5,914-bp sequenced are indicated, as are their translation directions.

quence of part of an *ampR* gene, but no evidence of a linkage between the presence of *ampR* and cephalosporinase regulation was reported (3). The purpose of our work was to identify *ampR* and *ampC* genes from an *M. morgani* isolate and correlate their presence with the regulatory properties of AmpR. The putative role of an AmpD-like protein was also investigated. A comparison with the plasmid-mediated *ampC-ampR* genes recently found in *Salmonella enteritidis* was also performed (4). Moreover, a comparison of *ampR*, *ampC*, and the surrounding sequences in *M. morgani* with those of other *ampC*-possessing enterobacterial species was undertaken.

MATERIALS AND METHODS

Bacterial strains and plasmid. *M. morgani* 1 was isolated at the Hôpital Antoine Bécélère (Clamart, France) in 1997 from a clinical specimen (dermatous ulcer) and was identified with the API 20E system (bioMérieux, Marcy l'Etoile). The other unrelated *M. morgani* clinical isolates (strains 2 to 16) were identified from patient specimens in 1997 at the Hôpital de Bicêtre (Le Kremlin-Bicêtre,

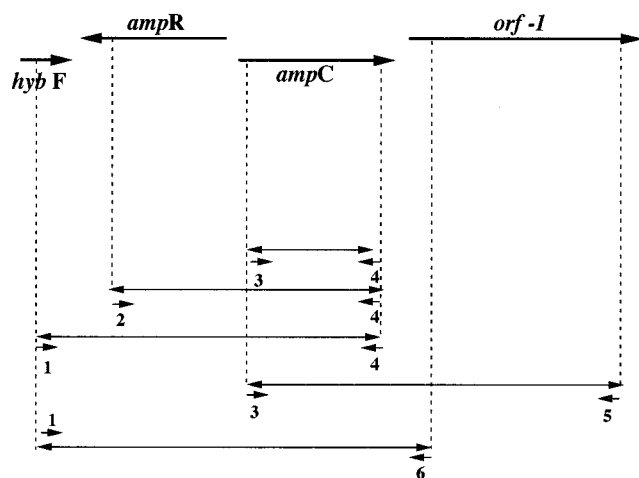


FIG. 2. Map of pPON-1 insert from *M. morgani* 1 and the primers used to PCR amplify the indicated genes from 15 *M. morgani* clinical isolates: primers 1 and 4 for *hybF*, *ampR*, and *ampC*; primers 2 and 4 for *ampR* and *ampC*; primers 3 and 4 for *ampC*; and primers 3 and 5 for *ampC* and *orf-1*. Cloning of *ampR-ampC* genes or of the *ampC* gene into pACYC184, giving recombinant plasmids pAC-1 or pAC-2, respectively, used similarly PCR-amplified fragments.

TABLE 1. MICs of β -lactams for clinical isolates *M. morgani* 1 and 5, *E. coli* DH10B harboring recombinant plasmid pPON-1, and the *E. coli* DH10B reference strain

β -Lactam	MIC (μ g/ml)			
	<i>M. morgani</i> 1	<i>E. coli</i> DH10B (pPON-1)	<i>E. coli</i> DH10B	<i>M. morgani</i> 5
Amoxicillin	>512	>512	2	512
Amoxicillin + Cla ^a	512	>512	2	512
Amoxicillin + Taz ^b	32	32	2	32
Ticarcillin	8	64	2	0.12
Ticarcillin + Cla	8	64	1	0.25
Ticarcillin + Taz	2	32	1	0.25
Piperacillin	32	32	1	0.5
Cephalothin	>512	>512	4	>512
Cefamandole	64	32	1	>512
Cefoxitin	16	128	8	16
Cefepime	0.06	0.06	0.03	<0.06
Cefpirome	0.5	0.06	0.03	1
Ceftazidime	4	16	0.12	<0.06
Ceftazidime + Clav	4	32	0.12	<0.06
Ceftazidime + Taz	0.06	2	0.12	<0.06
Cefotaxime	4	4	0.12	0.12
Ceftriaxone	2	1	0.06	<0.06
Imipenem	1	0.12	0.06	1
Aztreonam	1	1	0.12	<0.06
Moxalactam	0.125	0.5	0.25	<0.06

^a Cla, clavulanic acid at a fixed concentration of 2 μ g/ml.

^b Taz, tazobactam at a fixed concentration of 4 μ g/ml.

France). *Escherichia coli* DH10B (Life Technologies, Eragry, France) was used as the host strain for cloning experiments. β -Lactamase expression was studied by using *E. coli* MC4100 lacking an *ampC* gene or *E. coli* JRG582 from which its *ampDE* genes were deleted (12). The cloning vectors were either the pBKCVMV phagemid (Stratagene, La Jolla, Calif.), which confers kanamycin resistance, or pACYC184, which confers chloramphenicol and tetracycline resistance (7). Plasmid pNH5 containing an *HpaI* fragment with the *ampD* gene from *E. cloacae* into pBGS18 conferred kanamycin resistance (12).

Antimicrobial agents and MIC determinations. The agents and their sources were as follows: amoxicillin, clavulanic acid, and ticarcillin, SmithKline French-Beecham (Nanterre, France); aztreonam and cefepime, Bristol-Myers Squibb (Paris, France); ceftazidime, Glaxo (Paris, France); cephalothin, cefamandole, and moxalactam, Eli Lilly (Saint-Cloud, France); piperacillin and tazobactam, Lederle (Oullins, France); cefotaxime and cefpirome, Hoechst-Roussel (Paris, France); cefoxitin and imipenem, Merck Sharp & Dohme-Chibret (Paris, France); ceftriaxone (Roche, Neuilly, France); and kanamycin and chloramphenicol, Sigma (Saint-Quentin Falavier, France).

Antibiogram for the *M. morgani* clinical isolates and the recombinant *E. coli* strains were first done by a routine agar disk diffusion assay with Mueller-Hinton agar plates and antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). The MICs were then determined by an agar dilution technique on Mueller-Hinton agar plates with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot (26). All plates were incubated at 37°C for 18 h.

