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Altered Phenotypes Associated with *ampD* Mutations in *Enterobacter cloacae*

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A study was done to determine the genetic locus responsible for altered expression of AmpC β-lactamase in *Enterobacter cloacae* 1194E and several mutants derived from *E. cloacae* 029. These phenotypes were defined by units of enzyme activity found in sonic extracts of cells before and after induction with cefoxitin and included (units uninduced/units induced) the wild-type (7/219), high-level constitutive (10,911/10,862), temperature-sensitive (at 30°C 82/706 and at 42°C 5,031/6,020), and hyperinducible (19/1,688) phenotypes. When the *ampD* region of each *E. cloacae* strain was cloned and introduced into an *ampD* mutant *Escherichia coli* strain, the altered phenotypes were found to reside within this locus. Furthermore, transformants containing wild-type *ampD* were poorly inducible at 42°C while those with high-level constitutive or hyperinducible *ampD* were unaffected by temperature. Since the source of *ampD* was the only variable in these *E. coli* transformants, these results suggested that *ampD* encodes a protein that is involved in sensing the inducer. To test this possibility, the responses to different inducers of *E. coli* transformants containing various *ampD* regions were assessed. In the presence of wild-type *ampD*, transformants responded equally to cefoxitin and cefotetan, regardless of temperature. In the presence of temperature-sensitive *ampD*, induction by cefotetan was similar to that by cefoxitin at 30°C but greater than that by cefoxitin at 42°C. These results suggest that *ampD* encodes a protein involved in induction of AmpC β-lactamase in *E. cloacae*.

AmpC β-lactamase is inducible in a number of species belonging to the family *Enterobacteriaceae* (27). Four genes that control expression of this inducible enzyme have been identified (11, 12, 14, 17, 18, 20, 21). The exact function of these genes and their corresponding proteins is unknown, except for AmpR, a DNA-binding protein encoded by *ampR* (21). This protein has been shown to act as a transcriptional activator by binding to a DNA region immediately upstream of the *ampC* promoter. The exact function of the remaining three genes, *ampD*, *ampE*, and *ampG*, is unknown. However, mutations within any of these genes can lead to altered expression of AmpC β-lactamase.

Mutations within *ampR* usually lead to constitutive production of the enzyme at a level twofold above the normal basal (uninduced) level (19, 21). High-level constitutive or semiconstitutive production of the enzyme results from mutations within *ampD* (17, 24), while mutations within *ampG* produce basal, constitutive expression of the enzyme (14). Recently, Lindquist et al. (20) described hyperinducible expression of β-lactamase that was attributed to a mutation within *ampD* that did not affect transcription of *ampE*, a gene adjacent to *ampD* in *Escherichia coli* that encodes a transmembrane protein. Thus, it appears that different mutations within *ampD* are responsible for various altered phenotypes of AmpC expression.

Two other groups have recently reported altered AmpC expression in *Enterobacter cloacae*. In 1987, Curtis et al. (4) described spontaneous mutants of *E. cloacae* with temperature-sensitive expression of AmpC β-lactamase. Enzyme expression at the permissive temperature was similar to that of wild-type strains, while expression at restrictive temperatures was similar to that seen with *ampD* mutants (4). In 1988, Goering et al. (6) described a hyperinducible phenotype in *E. cloacae* 1194E that was thought to be due to an unusual *ampR* gene. However, subsequent studies with this strain revealed a normal *ampR ampC* region upon recloning and suggested that the abnormal phenotype resulted from a mutation within the *ampD* region (15). Therefore, studies were designed to confirm that the hyperinducible phenotype in *E. cloacae* 1194E was due to a mutation within *ampD* and determine whether the temperature-sensitive phenotype described by Curtis et al. (4) was also due to an *ampD* mutation.

