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# *ampG* Is Essential for High-Level Expression of AmpC β-Lactamase in *Enterobacter cloacae*

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**Mutants of *Enterobacter cloacae* 55 were studied to delineate more completely the genetics of inducible expression of AmpC β-lactamase. *E. cloacae* 55M-L, derived by mutagenesis from a mutant with high-level cefotaxime resistance (MIC, >64 μg/ml), *E. cloacae* 55M, demonstrated a novel phenotype by producing only low levels of AmpC constitutively. Neither the parental phenotype of *E. cloacae* 55M nor the wild-type phenotype of *E. cloacae* 55 could be restored in *E. cloacae* 55M-L by the introduction of functional *ampR*, *ampC*, or *ampD* genes. Cloning each of these genes from *E. cloacae* 55M-L confirmed the same genotype for this mutant as for its parental strain. Mutation of *E. cloacae* 55M-L to the *E. cloacae* 55M phenotype was found to occur spontaneously at a frequency of $10^{-8}$. All such revertants demonstrated an inducible wild-type phenotype after introduction of a functional *ampD*. These results suggested that the *E. cloacae* 55M-L phenotype was due to a mutation in an as yet unrecognized gene, designated *ampG*. Verification of this gene was obtained by the restoration of the *E. cloacae* 55M phenotype in *E. cloacae* 55M-L by introduction of a cloned 2.9-kilobase *Bam*HI fragment from the *E. cloacae* 55 chromosome. Transformation of both *ampG* and *ampD* into *E. cloacae* 55M-L reconstituted the inducible wild-type phenotype. These results indicate that *ampG* is required for the activation of *ampC* by AmpR. Without *ampG*, neither induction nor high-level expression of AmpC is possible. It is likely that the *ampG* gene product and AmpD together modulate the ability of AmpR to activate *ampC* expression.**

Most members of the family *Enterobacteriaceae* and other gram-negative bacteria possess a chromosomal class I cephalosporinase. In many of these species, expression of the enzyme is inducible by various β-lactam antibiotics (29, 32, 34). This inducibility can be altered by mutation resulting in elevated production of β-lactamase and resistance to many of the new β-lactam antibiotics (9, 15, 16, 21, 26, 31). To date the genetic apparatus responsible for both the inducible and the high-level expression of class I β-lactamase in *Enterobacter cloacae* and *Citrobacter freundii* has not been fully delineated. However, major advances have been made when the corresponding genes involved in regulation have been cloned (13, 15–17, 20–22, 26). From these studies, two regulatory genes affecting expression of *ampC*, the structural gene, have been identified. *ampR*, which is adjacent to *ampC*, appears to encode a DNA-binding protein that serves as the activator of *ampC* (13, 16, 20–22). A second regulatory gene, *ampD*, not closely linked to the *ampRampC* region, is responsible for low-level expression of *ampC* in uninduced *E. cloacae* wild-type strains. Inactivation of *ampD* by mutation occurs at a high frequency and results in high-level production of β-lactamase (16, 17, 21, 26). Therefore, *ampD* negatively regulates *ampC* expression. Exactly how or whether the product of *ampD* interacts with the AmpR protein is unknown. However, both *ampR* and *ampD* must be present to yield the inducible wild-type phenotype.

In a previous publication, Werner et al. described a mutant of *E. cloacae* 55 that expressed low levels of β-lactamase constitutively (36). Investigation of this unusual phenotype has led to the discovery of a new regulatory gene, *ampG*. We cloned *ampG*, and we propose a model for its role in *ampC* regulation.

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## MATERIALS AND METHODS

**Strains and plasmids.** The various strains and plasmids used in this study are described in Table 1. *E. cloacae* 55 is a wild-type clinical isolate that produces a class I type B (pI 7.8) chromosomal β-lactamase (9). *E. cloacae* 55M is a spontaneous mutant expressing high levels of the enzyme (9). *E. cloacae* 55M-L was derived from *E. cloacae* 55M by mutagenesis with nitrosoguanidine (36).