Genetic techniques. Genomic DNAs of *M. morgani* 1 to 16 were extracted as described previously (24). Fragments of genomic DNA from *M. morgani* 1 partially digested with *Sau3AI* (Pharmacia Biotech, Orsay, France) were ligated into the *Bam*HI site of the pBKCVMV phagemid. Ligation was performed at a 1:1 vector-insert ratio at a final concentration of 200 ng of DNA in a ligation mixture containing 1 U of T4 DNA ligase (Boehringer, Meylan, France) at 4°C for 18 h. Recombinant plasmids were transformed by electroporation (gene pulser II; Bio-Rad, Ivry-sur-Seine, France) into electrocompetent *E. coli* DH10B cells (Bio-Rad). Antibiotic-resistant colonies were selected on Trypticase soy agar plates containing 50 μ g of amoxicillin per ml and 30 μ g of kanamycin per ml.

Recombinant plasmid DNA was obtained from 100-ml Trypticase soy broth cultures grown with amoxicillin (100 μ g/ml) overnight at 37°C. The plasmid DNA was prepared with Qiagen columns (Qiagen, Courtaboeuf, France). Fragment sizes were estimated according to those on 1-kb DNA ladder (Pharmacia), which was used as a molecular size standard. A 5.9-kb cloned DNA fragment from pPON-1 (see Results section) was sequenced from both strands by using laboratory-designed successive primers and an Applied Biosystems sequencer (ABI 311). The nucleotide sequence and the deduced protein sequence were analyzed with Pedro's biomolecular tools (29). Multiple sequence alignment of deduced peptide sequences was carried out with the GCG program Pileup, which uses a simplification of the progressive alignment method of Feng and Doolittle (9).

	* A A A T N L L W R A F Q Q M A P T E T R S Q	
1	TTACGCCGCC GCCGTATTC A GCAGCCAGCG GCGCAATTGC TGCATGGCCG GGGTTTCCGT ACGGGACTGT	70
	L R T L W Y G G L T I E A A F P Q V L A G S Q L	
71	AACCGCTCA GCCAGTAGCC GCCGAGGGTG ATTTCTGCGG CAAACGGCTG TACCAGTGGC CCTGACTGTA	140
	L R S F M C V P A I A V G A G L Q A A E A M A	
141	ACAGGCGGCT GAACATACAT ACCGGTGCGA TCGCTACCCC GGCACCCAGT TGTGCCGCTC CGGCCATGGC	210
	L S T D F V M V P Q S P S P P T G G A C D L W	
211	CAGTGAGGTA TCGAACACCA TTACCGGCTG TGACGGGGAA GGCAGGTGTC CGCCCGCACA ATCCAGCCAG	280
	R S W E D R R F S R L L T F R H V D D P Q Q L Q	
281	CGGCTCCATT CATCCCGGCG GAATGAGCGC AGCAGGGTAA AGCGGTGAAC ATCATCCGGC TGCTGTAACT	350
	F A I A P S C L P A H A P S F I L E A D S E H	
351	GTTCTGCAAT GGCCGGTGAG CACAGCGGAG CGTGTGGTGC ACTGAAAATC AGTTCGCCAT CTGACTCATG	420
	W A G N G F R I T Y D H G E A A P D V H N N H	
421	CCAGCGCCCG TTACCGAAAC GGATCGTATA ATCATGCCCT FCCCGCCCGG GATCCACATG GTTATTATGG	490
	T S I H L D I F P H S D Y F G T L R P L L W G A	
491	GTGGAAATAT GCAGATCAAT ATGCGGATGG CTGTCAATAA ATCCGGTCAG ACGCGGCAGC AGCCAGCCCTG	560
	A F T G V A G V K V R E R V Q G H S F C E L T	
561	CGGCAATGT TCCCACCACA CCGACTTTCA CCCGCTCACG GAACTGCCCC TGAGAAAAAC ACTCCAGAGT	630
	A D I R D F A E N L V P L L G E G E H T L V L	
631	ATCCGCAATC CGGTCAAACG CCTCATPGAG CACCGGCAGT AATCCCTCAC CTTCATGGGT CAGCACCAGT	700
	G R S V R T F L V C G L Q E E L A R V Q Q S V A	
701	CCGCGTGAGA CGCGGGTAAA CAGCACACAG CCGAGTTGTT CTTCCAGAGC CCTGACCTGC TGGCTGACGG	770
	A H T V N L E I A A R T F S L H R A A A E F A	
771	CGGCATGGGT GACATTCAGC TCAATCGCCG CACGGGTAAA ACTGAGATGA CGGGCGGCGG CCTCAAAGGC	840
	R L P N L P L Y R R V M	
841	GCGCAGCGGG TTAAGGGGGA GATAACGTCT GACCATAATC CACCTGTAAG TTTTCTTTTA GGCTCTTGTT	910
911	<u>ATAATTA</u> ACC <u>GTGTTCTG</u> TCT <u>GTGAAT</u> CTGACGATAC TTGCCGCCGT CACTCACACA CGGAAGTTA	980
	<u>-10 ampC</u> <u>-35</u> <u>ampR -10</u>	
981	ATTCTGATGA AAAAATCGTT ATCTGCAACA CTGATTTCCG CTCGCTGGC GTTTTCCGCC CCGGGGTTTT	1050
	M K K S L S A T L I S A L L A F S A P G F	
1051	CTGCCGCTGA TAATGTCGCG CGCGTGGTGG ACAGCACCAT TAAACCGCTG ATGGCACAGC AGGATATTCC	1120
	S A A D N V A A V V D S T I K P L M A Q Q D I P	
1121	CGGATGGCG GTTCCGCTCT CCGTAAAGGG TAAGCCCTAT TATTTCAATT ACGGTTTTCG CGATATTTCAG	1190
	G M A V A V S V K G K P Y Y F N Y G F A D I Q	
1191	GCAAAACAGC CGGTCACTGA AAATACACTA TTTGAGCTCG GATCTGTAAG TAAACTTTC ACAGGTGTGC	1260
	A K Q P V T E N T L F E L G S V S K T F T G V	
1261	TGGGTGCGGT TTCTGTGGCG AAAAAGAGA TGGCGCTGAA TGATCCGGCG GCAAAATACC AGCCGAGGCT	1330
	L G A V S V A K K E M A L N D P A A K Y Q P E L	
1331	GGCTCTGCCG CAGTGAAGG GGATCACATT GCTGGATCTG GCTACCTATA CCGCAGCGCG ACTGCCGTTA	1400
	A L P Q W K G I T L L D L A T Y T A G G L P L	
1401	CAGGTGCCGG ATGCGGTAAG AAGCCGTGCG GATCTGCTGA ATTTCTATCA GCAGTGGCAG CCGTCCCGGA	1470
	Q V P D A V K S R A D L L N F Y Q Q W Q P S R	
1471	AACCGGGCGA TATGCTCTG TATGCAAACA GCAGTATCGG CCTGTTTGGT GCTCTGACCG CAAACGCGGC	1540
	K P G D M R L Y A N S S I G L F G A L T A N A A	
1541	GGGATGCCG TATGAGCAGT TGCTGACTGC ACGGATCCTG GCACCGCTGG GGTATCTCA CACCTTTATT	1610
	G M P Y E Q L L T A R I L A P L G L S H T F I	
1611	ACTGTGCCGG AAAGTGCGCA AAGCCAGTAT GCGTACGGTT ATAAAAACAA AAAACCGGTC CGCGTGTGCG	1680
	T V P E S A Q S Q Y A Y G Y K N K K P V R V S	
1681	CGGGACAGCT TGATGCGGAA TCTTACGGCG TGAATCCGC CTCAAAAGAT ATGCTGCGCT GGGCGGAAAT	1750
	P G Q L D A E S Y G V K S A S K D M L R W A E M	
1751	GAATATGGAG CCGTACCGGG CCGGTAATGC GGATCTGGAA ATGCAATGT ATCTTGACA GACCCGCTAC	1820
	N M E P S R A G N A D L E M A M Y L L A Q T R Y	
1821	TATAAAACCG CCGGATTA AAGGGGCTG GGCTGGGAAA TGTATGACTG GCGCAGCAGC AAAGATATGA	1890
	Y K T A A I N Q G L G W E M Y D W P Q Q K D M	
1891	TCATTAACGG CGTGACCAAC GAGGTGCGAT TGCAGCCGCA CCCGGTAAAC GACAACCAGG TTCAGCCGTA	1960
	I I N G V T N E V A L Q P H P V T D Q P Y	
1961	TAACCGTGCT TCCTGGGTGC ATAAAACGGG GGCAACAAC TGGTTCCGCG CCTATGTGGC CTTTATTCGG	2030
	N R A S W V H K T G A A T T G F G A Y V A F I P	
2031	GAAAAACAGG TGGCGATTGT GATCTGGCG AATAAAAACT ACCCGAATAC CGAAAGAGTC AAAGCCGCAC	2100
	E K Q V A I V I L A N K N Y P N T E R V K A A	
2101	AGGCTATTTT GAGTGCACCTG GAATAA	2126
	Q A I L S A L E *	