MATERIALS AND METHODS

Strains and plasmids. The various bacterial strains and plasmids used in this study are described in Table 1. *E. cloacae* 029 is a wild-type isolate that produces inducible type A (pI 8.8), class I chromosomal β-lactamase (4). *E. cloacae* 029-5 is a spontaneous temperature-sensitive mutant that is resistant to cefotaxime at 41°C but susceptible to the drug at 28°C (4). *E. cloacae* 029 and 029-5 were kindly provided by N. A. C. Curtis (Macclesfield, England). *E. cloacae* 029M is a spontaneous mutant resistant to cefotaxime regardless of temperature. This mutant was selected by using cefotaxime. *E. cloacae* 1194E also expresses type A (pI 8.8), class I chromosomal β-lactamase and is hyperinducible (15). *E. cloacae* 1194E was kindly provided by R. Guay (Quebec, Quebec, Canada).

Antibiotics and enzymes. Cefoxitin, cefotetan, and cefotaxime were kindly provided by their manufacturers (Merck Sharp & Dohme, West Point, Pa.; ICI Pharmaceuticals, Wilmington, Del.; and Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J., respectively). Kanamycin, tetracy-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Vector plasmid	Origin or reference
E. coli K-12			
FL01	*recA56*; derivative of *E. coli* MM294		18
JRG582	Hfr *azi* Δ(*nadC-aroP*)*2 thiA*		9
SN0302	*ampA1 ampC8 ampD2 pyrB recA rpsL*		17
E. cloacae			
55	*ampR*$^+$ *ampC*$^+$ *ampD*$^+$		7
029	*ampR*$^+$ *ampC*$^+$ *ampD*$^+$		4
029-5	*ampR*$^+$ *ampC*$^+$ *ampD*(Ts)[a]		4
029M	*ampR*$^+$ *ampC*$^+$ *ampD*		Present study
1194E	*ampR*$^+$ *ampC*$^+$ *ampD*(H)[b]		8
14	*ampR*$^+$ *ampC*$^+$ *ampD*$^+$		14
Plasmids			
pACYC184	Cm^r Tc^r		2, 28
pBP131	Su^r *ampR*$^+$ *ampC*$^+$ region of strain 14	RSF1010	16
pBP141-1-2	Km^r *ampD*$^+$ region of strain 14	pLG339	14
pBP141-3	Cm^r *nadC*$^+$ *ampD*$^+$ region of strain 14	pACYC184	13
pGKS141-3	Cm^r *nadC*$^+$ *ampD*$^+$ region of strain 55	pACYC184	Present study
pGKS146-3	Cm^r *nadC*$^+$ *ampD*$^+$ region of strain 029	pACYC184	Present study
pGKS147-3	Cm^r *nadC*$^+$ *ampD*(Ts) region of strain 029-5	pACYC184	Present study
pGKS148-3	Cm^r *nadC*$^+$ *ampD* region of strain 029M	pACYC184	Present study
pGKS165-4	Cm^r *nadC*$^+$ *ampD*(H) ampE$^+$ region of strain 1194E	pACYC184	Present study
pGKS145-4	Cm^r *nadC*$^+$ *ampD*(H) region of strain 1194E	pACYC184	Present study

[a] Ts, Temperature sensitive.
[b] H, Hyperinducible.

cline, chloramphenicol, sulfamethoxazole, and quinolinic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, Wis.) and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Alkaline phosphatase and a kit for labeling DNA with digoxigenin-dUTP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Media and growth conditions. For routine purposes, Mueller-Hinton broth or agar was used. M9 medium (23) containing 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.) and thiamine (1 μg/ml) were used for *ampD* cloning experiments. Quinolinic acid (30 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), sulfamethoxazole (300 μg/ml), or β-lactam antibiotics at various concentrations were added as required. Induction experiments were performed in Iso-Sensitest broth (Oxoid Ltd., Basingstoke, Hants, United Kingdom). For transformation experiments, SOB medium (20 g of Bacto-Tryptone [Difco], 5 g of yeast extract [Difco], 0.6 g of NaCl, 0.19 g of KCl, 10 ml of 1 M $MgSO_4$, 10 ml of 1 M $MgCl_2$) and SOC medium (SOB medium plus 20 ml of 20% glucose) were used as described by Hanahan (10).

