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Sequences of Wild-Type and Mutant *ampD* Genes of *Citrobacter freundii* and *Enterobacter cloacae*

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The *ampD* gene product regulates the expression of AmpC β-lactamase in gram-negative bacteria and is proposed to be involved in peptidoglycan metabolism. In this study, we sequenced the *ampD* wild type and three mutant genes of *Enterobacter cloacae* and *Citrobacter freundii*. They exhibited a high degree of homology with the corresponding gene of *Escherichia coli* except in the carboxy termini, where, in the wild-type genes of *E. cloacae* and *C. freundii*, four additional amino acids yielding the Ser-X-X-Lys motif were found. Evidence that this C-terminal region of the *ampD* gene product is necessary for activity was shown by constructing a deletion of the last 16 amino acids. The spontaneous mutation of *ampD02* is an out-of-frame insertion and yields an inactive AmpD protein. The single-base-pair substitution of Gly for Asp-121 in *ampD05* is responsible for a hyperinducible phenotype. These results demonstrate regions of the *ampD* gene and the corresponding protein which have functional importance for the induction of AmpC β-lactamase in *E. cloacae*.

Most gram-negative bacteria express chromosomally encoded β-lactamases (24). In *Enterobacter cloacae* and *Citrobacter freundii*, the expression of the *ampC* β-lactamase is inducible by β-lactams, whereas this enzyme is produced constitutively by *Escherichia coli*. The distinguishing feature of the inducible group is the presence of the *ampR* gene encoding a DNA-binding protein which acts as a transcriptional activator in the presence of β-lactam inducers (17). At least four other genes are involved in the expression of these β-lactamases: *ampD* (13), *ampE* (7, 16), *ampG* (10), and *pbpA* (22). The exact function of the corresponding proteins and the mechanism of the regulation of induction of the β-lactamase are still not fully understood.

Gram-negative bacteria with inducible chromosomally encoded β-lactamases mutate at a high frequency (10^{-5} to 10^{-7}) to become resistant to penicillins and cephalosporins (15). This resistance often results from overproduction of the *ampC* β-lactamase, which can be caused by mutations in the *ampD* gene (13). Studies on *ampD* mutants suggest that the *ampD* gene product acts as a negative regulator for *ampC* transcription. There is, however, no evidence that AmpD directly interacts with the DNA region upstream of *ampC* controlling its expression (16). Moreover, *ampD* mutants hyperproduce β-lactamase only in the presence of AmpR and AmpG. It has, therefore, been proposed that AmpD and AmpG together affect the activity of AmpR (10). Since *ampD* mutants of *E. coli* are more susceptible to β-lactam in the absence of β-lactamase (16) and since these mutants exhibit an increased release of [^{3}H]diaminopimelic acid-labelled cell wall fragments into the medium compared with wild-type *E. coli* strains, it has been argued that the cytosolic AmpD protein is somehow involved in the regulation of peptidoglycan metabolism (27).

An *E. coli ampD* mutant can be complemented by *C. freundii* or *E. cloacae ampD*, suggesting that the three allelic variants of the AmpD protein are functionally interchangeable (14). In this study, we compare the sequence of the *ampD* gene of the inducible group of species with the sequence of the corresponding *E. coli* gene which has already been published (7, 16). Moreover, we have determined the sequences of three mutant *ampD* genes from *E. cloacae* to define regions of functional importance.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. pBP20 was constructed by subcloning the *Eco*RV-*Bam*HI wild-type *ampD* from pBP141-3 into *Hin*DII-*Bam*HI-digested pUC19. Plasmid pBP19 is a kanamycin-resistant derivative of pUC19. It was constructed by inserting the 1.2-kb *Sma*I *kan* fragment from pUC4-KIXX into the unique *Sca*I site located in the *bla* gene of pUC19. Digestion of pBP20 with *Nla*IV yields a 1,050-bp *ampD* fragment. pBP19-4 was constructed by ligating this *Nla*IV fragment into pBP19 that had been digested with *Hin*dII. The insertion of the *Nla*IV *ampD* fragment in one orientation yields the stop codon TGA (*ampD04*) at the shortened end of *ampD* after Trp-171.

Microbiological techniques. For routine purposes, the complete medium standard NI broth (Merck AG, Darmstadt, Germany) was used. Iso-Sensitest broth (Oxoid, Wasel, Germany) was used in the determination of β-lactamase expression. When required, sulfamethoxazole (500 μg ml^{-1}), ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and chloramphenicol (30 μg ml^{-1}) were added. All growth was at 37°C.