FIG. 3. Nucleotide sequence of the 2,126-bp fragment of pPON-1 containing the *ampC*- and *ampR*-coding regions. The deduced amino acid sequences are designated in single-letter code. The putative promoter sequences are represented by -35 and -10 regions (boxed). The start and stop codons of these genes are underlined.

The deduced AmpC and AmpR proteins were compared with the corresponding proteins of the enterobacterial species possessing known *ampC* and/or *ampR* genes: *E. coli* (14), *C. freundii* (16), *E. cloacae* (11), *S. marcescens* (25), and *Y. enterocolitica* (28).

DNA-DNA hybridizations were performed as described by Maniatis et al. (19). Genomic DNA from *M. morgani* 1 was digested with either *EcoRI*, *EcoRV*, or *PstI*. The products of these digestions were separated on a 0.8% agarose gel prior to a Southern transfer onto an N^+ Hybond nylon membrane (Amersham) followed by UV light cross-linking. The membranes were incubated for 1 h at 42°C in a prehybridization solution made of 100 μ g of salmon sperm DNA per ml, 5 \times Denhardt's solution, 0.5% sodium dodecyl sulfate, 3 \times SSC (1 \times SSC is

0.15 M NaCl plus 0.015 M sodium citrate), and 30% formamide. The DNA probe used consisted of the 385-bp *SacI*-*Bss*III fragment from recombinant plasmid pPON-1 containing an internal part of the *ampC* gene. The probe was radiolabelled with [α - 32 P]dATP with a random-primer DNA labelling kit (Boehringer). Hybridization was revealed by autoradiography with Kodak films after exposure at -80°C for 18 h.

Comparison of the *ampC*-*ampR*-coding regions and of the surrounding sequences in the different expression phenotypes. By using the DNA sequences obtained from the recombinant plasmid pPON-1 (Fig. 1), sets of primers were designed to PCR amplify from *M. morgani* isolates of both inducible and non-inducible phenotypes either the *hybF*, *ampR*, and *ampC* genes (primer 1 [5'-TG