Susceptibility testing. MICs were determined in Iso-Sensitest agar by using an inoculum of 10^4 CFU per spot. The MIC was defined as the lowest concentration preventing growth after 18 to 24 h of incubation at 30 or 42°C in air.

β-Lactamase assays. β-Lactamase was induced over a 2-h period by using cefoxitin at a concentration of either 4 or 32 μg/ml, depending upon the susceptibility of the strain to the drug to ensure no significant killing during induction (14, 30). Usually, *E. cloacae* strains grown overnight at 37°C were diluted 1:20 into 100 ml of Iso-Sensitest broth at room temperature. *E. coli* strains were diluted 1:10 because of their slower growth rate. The strains were then incubated for 90 min at 30 or 42°C, respectively. After addition of the inducer, incubation was continued at the same temperature for another 2 h. β-Lactamase activity in sonic extracts was quantified in a UV spectrophotometric assay with 100 μM cephalothin as the substrate (30). The protein content was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Every induction experiment was performed in triplicate, and the results presented are averages of three determinations. Standard errors were generally not more than 20% of the mean. Means differing by greater than ±2 standard errors were considered significant (P = 0.05).

Nucleic acid techniques. Chromosomal DNA was isolated by a modified version of the protocol described by Edlund et al. (5). For rapid isolation of plasmid DNA, the alkaline extraction method of Birnboim and Doly was used (1). For cloning experiments, DNA was prepared as described by Clewell and Helinski (3). Restriction fragments were recovered from agarose gels by using the Geneclean system from Bio 101 Inc. (La Jolla, Calif.). In cloning experiments, vector DNA was usually treated with alkaline phosphatase to prevent self-ligation. Recombinant DNA techniques were performed as described by Maniatis et al. (22). The procedure used for *E. coli* transformation was described by Hanahan (10). Transformation of *E. cloacae* was performed by the $CaCl_2$ method described previously (14).

DNA-DNA hybridization. After electrophoresis, the gels were treated and blotted onto nylon filters as described previously (14). The filters were then rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried at 80°C for 30 min. Labeling of the probe DNA with digoxigenin-dUTP, prehybridization, hybridization, and detection of hybridized probe DNA were performed as recommended by the supplier (Boehringer Mannheim Biochemicals). Bands were usually visible after 5 to 30 min.

TABLE 2. β-Lactamase expression and cefotaxime susceptibility of *E. cloacae* strains

E. cloacae strain (*ampD* plasmid)	Cefotaxime MIC (μg/ml) at 30°C	Sp act[a] at 30°C		Cefotaxime MIC (μg/ml) at 42°C	Sp act[a] at 42°C	
		Uninduced	Induced		Uninduced	Induced
029	0.12	7	219	0.12	11	20
029M	32	10,911	10,862	64	10,228	12,209
029-5	0.5	82	706	32	5,031	6,020
1194E	32	19	1,688	32	67	1,009
029(pBP141-1-2)	0.06	6	63	0.06	9	12
029M(pBP141-1-2)	0.06	7	65	0.12	10	13
029-5(pBP141-1-2)	0.06	9	102	16	12	19
1194E(pBP141-1-2)	1	1	3	1	1	1

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

RESULTS

Characterization of *E. cloacae* strains. β-Lactamase expression and susceptibility to cefotaxime were determined for the *E. cloacae* strains at 30 and 42°C (Table 2). Wild-type *E. cloacae* 029 was inducible by cefoxitin at 30°C but not at 42°C. Mutant *E. cloacae* 029M expressed high levels of β-lactamase constitutively at both 30 and 42°C. At 30°C, the temperature-sensitive mutant *E. cloacae* 029-5 expressed a higher basal (uninduced) level of enzyme and a higher level of enzyme following induction by cefoxitin than did its wild-type parent *E. cloacae* 029. At 42°C, *E. cloacae* 029-5 expressed high levels of enzyme which were only slightly increased by cefoxitin. However, enzyme levels of *E. cloacae* 029-5 were only one-half those of *E. cloacae* 029M at 42°C. *E. cloacae* 1194E appeared to be hyperinducible by cefoxitin at both 30 and 42°C, and basal levels of enzyme at 42°C were higher than those produced at 30°C. MICs of cefotaxime generally paralleled basal levels of enzyme production for each strain, i.e., the lower the basal level of enzyme, the lower the MIC. Since *E. cloacae* 029 and 1194E are not isogenic, no comparisons of enzyme activities or MICs were made.

