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AmpD Is Required for Regulation of Expression of NmcA, a Carbapenem-Hydrolyzing β-Lactamase of *Enterobacter cloacae*

THIERRY NAAS,* SANDRINE MASSUARD, FABIEN GARNIER, AND PATRICE NORDMANN

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre, France

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To further elucidate the induction process of the carbapenem-hydrolyzing β-lactamase of Ambler class A, NmcA, *ampD* genes of the wild-type (WT) strain and of ceftazidime-resistant mutants of *Enterobacter cloacae* NOR-1 were cloned and tested in transcomplementation experiments. Ceftazidime-resistant *E. cloacae* NOR-1 mutants exhibited derepressed expression of the AmpC-type cephalosporinase and of the carbapenem-hydrolyzing β-lactamase NmcA. The *ampD* genes of *Escherichia coli* and *E. cloacae* WT NOR-1 transcomplemented the ceftazidime-resistant *E. cloacae* NOR-1 mutants to the WT level of β-lactamase expression, while the mutated *ampD* alleles of *E. cloacae* NOR-1 failed to do so. The deduced *E. cloacae* NOR-1 WT AmpD protein exhibited 95 and 91% amino acid identity with the *E. cloacae* O29 and *E. cloacae* 14 WT AmpD proteins, respectively. Of the 12 ceftazidime-resistant *E. cloacae* NOR-1 strains, 3 had AmpD proteins with amino acid changes, while the others had truncated AmpD proteins. Most of these mutations were located outside the conserved regions that link the AmpD proteins to the cell wall hydrolases. AmpD from *E. cloacae* NOR-1 is involved in the regulation of expression of both β-lactamases (NmcA and AmpC), suggesting that structurally unrelated genes may be under the control of an identical genetic system.

Several enterobacterial species express inducible, chromosomally encoded AmpC-type β-lactamases (cephalosporinase) (11, 37), which are class C enzymes according to Ambler's classification (2). The regulation of AmpC β-lactamase expression is intimately linked to cell wall recycling and involves at least three genes: *ampR*, which encodes a transcriptional regulator of the LysR family; *ampG*, which encodes a transmembrane permease; and *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase hydrolyzing 1,6-anhydromuropeptides (11, 12, 15, 16, 19, 31). In the absence of β-lactam inducer, AmpR is repressed by the murein precursor UDP-MurNAc-pentapeptide (uridine-pyrophosphoryl-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine) (14). Since β-lactams interfere with murein synthesis, their actions lead to an increased periplasmic accumulation of degradation products, such as 1,6-anhydromuropeptides, which are signal molecules for β-lactamase induction (8, 15). AmpG transports these products from periplasm to cytoplasm, where they are cleaved by AmpD, which acts as a negative regulator of AmpC β-lactamase expression (8, 15, 19, 31). In *ampD* mutants, the constitutive overproduction of AmpC β-lactamase is associated with an accumulation of an M tripeptide (monosaccharide tripeptide) (1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid) and an M pentapeptide (1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine) in the cytoplasm (8, 15). Jacobs et al. suggested that the M tripeptide could be the AmpR-activating ligand, since this product can relieve the repressed state of AmpR in vitro, resulting in the activation of β-lactamase expression (14). However, potential interactions of the M pentapeptide with AmpR have not been investigated. Another gene, *ampE*, which encodes a transmembrane protein, forms an operon with *ampD*, but this gene is not involved in β-lactamase expression (11, 13, 32).

Several chromosomally mediated Ambler class A β-lactamases are regulated in a manner similar to AmpC. In *Proteus vulgaris*, the chromosomal class A β-lactamase CumA is under the control of CumR, a LysR-type regulator, and is dependent on the presence of CumD (an AmpD analog) and CumG (an AmpG analog) for its induction (7). Similar observations have been made for *Citrobacter koseri* (formerly *Citrobacter diversus*) where CdiA, a class A β-lactamase, is under the control of CdiR (17) and for *Burkholderia cepacia*, which expresses a class A β-lactamase, PenA, that is under the control of a transcriptional regulator, PenR (41). *Serratia fonticola* expresses an inducible oxyimino cephalosporin-hydrolyzing class A β-lactamase named SFO-1 (33). This enzyme was recently found to be plasmid mediated in *Enterobacter cloacae* 8009 and regulated by an AmpR-type regulator (24). An inducible β-lactamase from *Proteus penneri* that is closely related to class A β-lactamases from a biochemical point of view has been described (25).