Putative signal peptide ↓		31		
DHA-1 (strain 1)	MKKSLSATLISALLAFSAPGFSAADNVAUVVSTIKPLMAQQDIPGMAVA			
AmpC (SLM01)	-----			
		50	64	81
DHA-1 (strain 1)	VSVKGPYYFYNGFADIQAKQPVTENTLFLGSSVSKTFTGVLGAVSVAKK			
AmpC (SLM01)	-----V-----			
		100		131
DHA-1 (strain 1)	EMALNDPAKYQPELALPQWKGITLLDLATYTAGGLPLQVPDAVKSRADDL			
AmpC (SLM01)	--T-----E-----			
		150		181
DHA-1 (strain 1)	LNFYQQWQSRKPGDMRLYANSSIGLFGALTANAAGMPYEQLLTARILAP			
AmpC (SLM01)	-H-----			
		200		231
DHA-1 (strain 1)	LGLSHTFIVTPEASQSQYAYGKKNKPVVSPGQLDAESYGVKSAKMDL			
AmpC (SLM01)	-----			
		250		281
DHA-1 (strain 1)	RWAEMNMEPSRAGNADLEMAMYLQTRYKTAAINQGLGWEMDWPQQKD			
AmpC (SLM01)	-----			
		300		331
DHA-1 (strain 1)	MIINGVTNEVALQPHFVTDNQVQPNRASVHWKGTATTGFGAYVAFIPEK			
AmpC (SLM01)	-----			
		350	360	
DHA-1 (strain 1)	QVAIVILANKNYPNTERVKAQAILLSALE			
AmpC (SLM01)	-----			

FIG. 4. Comparison of amino acid sequences of AmpC from *M. morgani* SLM01 and DHA-1 from *M. morgani* 1. Dotted lines indicate identical amino acids. The boldface amino acids are characteristic either of serine β -lactamases (S-V-S-K) or of class C β -lactamases.

AGTGCGGCGGACATTATC-3') and primer 4 [5'-GGCTTTGACTCTTTCCGTATTC-3'), the *ampR* and *ampC* genes (primer 2 [5'-GTTTCCGTACGGGACTGTAAC-3'), and primer 4), the *ampC* gene alone (primer 3 [5'-TTCTGC CGTGATAATGTGCG-3') and primer 4), or the *ampC* gene and *orf-1* (primer 3 and primer 5 [5'-ACCACCACAAAGCGGAGTC-3') (Fig. 2). Recombinant plasmids pAC-1 and pAC-2 were obtained by cloning PCR-amplified fragments (primers 1 and 6 and primers 2 and 6, respectively [primer 6, 5'-CCATAAAA CAGCCATAAAGC-3']) into the *EcoRV* site of pACYC184. pAC-1 contained the *ampR* and *ampC* genes, and pAC-2 contained only the *ampC* gene (Fig. 1 and 2). *E. coli* MC4100 was transformed with pAC-1 and pAC-2, *E. coli* JRG582 was transformed with pAC-1 with or without pNH5, and *M. morgani* 1 was transformed with pNH5 by the calcium chloride method as described elsewhere (19).

β -Lactamase assays. Cultures of *E. coli* harboring the pPON-1 recombinant plasmid and *M. morgani* 1 were grown overnight at 37°C in 200 ml of Trypticase soy broth with amoxicillin at 100 μ g/ml. After low-speed centrifugation, the bacterial pellet was resuspended prior to sonification (two times for 30 s each time at 20 Hz; phospholyser Vibra Cell 300; Bioblock, Illkirch, France) and subsequent high-speed centrifugation (twice, 30 min, 48,000 \times g, 4°C). The residual nucleic acids in the supernatant were precipitated by treatment with 7% (vol/vol) spermin (0.2 M; Sigma) for 2 h at 4°C. This suspension was ultracentrifuged at 100,000 \times g for 1 h at 4°C. The supernatants containing the crude enzyme extracts were subjected to isoelectric focusing.

TABLE 2. Kinetic parameters of various β -lactam antibiotics for AmpC β -lactamase (DHA-1) from *M. morgani* 1^a

Substrate	V_{max}^b	K_m (μ M)	V_{max}/K_m^b
Ampicillin	3.5	29	3.4
Aztreonam	<0.1	— ^c	—
Benzylpenicillin	100	28	100
Cefotaxime	<0.1	—	—
Cefepime	<0.1	—	—
Ceftazidime	0.5	27	0.5
Cephalothin	246	53	120
Cefoxitin	<0.1	—	—
Imipenem	<0.1	—	—
Piperacillin	2.4	22	3
Ticarcillin	<0.1	—	—

^a The β -lactamase extracts were from *E. coli* DH10B cultures harboring recombinant plasmid pPON-1.

^b V_{max} and V_{max}/K_m are expressed relative to that of benzylpenicillin, which was set equal to 100.

^c In these cases, the hydrolysis parameters could not be determined (V_{max} was too low or K_m was too high).

The β -lactamase activity of *E. coli* DH10B cultures harboring pPON-1 was assayed by UV spectrophotometry (spectrophotometer Ultrospec 2000; Pharmacia Biotech, Orsay, France) at 30°C in 100 mM phosphate buffer (pH 7.0). The antibiotic wavelengths were chosen as described previously by Matagne et al. (20). Antibiotic solutions were freshly prepared in 100 mM phosphate buffer (pH 7.0). Kinetic parameters were derived from the initial velocity obtained with four to six substrate concentrations. K_m values were determined according to the Eadie-Hofstee representation [$V = f(V_i/S)$, where V_i is the initial velocity and S is the substrate concentration]. V_{max} values were expressed relative to that of benzylpenicillin, which was set equal to 100. Enzyme inhibition was studied with cephalothin (100 μ M) as the substrate. Clavulanic acid and tazobactam, at various concentrations, were preincubated with enzyme for 3 min at 30°C before addition of the substrate.

Analytical isoelectric focusing was performed with a mini IEF 111 (Bio-Rad) with a pH gradient of 3.5 to 9.5 ampholine polyacrylamide gel according to the manufacturer's instructions. The focused β -lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Paris, France) in distilled water. The pI values were determined and compared to those from molecular standards and to those of known β -lactamases (26).