Introduction of a wild-type *ampD* (pBP141-1-2) by transformation into each of the *E. cloacae* strains altered expression of β-lactamase and, in some instances, susceptibility to cefotaxime (Table 2). Introduction of pBP141-1-2 into wild-type *E. cloacae* 029 diminished the inducibility of this strain at 30°C. This was most likely due to the gene dosage effect described previously for *ampD* (26). At 42°C, *E. cloacae* 029(pBP141-1-2) was poorly inducible, as it had been without cloned *ampD* (Table 2). β-Lactamase expression in *E. cloacae* 029M(pBP141-1-2) was essentially identical to that observed with *E. cloacae* 029(pBP141-1-2), as was susceptibility to cefotaxime. Introduction of pBP141-1-2 into *E. cloacae* 029-5 lowered the basal level of enzyme expression and the cefotaxime MIC at 30°C. Inducibility by cefoxitin at 30°C was about twofold higher for *E. cloacae* 029-5(pBP141-1-2) than for *E. cloacae* 029(pBP141-1-2) or *E. cloacae* 029M(pBP141-1-2). At 42°C, β-lactamase expression in *E. cloacae* 029-5(pBP141-1-2) was similar to that of *E. cloacae* 029(pBP141-1-2) and was only poorly inducible by cefoxitin. The MIC of cefotaxime for *E. cloacae* 029-5(pBP141-1-2) at 42°C was higher than that predicted by basal enzyme levels. β-Lactamase expression in *E. cloacae* 1194E(pBP141-1-2) was low and noninducible at both 30 and 42°C, and cefotaxime MICs were low at both temperatures.

The inability to induce *E. cloacae* 029 at 42°C in this study was in contrast to results reported by Curtis et al. (4). This discrepancy was perhaps due to the fact that in this study, cells were allowed to reach the temperature of induction before the inducer was added while Curtis et al. (4) added inducer immediately after switching temperatures. To test this possibility, *E. cloacae* 029 was induced with cefoxitin by using cells incubated at 42°C for 1.5 h prior to induction and cells incubated at 30°C and switched to 42°C immediately prior to induction. As was seen previously, cells at 42°C were weakly inducible (6 versus 78 nmol/min/mg for uninduced versus induced cells) while cells at 30°C were normally inducible (4 versus 295 nmol/min/mg). Cells switched from 30 to 42°C immediately prior to induction showed induction similar (4 versus 212 nmol/min/mg) to that of cells induced at 30°C.

Cloning and characterization of *ampD ampE* regions. Altered expression of β-lactamase toward a more wild-type phenotype by introduction of wild-type *ampD* into each mutant suggested that the mutant phenotypes were due to a lesion in *ampD*. Therefore the *ampD* region of each mutant was cloned. Since the influence of each *ampD* allele on β-lactamase expression could be assessed only in an identical host background, β-lactamase expression in *E. coli* SN0302 was examined. This host lacks functional *ampC*, *ampD*, and *ampE* regions and is therefore susceptible to β-lactam antibiotics. However, following introduction of the *ampR ampC* region (pBP131) from *E. cloacae* 14 into this strain, it closely resembles a high-level constitutive *E. cloacae* mutant (17). Therefore, *ampD* regions cloned from other organisms can be characterized by transforming them into this *E. coli* SN0302(pBP131) construct.

The cloning strategy for *ampD* used in this study has been described previously (16, 17). It requires the presence of the adjacent *nadC* gene, which is used as a selective marker in *E. coli* JRG582 recipients (the latter host lacks the *nadC ampD ampE* region) since *ampD* itself cannot be directly selected for. To ensure that both *nadC* and *ampD* are present on the same fragment, DNA-DNA hybridization is performed by using as a probe a 3.2-kb *Bam*HI insert of pBP141-3 which carries the *nadC ampD* region from *E. cloacae* 14. *ampD* has been localized on a 0.98-kb *Bgl*II-*Bam*HI fragment at the far left of the insert (13) (Fig. 1). This latter fragment is present in pBP141-1-2. Transcription of *ampD* occurs toward the end of the fragment, while the adjacent *nadC* gene is transcribed in the opposite direction. *ampE* is not present on this fragment, since the DNA downstream of *ampD* is not sufficient to encode another protein.