In *E. cloacae* NOR-1, in addition to the inducible AmpC β-lactamase that is under the control of the transcriptional regulator AmpR (29, 30), a second β-lactamase, NmcA, had been identified (30). NmcA, unlike most carbapenem-hydrolyzing β-lactamases that are involved in acquired carbapenem resistance, belongs to Ambler class A (26). NmcA expression is also regulated by the presence of a LysR-type regulator, NmcR, which is necessary for NmcA expression and induction (26, 30). To further elucidate the NmcA induction process,

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France. Phone: 33-1-45-21-20-19. Fax: 33-1-45-21-63-40. E-mail: thierry.naas@kb.u-psud.fr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference(s)
Strains		
E. coli DH10B	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ− *rpsL endA1 nupG*	Gibco-BRL-Life Technologies, Eragny, France
E. cloacae NOR-1	Inducible AmpC and NmcA β-lactamases	26, 30
E. cloacae NOR-1D$_{1\rightarrow12}$[b]	Ceftazidime-resistant mutants of *E. cloacae* NOR-1	This work
E. cloacae MHN1	Inducible AmpC β-lactamase	29
E. cloacae MHN2	Δ*ampDE* derivative of *E. cloacae* MHN1	29
E. coli MC4100	Δ*ampC*	13
E. coli JRG582	Δ*ampC* Δ*ampDE*	13
Plasmids		
pK19	Neo^r/Kan^r	36
pACYC184	Cm^r/Tet^r	5
pGB2	Str^r/Spc^r	6
pNH5	*ampD* of *E. coli*	13
pPCRScriptCm	Cm^r	Stratagene
pPTN-1	5.2-kb *Sau*3AI fragment from *E. cloacae* NOR-1 containing *nmcA* and *nmcR* into pACYC184 *Bam*HI	26, 30
pPTN-3	*Nae*I fragment from pPTN-1 containing *nmcA* and *nmcR* into pK19 *Sma*I	This report
pPTN-7	Blunt-ended *Nae*I fragment from pPTN-1 containing *nmcA* and *nmcR* into blunt-ended pACYC184 *Bam*HI	This report
pPTN-9	*Nae*I fragment from pPTN-1 containing *nmcA* and *nmcR* into pGB2	This report
pMS$_{1-12}$	*E. cloacae* NOR-1 *ampD*$_{1-12}$ mutant genes cloned into pPCRScript	This report
pMS$_{13}$	*E. cloacae* NOR-1 WT *ampD* gene cloned into pPCRScript	This report

[a] Antibiotic resistance phenotypes for plasmids: Neo^r/Kan^r, neomycin or kanamycin resistance; Cm^r/Tet^r, chloramphenicol or tetracycline resistance; Str^r/Spc^r, streptomycin or spectinomycin resistance.
[b] The subscript $_{1\rightarrow12}$ indicates the ceftazidime-resistant *E. cloacae* NOR-1D mutants.

ampD genes of the wild-type (WT) NOR-1 strain and of ceftazidime-resistant *E. cloacae* NOR-1 (NOR-1D) mutants were cloned and tested in transcomplementation experiments. Furthermore, the inducibility of NmcA expression was tested when *nmcA* and *nmcR* were located on a plasmid at different copy numbers.

MATERIALS AND METHODS

Bacterial strains, antimicrobial agents, and MIC determinations. The strains and plasmids used in this study are described in Table 1. Electrocompetent *Escherichia coli* DH10B (Life Technologies, Eragny, France) was used as host for construction and propagation of recombinant plasmids. Electrocompetent *E. coli* MC4100, *E. coli* JRG582, *E. cloacae* MHN1, *E. cloacae* MHN2, and *E. cloacae* NOR-1 variants were prepared as described previously (34). Bacterial cells were grown in Trypticase Soy (TS) broth or on TS agar plates (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). When required, kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), and spectinomycin (50 μg/ml) were added.

Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton (MH) agar plates (Sanofi Diagnostics Pasteur). The antimicrobial agents and their sources have been described elsewhere (27, 30). MICs of selected β-lactams were determined by an agar dilution technique on MH agar plates with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot (27, 30, 34). All plates were incubated at 37°C for 18 h. MICs of β-lactams were determined alone or with a fixed concentration of either clavulanic acid (2 μg/ml) or tazobactam (4 μg/ml). MIC results were interpreted according to the National Committee for Clinical Laboratory Standards guidelines (28).

In vitro selection of extended-spectrum cephalosporin-resistant mutants. Frequencies of in vitro selection of antibiotic-resistant mutants were determined by counting the number of colonies that arose by plating a large inoculum (10^9 CFU) of *E. cloacae* NOR-1 on MH agar plates containing ceftazidime (32 μg/ml).

Kinetic measurements. β-Lactamase extracts were obtained as described previously (34). The specific β-lactamase activity of the extracts was measured by UV spectrophotometry (ULTROSPEC 2000 spectrophotometer; Amersham Pharmacia Biotech, Orsay, France) as described previously (34). Basal and induced β-lactamase levels were determined as previously described (34). β-Lactamase activity was induced with imipenem (10 μg/ml) and cefoxitin (2 μg/ml for *E. cloacae* WT NOR-1 and 50 μg/ml for ceftazidime-resistant *E. cloacae* NOR-1D mutants). The specific β-lactamase activities were obtained as previously described with cephalothin and imipenem as substrates (30, 34). One unit of enzyme activity was defined as the activity which hydrolyzes 1 μmol of cephalothin or imipenem per min. The total protein content was measured with the Bio-Rad DC protein assay kit (Bio-Rad, Ivry/Seine, France).