Susceptibility testing was performed as described by Peter et al. (23). MICs of cefotaxime and cefoxitin were determined by microdilution tests in Iso-Sensitest medium with an inoculum of 10^5 cells per ml.

β-Lactamase-assay. The induction experiment was performed as described by Lindquist et al. (16). Cells were grown to mid-log phase, and cefoxitin and cefotaxime at various concentrations were added as inducers for the production of β-lactamase. After incubation at 37°C for 40 min, cells were harvested and frozen overnight. Treatment with lysozyme and sonication on ice with a Branson sonifier

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
JM83	F⁻ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ80d Δ(*lacZ*)M15]	28
HfrH	Hfr *thi-1 rel-1*	18
SN03	*ampA1 ampC8 pyrB recA13 rpsL*	21
SN0302	*ampA1 ampC8 ampD2 recA*	13
Plasmids		
pUC19	Ap	28
pUC4-KIXX	Ap Tn*5* Kan	20
pBP19	pUC19 (1.2 kb *Sma*I *kan* gene from pUC4-KIXX) Kan	This study
pBP131	RSF1010 (*ampR ampC* from *E. cloacae* 14) Su	12
pACYC184	Cm Tc	4
pBP141-3	pACYC184 (*ampD* from *E. cloacae* 14) Cm	9
pBP14102-3	pACYC184 (*ampD02* from *E. cloacae* 1402) Cm	9
pBP14105-3	pACAC184 (*ampD05* from *E. cloacae* 1405) Cm	9
pBP20	pUC19 (*ampD* from pBP141-3) Ap	This study
pBP19-4	pBP19 (1.05 kb *Nla*IV *ampD* fragment from pBP20) Kan	This study
pNU405	pBR322 (*ampD* from *C. freundii* OS60)	13

[a] Ap, ampicillin; Cm chloramphenicol; Kan, kanamycin; Su, sulfamethoxazole; Tc, tetracycline.

yielded the cell extract. The β-lactamase assay was performed as described by Peter et al. (23). The activity of the β-lactamase was quantified by using nitrocefin as the substrate (50 μM). The protein content of each sonic extract was determined by the method of Bradford (3) by using bovine serum albumin as the standard. Every induction experiment was performed in triplicate, and the results are averages.

Nucleic acid techniques. Recombinant DNA techniques and transformation of DNA were performed as described by Maniatis et al. (19).

Double-stranded DNA was prepared by using the protocols of Quiagen (Chatsworth, Calif.) and sequenced by using the dideoxy chain termination method of Sanger et al. (25).

For pUC sequencing, we used the 17mer reverse sequencing primer (Promega, Serva-group, Heidelberg, Germany) and the 17mer sequencing primer (Pharmacia, Freiburg, Germany). To sequence both strands of the complete wild-type and mutant *ampD* genes (*ampD02* and *ampD05*), we constructed sequence-specific oligonucleotides by using a model 391 DNA synthesizer (Applied Biosystems).

Computer techniques. Sequencing primers were constructed and the exclusion of homology to other parts of the gene or plasmid was controlled by using the DNASIS program (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). The nucleotide and protein sequences were aligned with the HUSAR program (Heidelberg Unix Sequence Analysis Resources, Heidelberg, Germany).

Nucleotide sequence accession numbers. These sequence data will appear in the EMBL/GenBank/DDBJ nucleotide sequence data bases under the accession number Z14003 for *ampD* from *E. cloacae* 14 and Z14002 for *ampD* from *C. freundii* OS60.

RESULTS AND DISCUSSION

Nucleotide and deduced amino acid sequences of *ampD* of *C. freundii* and *E. cloacae*. The expression of *E. cloacae ampC* β-lactamase yields high β-lactam resistance when present together with *ampR* on plasmid pBP131 in the *E. coli ampD2* mutant SN0302 (Table 2). Coexpressing either *E. cloacae ampD* on pBP20 or *C. freundii ampD* on pNU405 (13) complemented the chromosomal *E. coli ampD2* mutation to β-lactam susceptibility, showing that the cloned *ampD* genes express functional proteins in *E. coli*. The nucleotide sequences of *ampD* from *E. cloacae* 14 and *C. freundii* OS60 were determined by using the dideoxy nucleotide sequencing method (25) and compared with that of the corresponding gene of *E. coli* (Fig. 1). At the nucleotide level, the three genes exhibit a homology of 74 to 83%, which was similar to the homologies found for their respective *ampC* genes (5). The *ampR* genes from *E. cloacae* and *C. freundii* have also been determined (6, 17) and found to be 81% homologous at the nucleotide level. Thus, the sequence differences between the three *ampD* genes seem to reflect the evolutionary distance between the three species.