**Antibiotics and enzymes.** The following β-lactam antibiotics were used: cefoxitin and cefotaxime were kindly provided by their manufacturers (Merck, Sharp & Dohme, West Point, Pa., and Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J., respectively); kanamycin, tetracycline, chloramphenicol, sulfamethoxazole, and quinolinic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.); alkaline phosphatase and a kit for labeling of 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 (25) medium containing 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.) and thiamine (1 μg/ml) was used for some cloning experiments. Quinolinic acid (30 μg/ml), tetracycline (10 μg/ml), sulfamethoxazole (300 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 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 $MgCl_2$, and 10 ml of 1 M $MgSO_4$) and SOC medium (SOB medium plus 20 ml of 20% glucose) were used as described by Hanahan (11).

**Susceptibility testing.** Serial twofold dilution tests in Muel-

TABLE 1. Bacterial strains and plasmids used in the study

| Strain or plasmid | Description | Vector plasmid | Origin or reference |
|---|---|---|---|
| *E. coli* K-12 | | | |
| HB101 | *ara-14 galK2 hsdS20* ($r_B^- m_B^-$) *lacY1 leu mtl1 proA2 recA13F rpsL20 supE44 xyl5* $\lambda^-$ | | 2 |
| FL01 | *recA56*, derivative of *E. coli* MM294 | | 20 |
| JRG582 | Hfr *azi* Δ(*nadC-aroP*)*2 thiA* | | 10 |
| SN0302 | *ampA1 ampC8 ampD2 pyrB recA rpsL* | | 17 |
| *E. cloacae* | | | |
| 55WT | *ampR*$^+$ *ampC*$^+$ *ampD*$^+$ *ampG*$^+$ | | 9 |
| 55M | *ampR*$^+$ *ampC*$^+$ *ampD ampG*$^+$ | | 9 |
| 55M-L | *ampR*$^+$ *ampC*$^+$ *ampD ampG* | | 36 |
| Plasmids | | | |
| pACYC184 | Cm$^r$ Tc$^r$ | | 3, 30 |
| pBP131 | Su$^r$, *ampR*$^+$ *ampC*$^+$ region of strain 14[a] | RSF1010 | 16 |
| pBP132-3 | Tc$^r$, *ampR*$^+$ *ampC*$^+$ region of strain 7835[b] | pACYC184 | Korfmann (unpublished data) |
| pBP141-3 | Cm$^r$, *ampD*$^+$ *nadC*$^+$ region of strain 14 | pACYC184 | 16 |
| pBP141-1-2 | Km$^r$, *ampD*$^+$ region of strain 14 | pLG339 | Korfmann (unpublished data) |
| pGKS131-3 | Tc$^r$, *ampR*$^+$ *ampC*$^+$ region of strain 55 | pACYC184 | Present study |
| pGKS132-3 | Tc$^r$, *ampR*$^+$ *ampC*$^+$ region of strain 55M-L | pACYC184 | Present study |
| pGKS161-4 | Cm$^r$, *ampD*$^+$ *nadC*$^+$ region of strain 55 | pACYC184 | Present study |
| pGKS162-4 | Cm$^r$, *ampD nadC*$^+$ region of strain 55M-L | pACYC184 | Present study |
| pGKS171-3 | Cm$^r$, *ampG*$^+$ region of strain 55 | pACYC184 | Present study |

[a] Strain 14 is an *E. cloacae* wild-type strain with a class I chromosomal β-lactamase, pI 7.8 (16).
[b] Strain 7835 is an *E. cloacae* wild-type strain with a class I chromosomal β-lactamase, pI 9.1 (16).

ler-Hinton agar were performed with an inoculum of $10^4$ CFU per spot. The MIC was defined as the lowest concentration preventing growth after 18 h of incubation at 35°C in air.

**Induction of β-lactamase and determination of enzyme activity.** β-Lactamase was induced with cefoxitin in strains by using a 2-h induction assay (32). The concentration of cefoxitin varied from 1 to 64 μg/ml depending upon the susceptibility of the strain to the drug. β-Lactamase activity in sonic extracts was quantified by using a UV spectrophotometric assay with 100 μM nitrocefin as the substrate (32). The protein content of each sonic extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). For *Enterobacter* constructs containing two *ampC* genes, one encoding a type B enzyme (pI 7.8) and the other encoding a type C enzyme (pI 9.1), expression of each gene product was examined on isoelectric focusing gels (33). The various patterns obtained before and after induction made it possible to ascertain how both genes were expressed: inducibly or constitutively (8). Every induction experiment was performed at least in duplicate and usually in triplicate.