Hydridization, PCR analyses, and sequencing. Standard PCR experiments were performed as described previously (36). All the PCR amplifications were performed using the following amplification program: 10 min at 94°C; 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C; followed by a final extension step of 10 min at 72°C. In order to PCR amplify the *ampD* genes of *E. cloacae* WT NOR-1 and mutant strains, two primers derived from the *E. cloacae* O-14 *ampD* sequence (18, 23) were synthesized. The sequences of the primers were as follows: AmpDF, 5′-ATGTTGTTAGAAAACG GATG-3′; and AmpDB, 5′-TCATGTTATCTCCTTATCTG-3′. A 564-bp DNA fragment encompassing the entire *ampD* gene was amplified by PCR with the *Taq* DNA polymerase (PE Biosystems, Les Ulis, France) and whole-cell DNAs of *E. cloacae* WT NOR-1 and *E. cloacae* NOR-1D mutants.

The amplicons were purified using the Qiaquick PCR purification kit (Qiagen). The nucleotide and amino acid sequences were analyzed using the software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Multiple-sequence alignment of deduced peptide sequences was performed online at the University of Cambridge website using the ClustalW program (http://www.ebi.uk/clustallW).

In order to screen for the presence of the *ampD* gene in *E. cloacae* NOR-1D mutants that were negative by PCR analysis, 2-μg samples of heat-denatured whole-cell DNAs were spotted onto a nylon membrane (Hybond N$^+$; Amersham Pharmacia Biotech) and were subsequently UV cross-linked for 2 min (UV cross-linker; Stratagene, Amsterdam, The Netherlands). Hybridizations were performed as described by the manufacturer using the enhanced chemiluminescence nonradioactive labeling and detection kit (Amersham Pharmacia Biotech). The probe consisted of a 564-bp *ampD* PCR fragment from plasmid pMS13 (Table 1) that contained the entire *E. cloacae* NOR-1 *ampD* gene.

Recombinant DNA techniques and complementation experiments. Recombinant DNA techniques were performed mostly by standard procedures (36). Whole-cell DNAs from *E. cloacae* WT NOR-1 and ceftazidime-resistant *E. cloacae* NOR-1D mutants were prepared as previously described (27). The restriction enzymes, Klenow DNA polymerase, and ligase were from Amersham Pharmacia Biotech. The *Pfu* thermostable DNA polymerase was from Stratagene. Ligation products were subjected to electroporation into *E. coli* or *E. cloacae* strains according to the manufacturer's instructions (Gene Pulser II; Bio-Rad). Recombinant bacteria were plated onto TS agar plates containing the appropri-

TABLE 2. β-Lactamase specific activities from cultures of WT and ceftazidime-resistant *E. cloacae* NOR-1 strains

Substrate[a]	Strain[b]	Basal β-lactamase activity[c]	Inducer[d]	Induced β-lactamase activity[c]	Fold increase in activity	
					Induced/basal	Mutant/WT
Cephalothin	NOR-1	6	FOX	170	28	
			IMP	110	18	
	NOR-1D$_3$	1,400	FOX	3,930	3	230
			IMP	2,950	2	
	NOR-1D$_{1\rightarrow12}$	10,000 ± 1,200	FOX	9,900 ± 970	1	1,670
			IMP	10,500 ± 950	1	
Imipenem	NOR-1	0.85	FOX	27	31	
			IMP	11	13	
	NOR-1D$_3$	55	FOX	175	3	65
			IMP	170	3	
	NOR-1D$_{1\rightarrow12}$	475 ± 40	FOX	480 ± 70	1	560
			IMP	465 ± 70	1	

[a] A substrate concentration of 100 μM was used.
[b] NOR-1, the WT *E. cloacae* NOR-1 strain; NOR-1D$_3$, *E. cloacae* NOR-1D mutant strain 3; NOR-1D$_{1\rightarrow12}$, *E. cloacae* NOR-1D mutant strains 1 to 12 but not including strains 3 and 7.
[c] Specific activity (in micromoles per minute per milligram of protein). The β-lactamase activities are geometric mean determinations for three independent cultures. The standard deviations were within 10% for each individual strain. However, the standard deviations are shown whenever the values were those of cultures of several different strains.
[d] FOX, cefoxitin (50 μg/ml); IMP, imipenem (10 μg/ml).

ate antibiotic. Recombinant plasmid DNAs were prepared using Qiagen Mini and Maxi columns (Qiagen) (34). Plasmids were subsequently electroporated into the appropriate host (Table 1). Fragment sizes were estimated by comparison to the molecular size standard 1-kb DNA ladder (Life Technologies).