β -Lactamase basal level determination and induction assays were performed as suggested previously (32) with *M. morgani* 1 to 16 and *E. coli* MC4100 harboring either recombinant plasmid pAC-1 or pAC-2 (see Results section). Briefly, overnight cultures were diluted 1:10 and were grown for 1 h and 30 min in a preincubated Trypticase soy broth on a rotating shaker at 37°C. Then, the cultures were grown for an additional 2 h in the presence of an inducer. Imipenem (0.5 μ g/ml) was used as the inducer since this β -lactam was reported to be the most powerful cephalosporinase inducer for *M. morgani* (32), and cefoxitin (4 μ g/ml) was used for the induction of *E. coli* MC4100 with recombinant plasmids. One unit of enzyme activity was defined as the activity which hydro-

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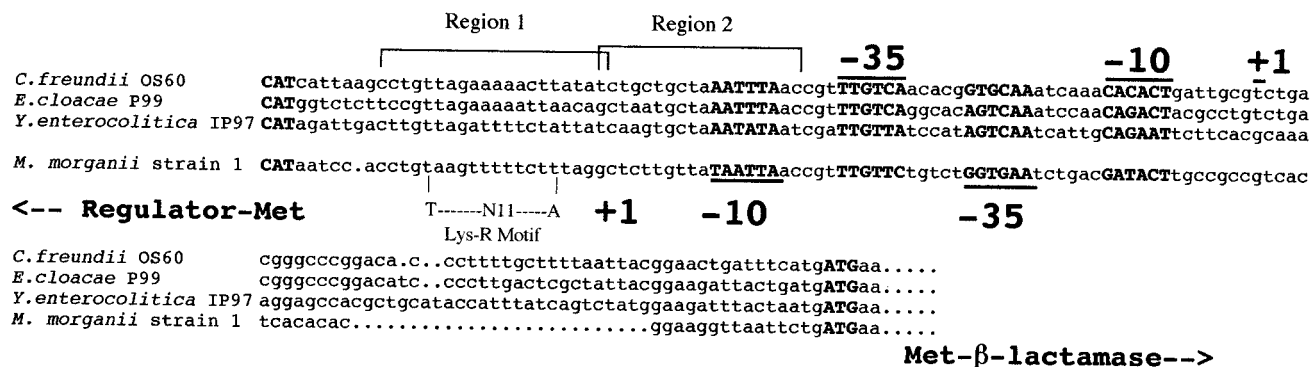


FIG. 5. Alignment of the intercistronic region of *ampC-ampR* from *M. morgani*, *C. freundii*, *Y. enterocolitica*, *S. marcescens*, and *E. cloacae*. The start codons and the -35 and -10 regions of the promoters are shown below the DNA sequences for *ampR* and above for the DNA sequences for *ampC*. The +1 sign indicates the putative mRNA transcription start site. The sequences marked Region 1 and Region 2 correspond to those conserved among *ampC-ampR* intercistronic regions. Region 1 and region 2 are the two components of the putative AmpR binding site. Only region 1 contains a LysR binding motif (T-N₁₁-A).

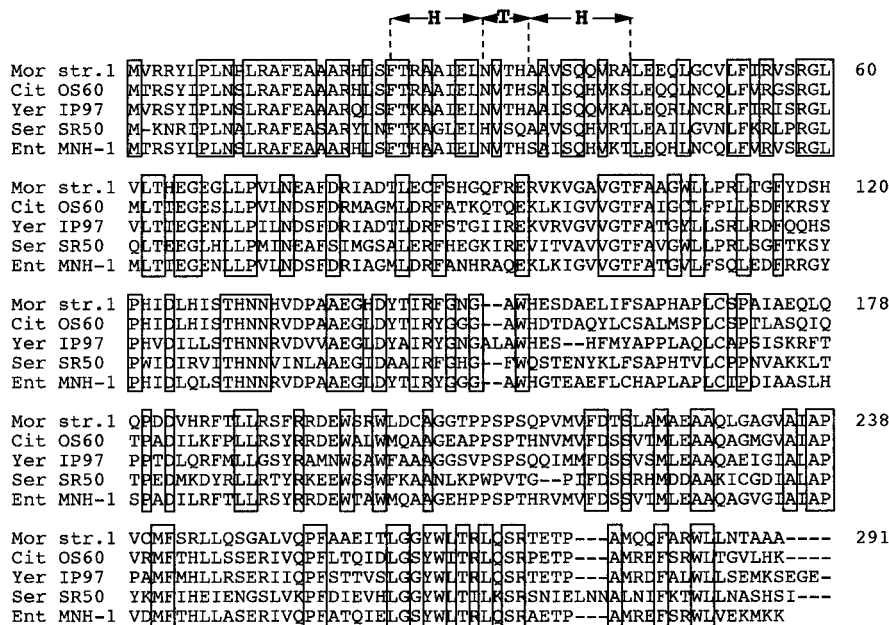


FIG. 6. Multiple-sequence alignment of amino acid sequences of AmpR regulating cephalosporinase expression. The origins of AmpR are as follows: *M. morganii* 1 (Mor str. 1), *C. freundii* OS60 (Cit OS60), *Y. enterocolitica* IP97 (Yer IP97), *S. marcescens* SR50 (Ser SR50), and *E. cloacae* MHN-1 (Ent MHN-1). Identical amino acids are boxed. The predicted helix-turn-helix DNA-binding motif of the LysR family is shown (HTH).

lyzed 1 μmol of cephalothin per min. The total protein content was measured with bovine albumin as the standard (Bio-Rad DC Protein assay kit).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the Genbank/EMBL nucleotide databases under the accession no. AF055067.

RESULTS AND DISCUSSION

Cloning of the cephalosporinase gene and susceptibility testing. Cloning from genomic DNA of *M. morganii* 1 gave several recombinant clones containing 5.9- to 12-kb inserts. One of them, which harbored recombinant plasmid pPON-1 with a 5.9-kb insert, was retained for further analysis (Fig. 1). MIC determinations showed that *M. morganii* 1 was resistant to amoxicillin, cephalothin, ceftazidime, and cefotaxime, of intermediate susceptibility to ticarcillin and ceftoxitin, and fully susceptible to moxalactam and imipenem (Table 1). This antibiotic resistance pattern corresponded to that of an *M. morganii* strain producing high levels of cephalosporinase. As reported previously (32) and as opposed to other enterobacterial species which possess a cephalosporinase, such a strain was, surprisingly, of intermediate susceptibility to ticarcillin and ceftoxitin, despite being resistant to expanded-spectrum cephalosporins. This strain remained susceptible to cefepime and ceftiprole, likely due to the poor affinity of the *M. morganii* cephalosporinase for these β-lactams and/or better intracellular penetration (27).