**Nucleic acid techniques.** Chromosomal DNA was prepared by a modified version of the protocol described by Edlund et al. (7). For rapid isolation of plasmid DNA, the alkaline extraction method of Birnboim and Doly (1) was used; for cloning experiments, DNA was isolated by the method of Clewell and Helinski (5). Restriction fragments were isolated out of an agarose gel by using the Geneclean system from Bio 101 Inc. (La Jolla, Calif.). Recombinant DNA techniques were performed as described by Maniatis et al. (23). Usually, vector DNA was treated with alkaline phosphatase to prevent self-ligation.

The procedure for transforming *Escherichia coli* was as described by Hanahan (11). Transformation of *E. cloacae* was performed as follows. Competent cells of *E. cloacae* were obtained by growing the cells in SOB medium to an $A_{546}$ of 0.3 to 0.4, centrifuging, and then suspending the cell pellet in 0.5 volume of 0.1 M $CaCl_2$. After incubation on ice for 1 to 1.5 h, the cells were concentrated 20-fold in 0.1 M $CaCl_2$, and samples of 0.2 ml were added to DNA. After 0.5 h on ice, the cells were incubated at 42°C for 1.5 min, cooled down on ice for 1 to 2 min, diluted with 3 ml of SOC medium, and incubated for 1 to 2 h with vigorous shaking at 37°C. Finally, the cells were plated on agar plates containing the appropriate selective antibiotic.

**DNA-DNA hybridization.** After electrophoresis, the gels were depurinated in 0.25 M HCl twice for 15 min each, denatured in 1.5 M NaCl–0.5 M NaOH twice for 30 min each, and neutralized in 1.5 M NaCl–1.0 M Tris hydrochloride (pH 8.0) twice for 30 min each. Gels were then blotted to nylon filters (Micron Separations Inc., Westboro, Mass.) in a Vacu-blot apparatus for 35 min with 2× SSC as buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). After blotting, filters were rinsed in 2× SSC 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.

## RESULTS

**Characterization of *E. cloacae* 55M-L.** The phenotype of *E. cloacae* 55M-L based upon expression of AmpC β-lactamase and susceptibility to cefotaxime is shown in Table 2 in comparison with those of *E. cloacae* 55 and *E. cloacae* 55M. Since *E. cloacae* 55M-L produced cephalosporinase constitutively at very low basal levels, it was susceptible to cefotaxime. *E. cloacae* 55 showed the normal wild-type phenotype of inducible enzyme expression. In *E. cloacae* 55M, which was resistant to cefotaxime, the enzyme was produced constitutively at high levels. Introduction of *ampD* (pBP141-1-2) by transformation into this latter mutant re-

TABLE 2. Cefotaxime susceptibility and β-lactamase expression of *E. cloacae* strains before and after transformation with *ampRampC* (pBP132-3) and *ampD* (pBP141-1-2)[a]

| *E. cloacae* strain | Cefotaxime MIC (μg/ml) | Enzyme sp act[b] | | Inducer concn of cefoxitin (μg/ml) |
|---|---|---|---|---|
| | | Uninduced | Induced | |
| 55M-L | 0.12 | 9 | 7 | 1 |
| 55 | 0.12 | 9 | 68 | 1 |
| 55M | 64 | 9,123 | 8,839 | 32 |
| 55M(pBP141-1-2) | 0.12 | 10 | 51 | 4 |
| 55(pBP141-1-2) | 0.12 | 8 | 39 | 4 |
| 55M-L(pBP132-3) | 0.25 | 55 | 50 | 2 |
| 55M-L(pBP141-1-2) | 0.12 | 6 | 6 | 1 or 4 |
| 55M-L(pBP141-1-2)(pBP132-3) | 0.25 | 62 | 64 | 2 or 16 |

[a] Results shown are means of replicate determinations.
[b] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

stored inducible expression of AmpC β-lactamase and susceptibility to cefotaxime (Table 2). This indicated that *E. cloacae* 55M was mutated in the *ampD* region. The lower inducibility of this construct and *E. cloacae* 55 (Table 2) containing a cloned *ampD* in comparison to *E. cloacae* 55 was probably due to the gene dosage effect described previously by Peter et al. (28), i.e., multiple copies of *ampD* produced by pBP141-1-2 provided greater negative influence over *ampC* expression than the single copy of *ampD* normally provided by the *Enterobacter* chromosome.