The *ampD* genes of *E. cloacae* WT NOR-1 and of 11 ceftazidime-resistant *E. cloacae* NOR-1D strains were PCR amplified using the *Pfu* thermostable polymerase (Stratagene). The two primers used were AmpD-RBS, which created a consensus ribosomal binding site (RBS) 5 bp upstream of the ATG start codon (AmpD-RBS; 5′-AAGGAGGATAC CATGTTGTTAGAAAACGGATGGC-3′) and AmpD-H3, which matched to the end of the *ampD* gene (AmpD-H3; 5′-AAAAAGCTTTCATGTTA-TCTCCTTATCTGACG-3′). These PCR fragments were then cloned into pPCRScript, downstream of the *lac* promoter, yielding plasmids pMS$_{1\rightarrow12}$ (plasmids pMS$_1$ to pMS$_{12}$) that contained the mutated *ampD*$_{1\rightarrow12}$ (*ampD*$_1$ to *ampD*$_{12}$) alleles and pMS$_{13}$ that contained the WT *ampD* allele (Table 1). The sequences of the cloned PCR-generated DNA fragments were confirmed by complete resequencing.

Plasmids pPTN-3, pPTN-7, and pPTN-9 were constructed by cloning a 2.2-kb *Nae*I fragment of plasmid pPTN-1 (26) into a *Sma*I-digested pK19 plasmid, a blunt-ended *Bam*HI site of pACYC184 plasmid, and a *Sma*I-digested pGB2 plasmid, respectively (Table 1). The recombinant plasmids were then used in induction experiments in host *E. coli* strains.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank-EMBL-DDBJ nucleotide databases under the accession nos. AF298868 to AF298879.

RESULTS AND DISCUSSION

Mutant selection and analysis of their biochemical properties. Twelve independent *E. cloacae* NOR-1 cultures were grown in 10-ml portions of TS broth for 16 h and were subsequently plated on ceftazidime-containing TS plates. An average of 10 to 100 colonies per 10^9 plated bacteria grew, resulting in a mutation frequency of 10^{-7} to 10^{-8}. From each independent experiment, the antibiotic susceptibility of three colonies was checked on a routine antibiogram. In addition to the usual phenotype observed in *E. cloacae* of a derepressed cephalosporinase expression, the inhibition zone around the imipenem disk was reduced, suggesting that the expression of the carbapenem-hydrolyzing β-lactamase NmcA might also have been modified. Furthermore, clavulanic acid restored part of the susceptibility to imipenem. One clone was retained for further analysis from each independent experiment. Subsequently, the MICs of selected β-lactams for the 12 ceftazidime-resistant strains of *E. cloacae* NOR-1D$_{1\rightarrow12}$ (strains NOR-1D$_1$ to NOR-1D$_{12}$) and the *E. cloacae* WT NOR-1 strains were determined. The ceftazidime and imipenem MICs confirmed the antibiogram observations (see Table 4).

In order to investigate the molecular mechanism of this increased imipenem resistance, enzymatic assays were performed with and without induction. The results clearly indicated that when cephalothin, which is a substrate of both the AmpC and NmcA β-lactamases (Table 2) was used, an increase in the specific activity of more than 1,000-fold was observed in the mutant strains compared to that of the WT strain. Furthermore, the mutants lost their ability of induction and had a high level of constitutive β-lactamase expression. When imipenem was used as a substrate, merely the activity of the carbapenem-hydrolyzing β-lactamase NmcA was measured. Again, the mutants had an increased specific activity towards imipenem (500-fold) with a loss of induction. Interestingly, *E. cloacae* NOR-1D$_3$ had only a partially derepressed expression of NmcA that was still induced (Table 2). Thus, the induction and derepression of NmcA seemed to follow the same regulation pathway as observed for cephalosporinase. In order to verify this hypothesis, the *ampD* genes of the mutants were sequenced and compared to the WT *ampD* gene.

Cloning and sequencing of the *ampD* alleles. Nucleotide sequence comparison revealed significant divergence between *E. cloacae ampD* genes similar to that found among the *Citrobacter freundii ampD* genes (40). The deduced amino acid sequence of *E. cloacae* NOR-1 AmpD exhibited 95, 91, 83, 82, 56, and 52% identity with AmpD of *E. cloacae* O29 (9), *E. cloacae* 14 (18), *E. coli* (13), *C. freundii* OS60 (18), *Pseudomonas aeruginosa* (21) and with the putative *Haemophilus influenzae* AmpD protein (10), respectively. Amino acid sequence alignment of the AmpD proteins revealed several conserved motifs (Fig. 1). The conserved core region and the four strictly conserved residues outside this region, which relate the AmpD proteins of members of the family *Enterobacteriaceae* to the