E. coli harboring pPON-1 showed a resistance profile similar to that of *M. morganii* 1 except for resistance to ceftoxitin (Table 1). The addition of clavulanic acid did not modify the resistance pattern. However, as described for other *M. morganii* cephalosporinases (1), the addition of tazobactam decreased the MICs of ticarcillin and ceftazidime, which is an uncommon property for cephalosporinases (Table 1).

Identification of the cephalosporinase. The entire 5.9-kb insert from recombinant plasmid pPON-1 was sequenced on both strands (Fig. 1). Analysis of this insert for coding regions revealed an open reading frame (ORF) of 1,137 bp encoding a

379-amino-acid protein. This ORF was preceded by -35 and a -10 regions consistent with a putative enterobacterial promoter (Fig. 3). The overall GC content of this ORF was 51%, which is within the expected range of the GC contents of enterobacterial genes. Within the deduced amino acid sequence of the protein, a serine-valine-phenylalanine-lysine tetrad (S-V-F-K) at positions 67 to 70 was found (Fig. 3); it included the conserved serine and lysine amino acid residues characteristic of β-lactamases possessing a serine active site (15). Three structural elements characteristic of class C β-lactamases (cephalosporinases) were found (21): YAN at positions 150 to 152, DAES at positions 217 to 220, and KTG at positions 314 to 316 (Fig. 4). This enzyme shared 56, 55, 54, 53, and 39% homologies with AmpC from *E. coli* K-12, *C. freundii* OS60, *E. cloacae* MHN-1, *Y. enterocolitica* IP97, and *S. marcescens* SR50, respectively.

Kinetic parameters of the identified β-lactamase (called DHA-1) revealed that it had strong activity against benzylpenicillin and cephalothin but poorly hydrolyzed ceftazidime, ceftoxime, and ticarcillin (Table 2). Its activity was strongly inhibited by tazobactam and was poorly inhibited by clavulanic acid. Their 50% inhibitory concentrations were 0.6 and >100 μM, respectively (28% inhibition for 300 μM clavulanic acid). These hydrolysis parameters correlated to those obtained with another *M. morganii* isolate described by Yang and Livermore (32). *M. morganii* 1 produced the same β-lactamase of pI 7.6 as the *E. coli* DH10B strain harboring recombinant plasmid pPON-1 (data not shown).

While this work was in progress, the sequence of an *ampC* gene from an *M. morganii* isolate (isolate SLM01) was published (3). It shares 98.9% amino acid identity with DHA-1 (Fig. 4). The cephalosporinases of *M. morganii* SLM01 and DHA-1 have pI values of 7.4 and 7.6, respectively. Their sequences differ at four positions for SLM01 and DHA-1: position 48, isoleucine versus valine, respectively; position 84, alanine versus threonine, respectively; position 90, alanine versus glutamic acid, respectively; and position 133, asparagine versus

TABLE 3. β -Lactamase activity of *M. morgani* 1 with or without pNH5 (*ampD* of *E. cloacae*) and *E. coli* MC4100 harboring either recombinant plasmid pAC-1 (*ampR* and *ampC* genes) or pAC-2 (*ampC* gene)

Strain	β -Lactamase activity (mU/mg of protein) ^a	
	Basal level	Induced
<i>M. morgani</i> 1 ^b	1,430	1,350
<i>M. morgani</i> 1(pNH5) ^b	8	400
<i>E. coli</i> MC4100(pAC-1) ^c	200	1,350
<i>E. coli</i> MC4100(pAC-2) ^c	1,160	1,100

^a One unit of β -lactamase is defined as 1 μ mol of cephalothin hydrolyzed per min. The β -lactamase activities are geometric mean determinations for three independent cultures. The standard deviations were within 10%.

^b Imipenem (0.5 μ g/ml) was used as the inducer.

^c Cefoxitin (4 μ g/ml) was used as the inducer.

histidine, respectively (Fig. 4). The first difference at position 48 is neutral. None of these amino acid changes are located within the putative catalytic site, as deduced from the closely related cephalosporinase from *E. cloacae* (18). These changes did not extend the hydrolysis profile of DHA-1 compared to that of a previously published cephalosporinase for another *M. morgani* isolate (3).

Hybridization experiments were then performed with genomic DNA from *M. morgani* 1 restricted with either *EcoRI*, *EcoRV*, or *PstI*. These restriction enzymes did not cut within the *bla*_{DHA-1} internal probe consisting of the 385-bp *SacI*-*BssHII* fragment from pPON-1 (Fig. 1). Single hybridization bands of various sizes (7, 3.2, and 9 kb, respectively) were obtained, thus indicating that *bla*_{DHA-1} is unique in *M. morgani* 1 chromosomal DNA, excluding any *ampC* duplication.

Genetic environment of the cephalosporinase gene. Downstream from the *ampC* gene, another ORF (*orf-1*) of unknown function was found. An identical *orf-1* was found downstream from *ampC* in *M. morgani* SLM01 (3), thus suggesting a conserved position of *ampC* in *M. morgani* species. Upstream from the *ampC* gene, three ORFs were also found in pPON-1 (Fig. 1). Two of them coded for HybF and HybE (partial sequence); the two proteins shared sequence homologies with hydrogenase subunits of *E. coli*, and HybF shared 66% homology with HybF from *E. coli* (23). Their functions in *M. morgani* have not yet been studied. Immediately upstream from the *ampC* gene, an *ampR* gene was found, and its deduced protein was 99% homologous to the partial sequence of AmpR reported from *M. morgani* SLM01. The *ampR* gene had an over-