The three AmpD proteins are highly homologous except for their carboxy termini, where *E. cloacae* and *C. freundii* carry four additional amino acids compared with *E. coli* AmpD, yielding a Ser-X-X-Lys motif typical of β-lactam binding proteins (8). However, none of the other six motifs characteristic of penicillin-binding proteins were found in AmpD, making it unlikely that AmpD belongs to this family of proteins. Most other differences between the three proteins were conservative replacements which are unlikely to affect protein function. The only single amino acid substitution so far reported to abolish AmpD activity is the substitution of Gly for Trp-7 in *E. coli* AmpD11 (7). As expected, Trp-7 was conserved in all AmpD proteins.

The carboxy-terminal region of *E. cloacae* AmpD is important for activity. To investigate whether the carboxy-terminal

TABLE 2. β-lactam resistance expressed from the cloned *ampC* gene of *E. cloacae* in *E. coli* SN0302 (*ampD2*)

Plasmid(s)	Plasmid[a] genotype	MIC (mg liter^{-1})	
		Cefoxitin	Cefotaxime
pBP131	*ampR ampC*	128	64
pBP131; pBP19	*ampR ampC*	128	32
pBP131; pBP20	*ampR ampC*; *ampD*	4	0.5
pBP131; pBP19-4	*ampR ampC*; *ampD04*	128	32
pBP131; pACYC184	*ampR ampC*	128	64
pBP131; pBP141-3	*ampR ampC*; *ampD*	8	0.25
pBP131; pBP14102-3	*ampR ampC*; *ampD02*	128	64
pBP131; pBP14105-3	*ampR ampC*; *ampD05*	128	0.25

[a] Plasmid-borne wild-type and mutant alleles of *ampR*, *ampC*, and *ampD* were from *E. cloacae*.