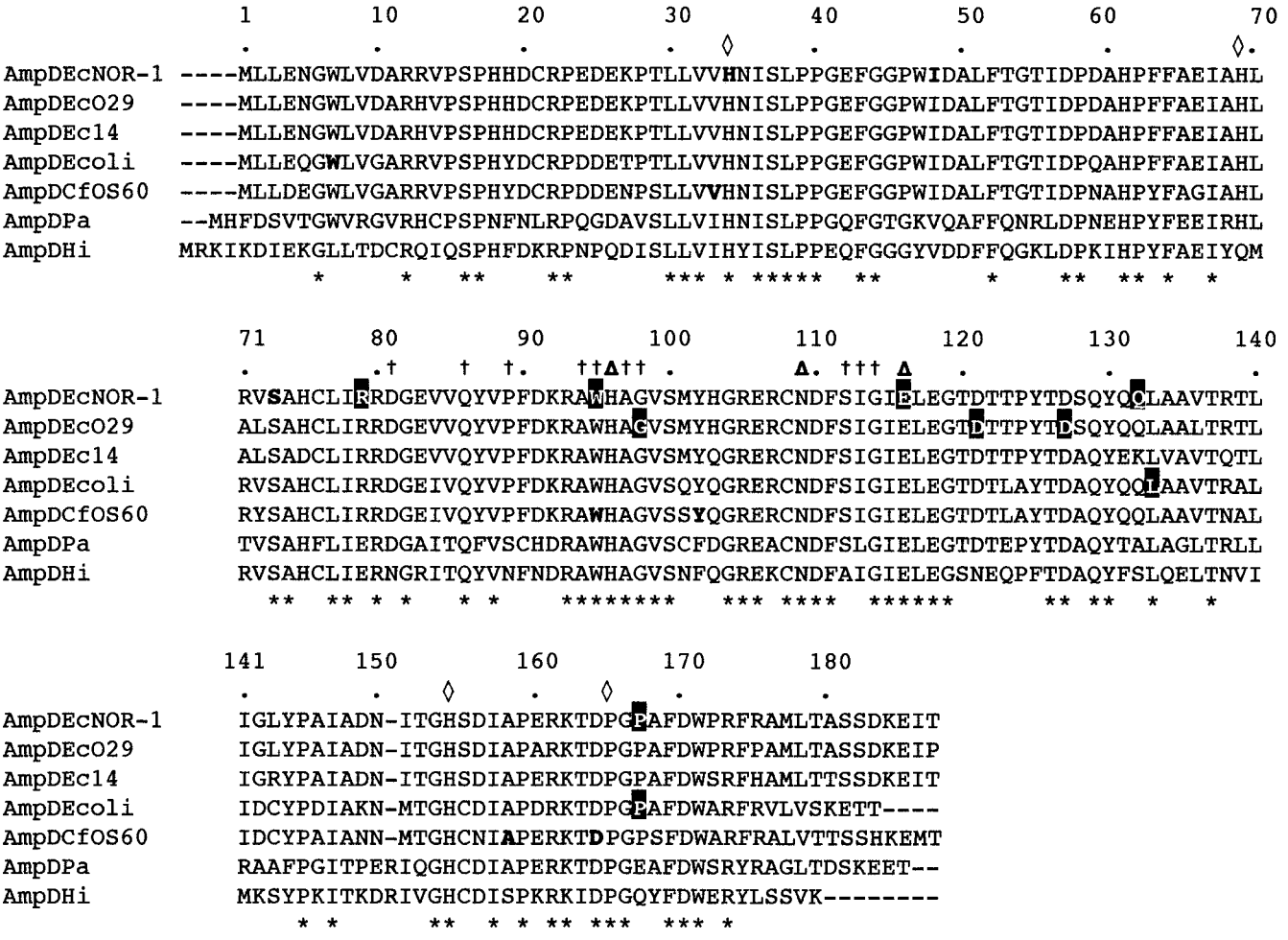

FIG. 1. Alignment of the AmpD amino acid sequence of *E. cloacae* NOR-1 (AmpDEcNOR-1) with those of *E. cloacae* O29 (AmpDEcO29) (9), *E. cloacae* 14 (AmpDEc14) (18), *E. coli* (AmpDEcoli) (13), *C. freundii* OS60 (AmpDCfOS600) (18), and *P. aeruginosa* (AmpDPa) (21) and a putative *H. influenzae* (AmpDHi) (10) AmpD protein. The identical amino acids are indicated by asterisks. The crosses and the open diamonds indicate the amino acids conserved in the core and outside region of the *Bacillus* cell wall hydrolases, respectively. The triangles show the amino acids strictly conserved in various cell wall hydrolases (16). The numbering used corresponds to the *E. cloacae* AmpD sequences. Amino acid substitutions that alter the activity of the AmpD protein (bold letters on gray shaded background) and mutated positions that yielded stop codons (white letters on black background) are indicated.

cell wall hydrolases of *Bacillus* spp. (16), were found in the *E. cloacae* NOR-1 AmpD. The high degree of identity of the AmpD proteins showed that AmpD of *E. cloacae* NOR-1 was closely related to its enterobacterial homologs and that they probably share a common mechanism of regulation of AmpC β-lactamase expression and murein metabolism.