For each *E. cloacae* strain, chromosomal DNA completely digested with *Bam*HI or *Hpa*I showed only one band that hybridized with the probe (data not shown). For *E. cloacae* 029 and its mutants, this band was a 3.2-kb *Bam*HI fragment very similar to the probe. For *E. cloacae* 1194E,

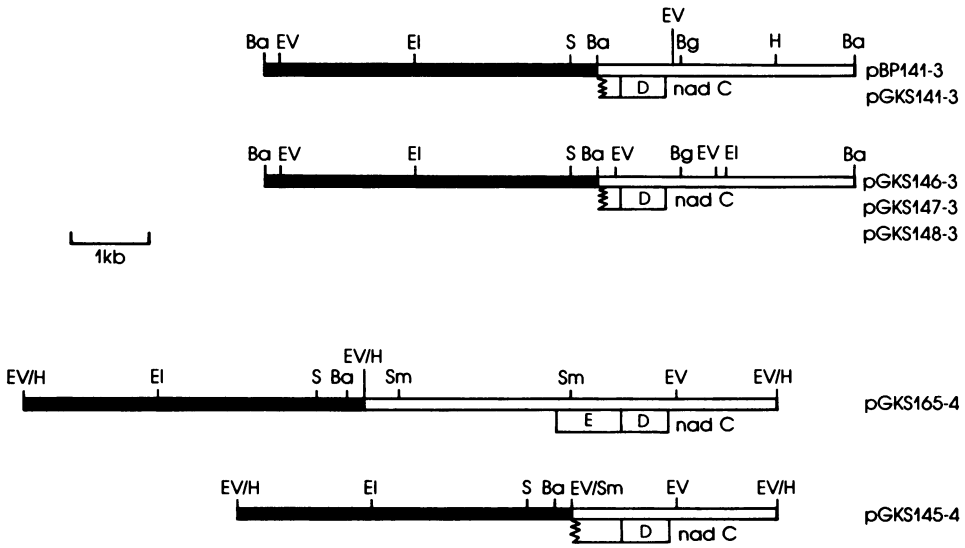

FIG. 1. Restriction maps of recombinant plasmids used in this study. Solid areas indicate vector DNAs. Enzyme abbreviations: Ba, *Bam*HI; EV, *Eco*RV; EI, *Eco*RI; S, *Sal*I; Bg, *Bgl*II; H, *Hpa*I; Sm, *Sma*I.

this band was a 5.4-kb *Hpa*I fragment. These fragments were then cloned into pACYC184 cut with *Bam*HI or *Eco*RV. Restriction maps of the resulting recombinant plasmids were then compared with that of pBP141-3 (Fig. 1). The 3.2-kb *Bam*HI inserts in plasmids pGKS146-3, pGKS147-3, and pGKS148-3 appeared to be identical to the insert in pBP141-3 except for some restriction sites. pGKS165-4 prepared from chromosomal DNA of *E. cloacae* 1194E carried additional sequences of at least 2.5-kb downstream of *ampD*. This additional region would probably contain *ampE* (12, 20). However, since recent studies by Lindquist et al. (20) have suggested that hyperinducibility can result from mutations within *ampD* that do not affect *ampE*, it was important to include clones from *E. cloacae* 1194E with and without *ampE*. Since the host, *E. coli* SN0302, does not contain a functional *ampE* region, an examination of the phenotype observed with transformants containing *ampD* and *ampD ampE* from *E. cloacae* 1194E would determine whether hyperinducibility in this mutant also required *ampE*. Therefore, a recombinant plasmid containing only *nadC ampD* was also prepared by using chromosomal DNA from *E. cloacae* 1194E. This plasmid (pGKS145-4) lacked most of the sequence downstream from *ampD* (Fig. 1). All recombinant plasmids containing either the *nadC ampD* region or the *nadC ampD ampE* region in the same orientation were finally transformed into *E. coli* SN0302 containing the *ampR ampC* region (pBP131) of *E. cloacae* 14. Plasmid pGKS141-3, carrying the *nadC ampD* region of *E. cloacae* 55, a well-characterized wild-type strain, served as a control (Fig. 1).