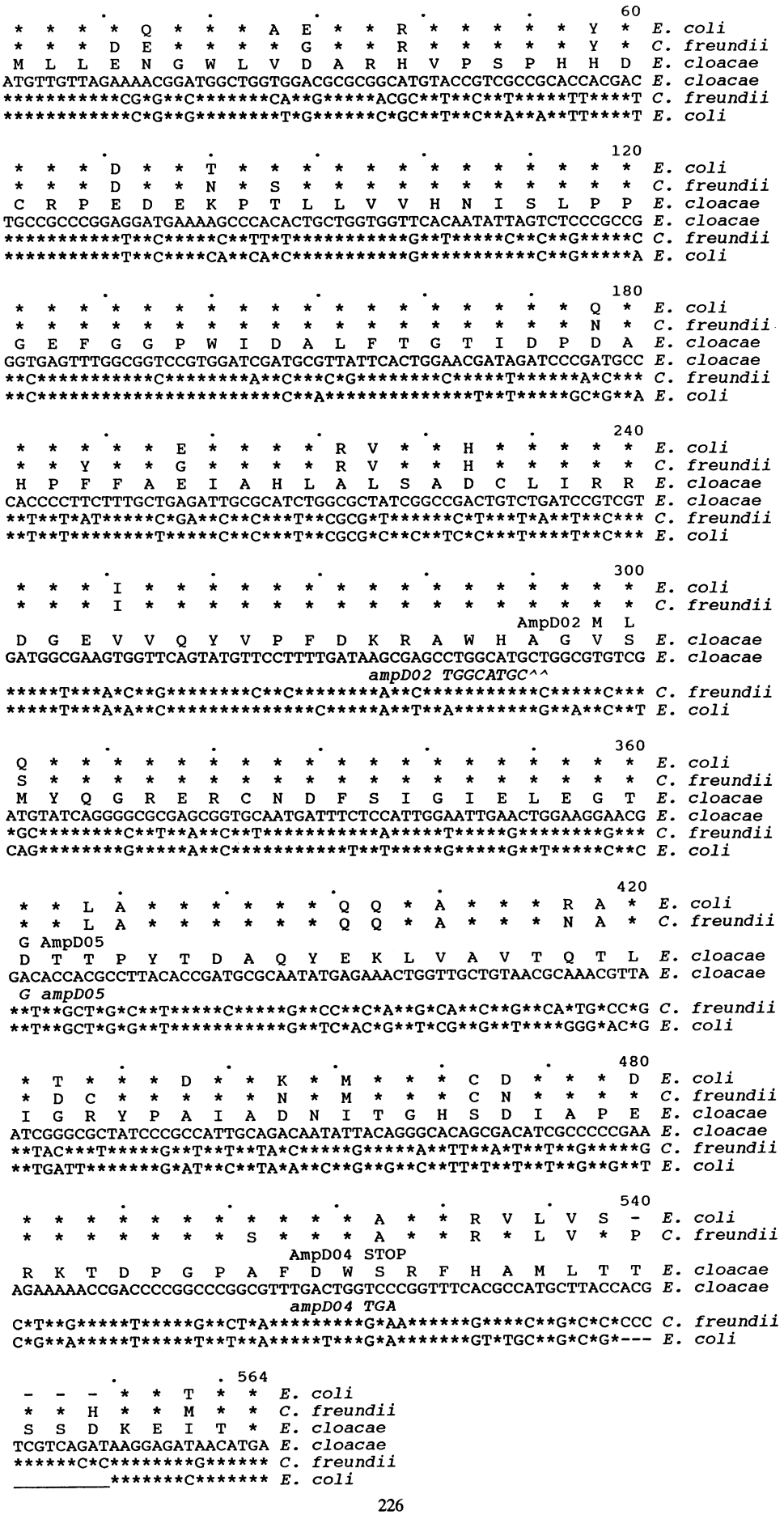

TABLE 3. β-Lactamase expression of SN0302/pBP131 and SN03/pBP131 containing wild-type *ampD* and *ampD05* on pACYC184

E. coli strain/ *ampR*$^+$ *ampC*$^+$ plasmid	*ampD* plasmid	Sp act[a]			
		Uninduced	Induced with cefoxitin at concn (μg/ml) of:		
			8	16	128
SN0302/pBP131	Wild type	4	23	44	40
SN0302/pBP131	*ampD05*	3	8	102	135
SN03/pBP131	Wild type	1	6	6	19
SN03/pBP131	*ampD05*	2	6	13	106

[a] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

region of AmpD has functional importance, a stop codon (TGA) was introduced (as described in Materials and Methods) in *E. cloacae ampD*, yielding a truncated *ampD* gene product lacking the 16 carboxy-terminal residues. Plasmid pBP19-4 carrying this *ampD04* allele did not complement the *E. coli ampD2* mutation (Table 1), showing that AmpD04 is a functionally inactive protein.

To prove the stability of this truncated protein, we cloned *ampD04* and wild-type *ampD* in pT7-7. By using the T7-System of Tabor and Richardson (26), we could overproduce both the truncated and the wild-type AmpD proteins. These plasmid-encoded proteins with calculated molecular masses of 18.98 (AmpD04) and 20.84 (wild-type AmpD) kDa, respectively, were labelled with [^{35}S]methionine and separated on linear 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Determination of the mutations in *E. cloacae ampD02* and *ampD05*. In a study by Korfmann and Wiedemann (12), cefotaxime-resistant mutants of *E. cloacae* strains were selected and studied. The *ampD* mutants from their study, *ampD02* and *ampD05*, were sequenced in the present study.

The *ampD02* mutation is a tandem duplication of the sequence TGGCATGC, yielding a frameshift mutation after codon 98 (Fig. 1). Since plasmid pBP14102-3 carrying this mutation did not complement *ampD2* of *E. coli* SN0302 (Table 2), the *ampD02* mutation resulted in a nonfunctional AmpD protein.

The *ampD05* mutation was found to be an A-to-G transition in codon 121 which creates an additional *Ban*I restriction site and changes Asp-121 into Gly. Asp-121 is found in the sequences of all three AmpD proteins and is located in a highly conserved region of AmpD (Fig. 1). Interestingly, this alteration in *ampD* seems to function like the wild-type *ampD*, as far as the MIC of cefotaxime is concerned, but results in an increased MIC of cefoxitin (Table 2).