Of the 12 ceftazidime-resistant *E. cloacae* NOR-1D mutants, 3 had single amino acid changes in AmpD. Eight mutations resulted in premature termination of the protein by either creation of a stop codon at the site of mutation or by introducing a frameshift mutation leading to a stop codon located further downstream (Table 3 and Fig. 1). PCR amplification of the *ampD* gene of *E. cloacae* NOR-1D$_7$ failed despite the use of different primer combinations. Dot blot hydridization experiments with an internal *E. cloacae* NOR-1 *ampD* probe indicated that *E. cloacae* NOR-1D$_7$ still possessed an *ampD* gene. However, this gene may be mutated at the site of primer binding or may be partially deleted, thus lacking one primer-binding site. Interestingly, the amino acid substitutions were found in the N terminus of the protein, while the stop codons were mostly located within the C terminus. Sequence data from the literature indicated the importance of the carboxy portion of the AmpD protein for the inducible AmpC phenotype (11). Two mutations in the amino terminus which correlated with a fully derepressed phenotype have been characterized, a valine-to-glycine change at position 33 in *E. cloacae* (40) and a tryptophan-to-glycine change at position 7 in *E. coli* (13).

Here, we present further evidence that point mutations within the amino terminus lead to a fully derepressed phenotype for AmpC and NmcA expression. The histidine 34 residue, which is conserved among the AmpD proteins and also among the hydrolases, when replaced by tyrosine led to a fully derepressed phenotype (Fig. 1). A serine-to-leucine replacement at position 73 gave a similar phenotype. This last serine, even though conserved among the AmpD proteins, lies outside the conserved boxes shared with the hydrolases and thus may represent an AmpD-specific amino acid important for its activity. The isoleucine-to-serine substitution at position 48 gave

TABLE 3. Amino acid differences among *ampD* gene products of ceftazidime-resistant *E. cloacae* NOR-1 mutants

AmpD protein	Nucleotide and amino acid differences at positions[a]:								Type of mutation
	100 **34**	143 **48**	218 **73**	235–236 **79**	284–285 **95**	346 **116**	394 **132**	496–502 **166–167**	
WT AmpD	C **H**	T **I**	C **S**	C–G **R**	GG **W**	G **E**	C **Q**	GGCCCGG **GP**	
AmpD1			T **L**						Missense
AmpD2	T **Y**								Missense
AmpD3		G **S**							Missense
AmpD4						T *			Nonsense
AmpD5					A *				Nonsense
AmpD6								G−−−−−G **GL***	5-bp deletion
AmpD7									ND[b]
AmpD8				CAG **QSRW***					1-bp insertion
AmpD9							T *		Nonsense
AmpD10							T *		Nonsense
AmpD11					A *				Nonsense
AmpD12					A *				Nonsense

[a] The nucleotide and amino acid differences from the wild type at nucleotide and amino acid positions are shown in the pair of lines for each AmpD protein. For each pair of lines, the first line shows the nucleotide difference for nucleotide positions 100 to 502 (nucleotide numbering according to the *E. cloacae* NOR-1 WT *ampD* gene) and the second line shows the amino acid difference for amino acid positions 34 to 167 (in bold type) (amino acid numbering according to *E. cloacae* NOR-1 WT AmpD protein). *, stop codon; –, deletion.

[b] ND, not determined. No PCR product obtained. Hybridization positive.

a high basal level and still inducible phenotype. This position is not strictly conserved among AmpD proteins but contains a neutral amino acid residue, which may be involved in the proper folding of the enzyme.

The remaining mutations led to premature termination of AmpD proteins. Even though these mutations were scattered throughout the entire protein sequence, three were located at amino acid position 95. Indeed, of 12 independent mutations, 3 were located at this position. In *C. freundii*, this position was also found to be a site of mutation (40). The last 16 amino acids are important for the activity of AmpD, since the deletion of these amino acids led to a derepressed phenotype. This observation has been made with an AmpD mutant of *E. coli* (13). Most mutations identified in the present work were located at positions that have never been reported and were mostly located outside of the known conserved motifs. The 11 *ampD* mutations identified in this work expand the number of known mutations leading to altered Bush group 1 and 2e β-lactamase expression (4). They support previous findings demonstrating the essential nature of the carboxy terminus of the mature protein but also revealed key positions at the amino terminus of the protein that are also important for the activity. Mutations introducing stop codons are primarily encountered in the C-terminal portion, while mutations introducing amino acid substitutions are mostly encountered in the N-terminal portion.

In clinical and laboratory isolates of *E. cloacae*, *C. freundii,* and *P. aeruginosa*, several phenotypes of altered β-lactamase expression have also been described (11, 37). In enterobacteria, three of four phenotypes of altered β-lactamase expression have been associated with mutations in *ampD* (3, 9, 13, 18, 23, 40). They include wild-type (normal induction), hyperinducible (higher basal level of AmpC expression and high-level induction in the presence of low levels of inducing drugs), and stably derepressed. In our study, these three phenotypes were found. Kuga et al. have shown that mutations in *ampR* may also lead to high-level AmpC expression, and the mutation frequency was 10^{-6} as tested from a plasmid carrying *ampC* or *ampR* in an *ampD*-deficient *E. coli* strain (20). Since the mutation frequencies of *ampD* were 10^{-7} to 10^{-8}, mutations in *nmcR* and/or *ampR* of *E. cloacae* NOR-1 also should have been detected. However, of the 12 mutants studied, all had a mutation in the *ampD* sequence. Sequencing of *nmcR* from *E. cloacae* NOR-$1D_1$ and NOR-$1D_3$ revealed WT sequences (data not shown). *nmcR* mutations may occur only after *ampD* mutations and thus may increase the resistance of the strains even more. The frequency of mutation to beta-lactam resistance via mutations in *nmcR* is probably always at least 10-fold lower than mutations in *ampD*, since the former type of mutation must not affect the ability of the regulator to interact properly with its target sequence, while any mutations affecting the amidase activity of AmpD will have a phenotype. This issue will be addressed in future investigations. In addition, it is not known whether *nmcR* mutations have an impact on the expression of a single copy of the *nmcA* gene present on the chromosome in the absence of an *ampD* mutation. Using the *E. coli*