The β-lactamase expression and cefotaxime susceptibility of *E. coli* SN0302(pBP131) containing the cloned *nadC ampD* regions from the *E. cloacae* strains are shown in Table 3. At both of the temperatures examined, the phenotype conferred by the *ampD* region (pGKS141-3) of *E. cloacae* 55 was identical to that conferred by the *ampD* region (pGKS146-3) of *E. cloacae* 029; i.e., wild-type inducibility at 30°C and poor inducibility at 42°C. Introduction of the *ampD* region (pGKS148-3) of *E. cloacae* 029M into *E. coli* SN0302 (pBP131) altered neither β-lactamase expression nor cefotaxime susceptibility. Introduction of the *ampD* region (pGKS147-3) of temperature-sensitive mutant *E. cloacae* 029-5 into *E. coli* SN0302(pBP131) produced wild-type inducibility at 30°C but hyperinducibility at 42°C (Table 3). The cefotaxime MIC obtained at 42°C was somewhat higher than that expected on the basis of the basal enzyme level produced at that temperature. *E. coli* SN0302(pBP131) containing the *ampD* region (pGKS145-4) of *E. cloacae* 1194E was hyperinducible at both temperatures, with somewhat elevated basal levels of β-lactamase. Cefotaxime MICs were also above that obtained with *E. coli* SN0302(pBP131) containing either of the cloned wild-type *ampD* regions

TABLE 3. β-Lactamase expression and cefotaxime susceptibility of *E. coli* SN0302(pBP131) containing *ampD* from various *E. cloacae* strains

E. coli (*ampR*$^+$ *ampC*$^+$ plasmid) (*ampD* plasmid)	*E. cloacae* *ampD* donor strain	Cefotaxime MIC (μg/ml) at 30°C	Sp act[a] at 30°C		Cefotaxime MIC (μg/ml) at 42°C	Sp act[a] at 42°C	
			Uninduced	Induced		Uninduced	Induced
SN0302(pBP131)		64	4,230	3,941	32	2,007	4,389
SN0302(pBP131)(pGKS141-3)	55	0.12	6	207	0.25	8	22
SN0302(pBP131)(pGKS146-3)	029	0.12	7	205	0.25	8	16
SN0302(pBP131)(pGKS148-3)	029M	64	4,240	4,366	32	1,776	3,999
SN0302(pBP131)(pGKS147-3)	029-5	0.12	7	282	4	12	2,066
SN0302(pBP131)(pGKS145-4)	1194E	1	28	1,146	8	36	1,924

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

TABLE 4. Induction of AmpC β-lactamases by cefoxitin and cefotetan in *E. coli* SN0302(pBP131) containing *ampD* from various *E. cloacae* strains

E. coli (*ampR*$^+$ *ampC*$^+$ plasmid) (*ampD* plasmid)	*E. cloacae* donor strain	Sp act[a] at:	
		30°C	42°C
SN0302(pBP131)(pGKS146-3)	029	6/218/217	11/39/36
SN0302(pBP131)(pGKS147-3)	029-5	7/290/281	18/2,898/4,978
SN0302(pBP131)(pGKS145-4)	1194E	10/1,462/2,587	36/2,853/3,792

[a] Nanomoles of cephalothin hydrolyzed per minute per milligram of protein; uninduced/induced with 4 μg of cefoxitin per ml/induced with 4 μg of cefotetan per ml.

(pGKS141-3 or pGKS146-3). However, they were lower than that obtained with *E. coli* SN0302 with no *ampD* or with *ampD* (pGKS148-3) from *E. cloacae* 029M. The results were the same when pGKS165-4 containing the *nadC ampD ampE* region of *E. cloacae* 1194E was examined in *E. coli* SN0302(pBP131) (data not shown).