lapping and divergently oriented promoter, as described for other *ampC-ampR* regulatory systems (Fig. 5) (5). Analysis of the intercistronic region revealed close similarity with those of the other *ampC-ampR* systems of *C. freundii*, *E. cloacae*, *S. marcescens*, and *Y. enterocolitica* (Fig. 5) (5, 11, 17, 28). Upstream from the -35 and -10 consensus sequences for the *ampC* promoter, a deletion has occurred in *M. morgani* species compared to those of the other enterobacterial species. The significance of this result remains unknown; it is, however, unlikely that it may significantly influence the effect of AmpR binding, but it may affect AmpC expression. The deduced AmpR protein of *M. morgani* 1 shared homology with AmpR proteins of other species: 81, 62, 60, and 48% for *E. cloacae* MHN-1, *C. freundii* OS60, *Y. enterocolitica* IP97, and *S. marcescens* S50, respectively (25). This homology was mainly within the N-terminal sequence, where a helix-turn-helix motif is required for binding to the intercistronic regions between *ampR* and *ampC* (Fig. 6). The homologies among the AmpR proteins were different from those found for AmpC proteins, although AmpR and AmpC from *S. marcescens* SR50 shared the least homology with both AmpR and AmpC from *M. morgani* 1.

Regulation of cephalosporinase expression. Since pBKCMV is a multicopy vector (200 to 300 copies), expression of *ampC* was studied after its cloning into a plasmid, pACYC184, of lower copy number (20 to 30 copies). pAC-1 and pAC-2 were obtained by cloning the PCR-amplified *ampC* and *ampR* genes and the *ampC* gene, respectively. *E. coli* MC4100(pAC-1) had a cephalosporinase inducible phenotype in the presence of cefoxitin (6.7-fold increase), while *E. coli* MC4100(pAC-2), which lacked the *ampR* gene, showed an increase in its level of basal cephalosporinase expression (5.8-fold), together with a loss of inducibility (Table 3). Similarly, the MICs of the β -lactams were higher for *E. coli* MC4100(pAC-2) than for *E. coli* MC4100(pAC-1), and this was most noticeable for cefotaxime (Table 4). Similar results were obtained with the *C. freundii* and *E. cloacae* cephalosporinases, for which *ampR* deletion results in an increase in β -lactamase expression (2.4-fold) and a lack of induction (5, 17). Thus, AmpR seems to act in *M. morgani* like it does in other enterobacterial species: as a negative regulator of cephalosporinase expression in the absence of a β -lactam inducer and as an activator in the presence of an inducer.

The level of cephalosporinase from *M. morgani* 1 indicated high-level and constitutive expression of this enzyme, as inferred by negative results of induction assays (Table 3). *M. morgani* 1 might possess mutations either in the promoter of an *ampD*-like gene or within the structural gene. To test this

TABLE 4. MICs of β -lactams for *E. coli* strains and *M. morgani* 1 with different genotypes

Strain	Relevant genotype	MIC (μ g/ml)			
		Amoxicillin	Piperacillin	Ceftazidime	Cefotaxime
<i>E. coli</i>					
MC4100	<i>ampD</i> ⁺ <i>ampE</i> ⁺ <i>ampC</i>	4	0.5	0.5	<0.06
MC4100(pAC-1)	<i>ampD</i> ⁺ <i>ampE</i> ⁺ <i>ampC</i> ⁺ <i>ampR</i> ⁺	128	8	2	0.5
MC4100(pAC-2)	<i>ampD</i> ⁺ <i>ampE</i> ⁺ <i>ampC</i> ⁺ <i>ampR</i>	512	32	16	2
JRG582	Δ (<i>ampDE</i>)	1	<0.5	0.5	<0.06
JRG582(pAC-1)	Δ (<i>ampDE</i>) <i>ampC</i> ⁺ <i>ampR</i> ⁺	256	8	8	1
JRG582(pAC-1)(pNH5)	Δ (<i>ampDE</i>) <i>ampC</i> ⁺ <i>ampR</i> ⁺ <i>ampD</i> ⁺	128	8	4	0.25
<i>M. morgani</i>					
1		>512	32	4	4
1(pNH5)	<i>ampD</i> ⁺	16	2	0.5	0.12

TABLE 5. β -Lactamase expression phenotypes and MICs at which 90% of strains are inhibited for selected β -lactams for 16 *M. morganii* clinical strains

Phenotype	Isolate	β -Lactamase activity (mU/mg of protein) ^a		MIC ₉₀ (μ g/ml) ^b		
		Basal	Induced ^c	Ceftazidime	Cefotaxime	Piperacillin
High level, constitutive	1	1,430	1,350	4	4	32
	2	5,031	10,487	256	32	64
	3	8,761	6,113	8	4	16
	4	960	1,990	32	8	64
Low level, inducible	5	3	41	0.06	0.12	0.25
	6	3	98	<0.06	<0.06	0.25
	7	3	229	<0.06	<0.06	0.25
	8	3	242	<0.06	<0.06	0.25
	9	4	50	<0.06	<0.06	0.25
	10	4	93	<0.06	<0.06	0.25
	11	5	16	0.06	0.06	0.12
	12	5	3,644	<0.06	<0.06	0.50
	13	10	430	<0.06	<0.06	0.50
	14	35	5,532	<0.06	<0.06	0.50
	15	70	3,165	<0.06	<0.06	0.50
	16	365	2,774	1	1	8

^a One unit of β -lactamase is defined as 1 μ mol of cephalothin hydrolyzed per min.

^b MIC₉₀, MIC at which 90% of isolates are inhibited.

^c Imipenem (0.5 μ g/ml) was used as the inducer.

hypothesis, *M. morganii* 1 was transformed with plasmid pNH5 containing an *ampD* gene from *E. cloacae*. A decrease in the β -lactam MICs was obtained (Table 4). Similarly, the β -lactam MICs for *E. coli* JRG582 (Δ *ampDE*) harboring the *ampC* and *ampR* genes from *M. morganii* 1 with or without the same *ampD* gene were lower in the presence of *ampD* (Table 4), and *E. coli* JRG582 had an inducible cephalosporinase expression phenotype. Additionally, *M. morganii* 1 transformed with pNH5 (*ampD* gene) recovered inducible cephalosporinase expression (50-fold increase) (Table 3). Moreover, these results indicated that an AmpD from *E. cloacae* may act in *trans* as a regulatory protein for expression of *M. morganii* AmpC in a similar manner as for the AmpC of *E. cloacae*.