TABLE 4. MICs of selected β-lactams and *E. cloacae* NmcA specific β-lactamase activities in different host strains

Strain	Relevant genotype	MIC (μg/ml)[a]						β-Lactamase activity[b]		
		ATM	ATM-CA	CAZ	CAZ-CA	IMP	IMP-CA	Basal	Induced[c]	Ratio
E. cloacae										
NOR-1	WT	16	0.12	0.5	0.5	8	1	0.85	27	31
NOR-$1D_3$	CAZ^r mutant	128	128	32	32	16	2	55	175	3
NOR-$1D_{1\rightarrow12}$	CAZ^r mutant	256	128	256	256	32	2	475	480	1
NOR-$1D_1$/pNH5	CAZ^r mutant with *E. coli ampD*	128	64	32	32	16	1	29	285	10
NOR-$1D_1$/pSM_{13}	CAZ^r mutant with *ampD* from NOR-1	64	32	8	8	8	1	4.5	75	16
NOR-$1D_1$/pSM_3	CAZ^r mutant with *ampD* from NOR-$1D_3$	128	128	64	64	16	2	320	350	1
NOR-$1D_1$/$pSM_{1\rightarrow12}$	CAZ^r mutant with *ampD* from NOR-$1D_{1\rightarrow12}$	256	128	256	256	32	1	470	465	1
MHN2[d]	Δ*ampD*/*E*[e]	64	64	256	256	0.5	0.5	20,000	19,000	1
MHN2/pNH5[d]	Δ*ampD*/*E, E. coli ampD*	32	32	32	32	0.5	0.5	685	4,890	7
MHN2/pSM_{13}[d]	Δ*ampD*/*E, ampD* from NOR-1	1	1	0.5	0.5	0.25	0.25	0.75	20	27
MHN2/$pSM_{1\rightarrow12}$[d]	Δ*ampD*/*E, ampD* from NOR-$1D_{1\rightarrow12}$	128	64	256	256	0.5	0.5	20,500	18,300	1
E. coli										
MC4100	WT	0.06	0.06	0.25	0.12	0.12	0.12	ND[f]	ND	
MC4100/pPTN-3	WT strain with *nmcA* and *nmcR* (400 copies)	512	128	2	0.25	>128	8	1,220	1,435	1
MC4100/pPTN-7	WT strain with *nmcA* and *nmcR* (20–40 copies)	128	32	1	0.25	128	4	455	910	2
MC4100/pPTN-9	WT strain with *nmcA* and *nmcR* (1–5 copies)	64	4	0.5	0.25	64	2	200	710	4
JRG582	Δ*ampD* mutant	0.06	0.06	0.03	0.03	0.12	0.12	ND	ND	
JRG582/pPTN-3	Δ*ampD* mutant with *nmcA* and *nmcR* (400 copies)	>512	256	1	0.25	>128	32	2,720	1,690	1
JRG582/pPTN-7	Δ*ampD* mutant with *nmcA* and *nmcR* (20–40 copies)	>512	128	2	0.5	>128	32	2,400	2,090	1
JRG582/pPTN-9	Δ*ampD* mutant with *nmcA* and *nmcR* (1–5 copies)	256	64	2	0.5	>128	16	1,150	1,020	1
JRG582/pPTN-9/pSM_{13}	Δ*ampD* mutant with *nmcA* and *nmcR* and *ampD*NOR-1	64	16	0.25	0.12	8	1	9.5	120	12

[a] Drug abbreviations: ATM, aztreonam; CAZ, ceftazidime; IPM, imipenem; CA, clavulanic acid.
[b] Specific activity (in micromoles per minute per milligram of protein). Imipenem was used as a substrate, unless indicated otherwise. The β-lactamase activities are geometric mean determinations for three independent cultures. The standard deviations were within 10% for each strain.
[c] Induced with cefoxitin.
[d] Cephalothin was used as the substrate for the determination of the specific activities for these strains.
[e] Δ*ampD*/*E*, deletion of *ampD* or *ampE*.
[f] ND, not determined.

host strains and the induction assay based on a low-copy-number plasmid carrying *nmcA* and *nmcR*, we will be able to investigate the role of *nmcR* mutations.