Involvement of AmpD in induction. The results shown in Tables 2 and 3 not only identified *ampD* as the locus responsible for the abnormal phenotypes of the *E. cloacae* strains; they also suggested that *ampD* is involved in the induction process. For example, the poor inducibility at 42°C seen in both *E. cloacae* and *E. coli* hosts in the presence of any wild-type *ampD* strongly suggested that AmpD is involved in sensing of the inducer. Were another protein responsible, poor inducibility would have been observed in all strains at 42°C regardless of the source of *ampD*. This was clearly not the case.

If AmpD were involved in sensing the inducer, it might be possible to show differential responses to β-lactam inducers by using the various *ampD* clones constructed in this study. Therefore, the response of *E. coli* SN0302(pBP131) containing recombinant plasmids with various *ampD* regions was assessed by using cefoxitin and cefotetan as inducers. For these studies, both inducers were examined at 4 μg/ml. As shown in Table 4, the response of *E. coli* SN0302(pBP131) with wild-type *ampD* (pGKS146-3) was the same with both inducers at 30 and 42°C. Both were weaker inducers at 42°C than at 30°C for this construct. In the presence of temperature-sensitive *ampD* (pGKS147-3), the responses of *E. coli* SN0302(pBP131) to cefoxitin and cefotetan were similar at 30°C but cefotetan became a significantly more potent inducer than cefoxitin at 42°C ($P = 0.05$). In the presence of hyperinducible *ampD* (pGKS145-4), cefotetan was a more potent inducer than cefoxitin at both 30 and 42°C, although the difference was not statistically significant.

DISCUSSION

Although the precise mechanism responsible for expression of AmpC β-lactamase is unknown, it is clear from the results of this and previous studies that multiple mutant phenotypes of enzyme expression can arise from mutations within *ampD*. These phenotypes include semiconstitutive to constitutive (11, 14, 17, 20), hyperinducible (20), and temperature-sensitive (4) expression.

The semiconstitutive phenotype of *E. coli* SN0302 originally reported by Lindberg et al. (17) was confirmed in this study, although enzyme expression was found to be constitutive at 30°C and semiconstitutive at 42°C. This phenotype was later shown by Lindquist et al. (20) to result from an IS*1* insertion in *ampD* that had polar effects on *ampE*. Thus, *ampE* in *E. coli* SN0302 was essentially nonfunctional. This made the strain ideal for our studies, since it allowed us to examine the effects of different *ampD* alleles in the absence of *ampE*. Were any of our mutant phenotypes to reside in or require the presence of *ampE*, complementation tests with *E. coli* SN0302(pBP131) should have given negative results because of the absence of *ampE* on our clones. Therefore, transfer of hyperinducible *ampD* into *E. coli* SN0302 (pBP131) was not expected to produce a hyperinducible phenotype, since this cloned DNA lacked *ampE*—a requirement for the phenotype according to data presented by Lindquist et al. (20). However, transfer of a hyperinducible phenotype was observed with both pGKS165-4 (*ampD ampE* region of *E. cloacae* 1194E) and pGKS145-4 (*ampD* region of *E. cloacae* 1194E), suggesting that a hyperinducible phenotype can be produced by different mutations within *ampD* not all of which require *ampE* to remain functional.

The results of this study with temperature-sensitive *E. cloacae* 029-5 clearly localized this mutant phenotype within *ampD*. Although it had been originally hypothesized that the lesion resided in *ampR* (4), this theory arose before the existence of *ampD* was known. In general, the results of our studies on β-lactamase expression in *E. cloacae* 029 and 029-5 are in agreement with those published by Curtis et al. (4). The one major discrepancy concerns the inducibility of wild-type *E. cloacae* 029 at 42°C, which was found to be due to differences between the methods used for induction. Curtis et al. incubated cells at 28°C and switched them to 41°C immediately before adding inducer (4). Cells were at 42°C for 1.5 h prior to exposure to inducer in our study. It appears that the time delay before the cells actually reached 41°C in the study by Curtis et al. (4) was sufficient to allow nearly normal induction to occur.