Induction experiments and basal cephalosporinase levels divided the 15 other *M. morganii* isolates into two groups: those with low-level (although variable) and inducible expression of cephalosporinase and those with high-level and constitutive expression of cephalosporinase (Table 5). Detailed MICs of

the β -lactams for *M. morganii* 5 (low-level and inducible cephalosporinase expression) confirmed these results (Table 1). Both groups correlated with strains with oxyiminocephalosporin-susceptible and -resistant phenotypes, respectively. The only exception was *M. morganii* 16, which had a relatively high level of cephalosporinase expression, but it remained inducible (Table 5). Therefore, it is likely that *M. morganii* strains with high-level and constitutive cephalosporinase expression, like *M. morganii* 1, corresponded to those possessing a nonfunctional AmpD-like protein.

Comparison of the *ampC*- and *ampR*-coding regions and surrounding sequences in *M. morganii* isolates with different β -lactamase expression phenotypes. Using different pairs of primers designed from the pPON-1 sequence (Fig. 1), we amplified *hybF-ampR*, *ampC-ampR*, *ampC*, and *ampC-orf-1* DNA fragments from the genomic DNAs of 15 *M. morganii* isolates, strains 2 to 16. In each case, the same fragments expected from the pPON-1 sequence were obtained, i.e., 2,418 bp for the *hybF-ampR-ampC* fragment, 2,043 bp for the *ampR-ampC* fragment, 1,047 bp for the *ampC* fragment, and 3,073 bp for the *ampC-hybF* fragment. In all *M. morganii* strains studied, *ampR*, *orf-1*, and *hybF* were found at the same locations at which they were found in *M. morganii* 1, whatever the β -lactamase expression phenotype was. Compared to other enterobacterial species in which an *ampC* gene is found (8). *M. morganii* species did not possess a fumarate operon upstream from the *ampC* gene (Fig. 7). This result may infer that *M. morganii* species are more distantly related to *E. cloacae* species and *C. freundii* species than the latter two species are to one another. Surprisingly, HybF shared 53% homology with an ORF, ORFB, of unknown function, with its gene being located just downstream from the fumarate operon in *Proteus vulgaris*, an *ampC*-negative enterobacterial species. It remained intriguing that HybF is a protein involved in dihydrogen uptake in *E. coli*, with its gene being a member of an operon which can be differentially induced to high levels when cells are grown in medium containing dihydrogen as an electron donor and fumarate as an electron acceptor (23).

Finally, Barnaud et al. (4) reported on an inducible plasmid-mediated cephalosporinase gene along with a functional *ampR* gene from *Salmonella enteritidis* (4). Sequence analysis revealed that this plasmid-mediated cephalosporinase was 100% homologous to DHA-1 (the reason why the same name was retained), with their genes differing by only seven base-pair substitutions (data not shown). The AmpR sequences were 98% identical at the DNA level and 99% identical at the protein level. The investigators reported an unusual integron carrying *aadA2*, *sull* along with *bla*_{DHA-1}, and *ampR* (30). In

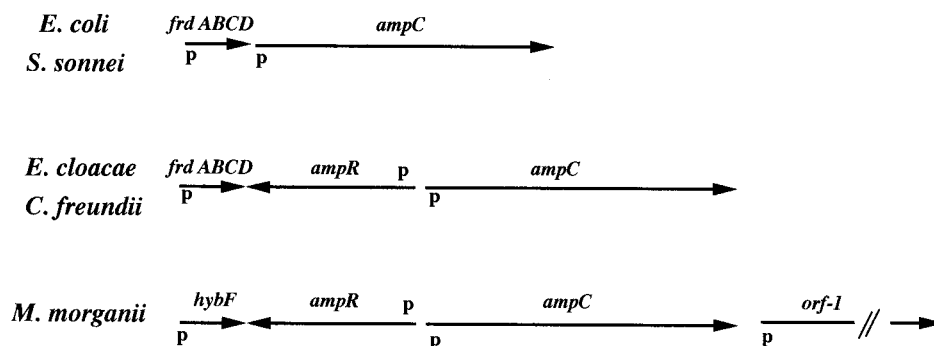


FIG. 7. Organization of the sequences surrounding *ampC* in various enterobacterial species. The positions and directions of the fumarate operon (*frdABCD*), *hybF*, *hybE*, *orf-1*, *ampC*, and *ampR* genes are indicated with arrows. Also indicated are the locations of the putative promoters.

this integron, no 59-bp element typical of an integron recombination site was located closed to *bla*_{DHA-1} or *ampR* (30). *aadA2* and *sull* were not chromosomally located around the *ampR-ampC* genes in our *M. morgani* strains. In contrast, the three chromosomally located ORFs found around *ampR-ampC* genes in *M. morgani* strains were not found to be located on an integron in *S. enteritidis* (30). Analysis of the DNA sequence located on each side of the *ampC-ampR* sequence in *M. morgani* strains and in the *ampC-ampR*-possessing *S. enteritidis* strain did not locate any hot-spot recombination site, therefore giving no explanation for the presence of *ampC-ampR* within this integron.

Taken together, the results indicating a plasmid location of *bla*_{DHA-1} in *S. enteritidis* and the results of our work indicate that almost identical *ampR-ampC* operons may be located in either the chromosome or the plasmid for different enterobacterial species. The encoded AmpC sequences were 100% identical, in contrast to other reported plasmid-mediated cephalosporinases, which share at most 96% homology with any chromosomal cephalosporinases (10). This is therefore confirmatory evidence of the chromosomal origin of the plasmid-located cephalosporinases which are now spread worldwide (22).

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