Complementation of ceftazidime-resistant *E. cloacae* NOR-1 strains. In order to know whether the *ampD* mutations were responsible for the observed phenotype, the *ampD* genes were cloned onto a high-copy-number plasmid, resulting in plasmids $pMS_{1\rightarrow12}$ and tested in transcomplementation experiments. The WT *ampD* gene of *E. coli* and *E. cloacae* NOR-1, as expressed from plasmids pNH5 (13) and pMS-13, respectively, transcomplemented the ceftazidime-resistant *E. cloacae* NOR-1D strains to low-level β-lactam resistance (decrease in ceftazidime and imipenem MICs) and restored WT β-lactamase expression (low basal level and inducibility) (Table 4). The induced/noninduced ratio of β-lactamase activity was 9.5 in cells producing the *E. coli* AmpD protein, while in cells expressing the *E. cloacae* NOR-1 AmpD protein, this ratio was 16. This difference could reflect differences in expression levels of the AmpD proteins or species-specific differences in activity.

The 11 mutated *ampD* alleles failed to restore a low-level and inducible phenotype, indicating that the single *ampD* mutation was sufficient to inactivate AmpD. The mutated *ampD3* allele was capable of partial recovering of an inducible phenotype. These results showed that the cloned WT *ampD* allele is sufficient to restore the WT NmcA expression level, thus confirming that AmpD is involved in the induction of NmcA.

Similarly, the WT *ampD* genes of *E. coli* and *E. cloacae* NOR-1, as expressed from plasmids pNH5 and pMS-13, respectively, transcomplemented the derepressed *E. cloacae* MNH2 mutants to low-level β-lactam resistance and WT β-lactamase expression (low basal level and inducibility). Taken together, these results clearly showed that the WT AmpD protein was sufficient to restore basal and inducible β-lactamase expression of NmcA and AmpC β-lactamases.

Complementation of *E. coli ampD* mutants. To determine the ability of the *E. cloacae* NOR-1 *ampD* gene to complement *E. coli ampD* mutations, plasmids pSM_{13} and $pSM_{1\rightarrow12}$ were transformed into *E. coli* JRG582 (Δ*ampDE*) and *E. coli* MC4100 (*ampDE*$^+$) (13) containing plasmid pPTN-9. pPTN-9 is a low-copy-number plasmid that carries the *nmcA* and *nmcR* genes from *E. cloacae* NOR-1. *E. coli* MC4100/pPTN-9 exhibits a high basal β-lactamase activity and is inducible, while *E. coli* JRG582/pPTN-9 has a fully derepressed phenotype (Table 4). These two strains were resistant to aztreonam and imipenem (Table 4). The *ampD* genes of *E. coli* and *E. cloacae*, as expressed from pNH5 and pMS-13, respectively, transcomplemented the *E. coli ampDE* mutant to low-level β-lactam resistance and WT β-lactamase expression (low basal level and inducibility) (Table 4). In addition, the 11 mutated alleles of *ampD* failed to restore an inducible phenotype of carbapenem-hydrolyzing β-lactamase (data not shown). These results showed that the cloned *E. cloacae* NOR-1 *ampD* gene expresses a functional AmpD protein in *E. coli* cells and that the mutations observed in the *ampD* gene also account for the observed phenotype in *E. coli*.

Recombinant plasmids pPTN-3, -7, and -9 carry the same *nmcA* and *nmcR* genes but at different copy numbers (Table 1). Induction assays and MICs (Table 4) revealed that β-lactamase activity was directly related to the plasmid copy number measured. However, the activity increase was not linearly related to the theoretical copy number of the plasmid. Further-

more, the induced/noninduced ratio is conversely related to its theoretical copy number.

Conclusions. In order to determine whether genetic alterations leading to a derepressed NmcA expression phenotype of *E. cloacae* NOR-1 could be linked to *ampD* mutations, 12 independent ceftazidime-resistant *E. cloacae* NOR-1D strains were investigated. Of these 12 strains, 11 had point mutations in the *ampD* coding sequence leading to nonfunctional AmpD. These results along with the high percentage of identity observed among enterobacterial AmpD proteins strongly suggest that *E. cloacae* NOR-1 AmpD acts as an *N*-acetyl-anhydromuramyl-L-alanine amidase, which leads to a decreased amount of anhydromuropeptide (MTp), the signal molecule for β-lactamase expression (17).

Our data indicate that controls of the induction process are similar for NmcA and AmpC β-lactamases, suggesting that different structural genes may be under the control of identical regulatory systems. Such an observation has already been made for *Aeromonas sobria*, where three different β-lactamase genes are under the same two-component regulatory pathway that is not related to LysR-type regulation (1). It would be interesting to study the regulation of β-lactamase expression in *Yersinia enterocolitica,* an enterobacterial species that naturally contains an AmpC-type cephalosporinase and an Ambler class A β-lactamase (38, 39).

Finally, from a clinical point of view, our results showed that treatment with ceftazidime might select strains that are resistant to all available β-lactams through a single genetic event affecting the expression of two unrelated broad-spectrum β-lactamases.

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