To further characterize the *ampD05* mutant phenotype, β-lactamase expression in the mutant was monitored with and without cefoxitin and cefotaxime as inducers in comparison with its expression in the wild-type strain. The basal level of β-lactamase was identical in both strains, in SN0302/pBP131/pBP14105-3 and in SN302/pBP131/pBP141-3, suggesting that the Gly-121 AmpD mutant protein acts as a negative effector on AmpC β-lactamase expression in the absence of an inducer. Induction experiments with different concentrations of cefoxitin revealed that the degrees of β-lactamase expression were similar for both strains with 8 μg of cefoxitin per ml but considerably higher with the mutant *ampD* when the inducer concentration was raised to 16 μg/ml or higher (Table 3). The hyperinducible phenotype exhibited by the Gly-121 mutant was also observed in *E. coli* SN03 expressing a wild-type *E. coli* AmpD protein and containing *ampR ampC* on plasmid pBP131 (Table 3). Thus, the mutant allele was dominant over the wild type.

A hyperinducible phenotype was previously reported for an *E. coli ampD* mutant (*ampD1*) carrying a frameshifting deletion of 52 bp after codon 23 (16). The mutant phenotype and the nature of the mutation led the authors to suggest that the AmpD protein was not essential for induction. The different expression of β-lactamase in an *ampD1* and *ampD2* *E. coli* background was suggested to be due to a different level of expression of the distally located *ampE* gene. However, since plasmid-borne *ampD* with or without *ampE* complements chromosomal *ampD* mutations, the different phenotypes of *E. coli ampD1* and *ampD2* may have other explanations. One attractive possibility is that translational frameshifting can occur at the ribosomal level, yielding low levels of an AmpD protein with an internal 17-amino-acid deletion. This second hypothesis is supported by the data presented here. Hyperinducibility was generated by expressing only the AmpD Gly-121 mutant protein in trans. Since none of the plasmids carry the *E. cloacae ampE* gene, the mutant phenotype must therefore be due solely to the AmpD protein. An *ampD* gene which yields a hyperinducible phenotype when expressed on plasmid pGKS145-4 in SN0302/pBP131 was also described by Korfmann et al. (11). At 30°C, the MIC of cefotaxime for this strain is lower than that obtained for SN0302/pBP131 but three times higher than that obtained for SN0302/pBP131 containing the cloned wild-type *ampD* gene. Because of these different MICs of cefotaxime, the mutation must be different from the Gly-121 described in this article.

Since both the mutant AmpD protein expressed from plasmid pGKS145-4 and the AmpD Gly-121 mutant protein described in this article were dominant over the wild type, the hyperinducible phenotype is likely not explained by a reduced expression of AmpD but by an altered function of these AmpD mutant proteins.

A tentative model for the regulation of class I chromosomally encoded β-lactamases has been proposed (2). This model implies that *ampC* transcription is dependent on the activation state of AmpR. It has been proposed that in *ampD* mutants a positive endogenous ligand is generated even in the absence of a β-lactam inducer. As suggested by Bartowsky and Normark (1), who quoted unpublished data by S. Lindquist et al., the *ampG* gene product is a transmembrane protein which is involved in signal transmission across the cytoplasmic membrane. In the cytosol, this ligand can bind to AmpR and convert the protein into a transcriptional activator. It is believed that the AmpD protein is involved in peptidoglycan metabolism, possibly by acting negatively on the carboxypeptidase activity (27). Since carboxypeptidases hydrolyze the terminal D-alanine from the cell wall stem

FIG. 1. Nucleotide sequence from *E. cloacae* 14 *ampD* along with deduced amino acid sequence of AmpD. Both the nucleotide and amino acid sequences are compared with the corresponding sequences from *E. coli* SN03 and *C. freundii* OS60. The three *E. cloacae ampD* mutations discussed in this article are shown. An asterisk (*) marks the nucleotides or amino acids that are identical to those in the sequence from *E. cloacae* 14.

peptide, one possibility is that the activating ligand for AmpR involves D-alanine-containing cell wall peptides. However, at this stage it is better to be cautious and conclude that the chemical nature of the ligand(s) activating AmpR in an *ampD* mutant background is not known. It may be that the hyperinducible phenotype exerted by the Gly-121 AmpD mutant protein can be explained by a decreased ability to interact with such a ligand while retaining the ability to affect peptidoglycan metabolism as in the case of the wild-type AmpD protein. This would lead to an increased availability for ligand interaction with AmpR and subsequent activation of *ampC* transcription. Further studies are needed to prove or disprove such a hypothesis.

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