The temperature-sensitive phenotype observed in *E. cloacae* 029-5 was not the same as that observed in *E. coli* SN0302(pBP131) containing *ampD* from *E. cloacae* 029-5. In the former, enzyme expression was inducible at 30°C and constitutive at 42°C, while in the latter, enzyme expression was almost wild type at 30°C but hyperinducible at 42°C. This change in phenotype was probably due to a gene dosage effect. With cloned wild-type *ampD*, weaker inducibility is often seen in constructs with multiple copies of the gene because of the strong negative effects of the gene product (26). The results obtained with *E. cloacae* 029-5 make it clear that temperature-sensitive *ampD* still imparts a negative effect on enzyme expression at both temperatures, although this effect is not as strong as that seen in the wild type. When temperature-sensitive *ampD* is present in multiple copies in *E. coli* SN0302(pBP131), its negative impact on enzyme expression is even greater because of the gene dosage effect. Thus, basal levels are lower at both temperatures in *E. coli* SN0302(pBP131) than in *E. cloacae* 029-5 because of this gene dosage effect. Since *ampD* is temperature sensitive, its

negative impact on enzyme expression is minimized at 42°C in both strains.

Several pieces of evidence emerged from this study that strongly implicate *ampD* in the induction process itself. The poor inducibility at 42°C of any strain possessing wild-type *ampD* suggested involvement of this gene in the induction process. The differential responses of *E. coli* SN0302 (pBP131) containing *ampD* from *E. cloacae* 029-5 to cefoxitin and cefotetan also implicated this gene in the induction process. Earlier, Curtis et al. (4) showed that cefotetan was a better inducer for *E. cloacae* 029-5 than for *E. cloacae* 029, while the inducer potency of cefoxitin was just the reverse. These observations, coupled with our own, clearly demonstrate that alterations within *ampD* can change the responses of an organism to different inducers.

Whether or not the AmpD protein is itself the sensor for the inducer remains to be investigated. There are several lines of evidence against this possibility. Reports by Honoré et al. (12) and Oliva et al. (25) suggest that AmpE and/or PBP 2, two transmembrane proteins, may be the sensor(s) for the inducer. However, these reports do not preclude the possibility that there are multiple distinct sensors. Also, the actual role of these two proteins in β-lactamase expression is still uncertain. The report by Lindquist et al. (20) that AmpD is a 25-kDa cytoplasmic protein also argues against this protein as a sensor if the sensor must be a membrane-associated protein to detect β-lactam drugs in the periplasm, as has been assumed. However, it should be noted that in the minicell assays performed by Lindquist et al. (20), radioactivity was found in a 25-kDa membrane protein, as well as in a 25-kDa cytoplasmic protein.

Several data produced by the current study suggest that AmpD is the sensor. Introduction of wild-type *ampD* failed to normalize expression of AmpC β-lactamase in *E. cloacae* 029-5 and 1194E completely. This is in contrast to previous studies, in which the wild-type *ampD* allele had always been dominant over any mutant allele (14, 17, 20). Thus, this suggested that the AmpD proteins from the temperature-sensitive and hyperinducible mutants somehow interfered with the normal AmpD protein. One possible mechanism for such interference could be occupation of binding sites by the mutant proteins. A requirement for binding to a membrane site for proper functioning (i.e., sensing of an inducer) and occupation of some of those sites by these abnormal AmpD proteins could explain the mosaic phenotype observed. Clearly, further investigations into the role of the AmpD protein in the induction of AmpC β-lactamase are needed. Careful examination of the penicillin-binding ability of the protein and its native location within the cell are needed in future studies, especially in light of the fact that preliminary studies reported recently (29) indicate that AmpD indeed has penicillin-binding activity. Therefore, it is possible that AmpD is directly involved in sensing of the β-lactam inducer. Further studies are clearly needed to determine precisely how the various regulatory proteins affect β-lactamase expression in *E. cloacae*.

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