To determine whether a mutation in one of the known regulatory genes or in the promoter region of *ampC* was responsible for the low-level constitutive phenotype of *E. cloacae* 55M-L, the corresponding regions of a wild-type *E. cloacae* strain were transformed into this strain. Introduction of *ampD* had no effect on the phenotype of *E. cloacae* 55M-L (Table 2). The *ampRampC* region from *E. cloacae* 7835(pBP132-3), which encoded a type C enzyme (pI 9.1), produced an elevated level of AmpC β-lactamase due to the higher copy number of the cloned *ampC* gene versus the chromosomally located one. Enzyme expression, however, was still constitutive. Introduction of *ampD* (pBP141-1-2) into this construct by transformation still failed to provide an inducible phenotype (Table 2). To exclude the possibility that induction was masked by the amount of β-lactamase produced in these constructs, higher concentrations of inducers were examined. However, even when concentrations as high as 16 μg of cefoxitin per ml were used, no evidence of inducibility was observed. From these results, we concluded that the unusual phenotype of *E. cloacae* 55M-L was not due to a mutation in one of the known genes.

**Genotype of *E. cloacae* 55M-L.** To determine the genotype of *E. cloacae* 55M-L directly, several cloning experiments were performed with wild-type strain *E. cloacae* 55 as a positive control. The *ampRampC* region of both strains was cloned on an 8.8-kilobase (kb) *Eco*RI fragment into pACYC184, resulting in plasmids pGKS131-3 and pGKS132-3. In *E. coli* HB101, both constructs showed identical inducible expression of AmpC β-lactamase, indicating that the *ampRampC* region of *E. cloacae* 55M-L was functional in an *ampD*$^+$ background (Table 3). The *ampD* gene was cloned by using a procedure that used the expression of the adjacent *nadC* gene as a selective marker in a *nadC* mutant strain (*E. coli* JRG582) as described previously (16, 17). First, however, a DNA-DNA-hybridization experiment was performed with the *ampDnadC* region from pBP141-3 as a probe. According to this blot (data not shown), *nadC*, *ampD*, and ca. 2.8 kb of additional sequences downstream of *ampD* were cloned on a 5-kb *Hpa*I fragment into pACYC184, cut with *Eco*RV. The resulting recombinant plasmids (pGKS161-4 and pGKS162-4) containing either the *ampD* region of *E. cloacae* 55 or the corresponding region of *E. cloacae* 55M-L were finally transformed into *E. coli* SN0302 that also contained the *ampRampC* region of *E. cloacae* 14(pBP131). In contrast to the *ampD* region of the wild-type *E. cloacae* 55, the corresponding region from *E. cloacae* 55M-L could not *trans*-complement the *ampD2* mutation of the *E. coli* host back to the wild-type phenotype of inducible enzyme synthesis (Table 3). The latter construct showed an induction ratio of AmpC β-lactamase of 2.9, which was similar to that reported previously for *E. coli* SN0302 containing an *ampRampC* and an *ampD* mutant plasmid (17). Therefore, we concluded that *E. cloacae* 55M-L was mutated in *ampD* like its parent *E. cloacae* 55M, but only the latter expressed high levels of AmpC constitutively. These cloning experiments suggested that despite its unusual phenotype, *E. cloacae* 55M-L had the same genotype for all of the known *amp* genes as its parent, *E. cloacae* 55M.

TABLE 3. Cefotaxime susceptibility and β-lactamase expression of *E. coli* strains containing *ampRampC* or *ampD* from various *E. cloacae* strains[a]

| *E. coli* strain | Cefotaxime MIC (μg/ml) | Enzyme sp act[b] | | Inducer concentration of cefoxitin (μg/ml) |
|---|---|---|---|---|
| | | Uninduced | Induced | |
| HB101(pGKS131-3) | 0.12 | 27 | 162 | 4 |
| HB101(pGKS132-3) | 0.06 | 31 | 139 | 4 |
| SN0302(pBP131)(pGKS162-4) | 64 | 5,026 | 14,618 | 32 or 64 |
| SN0302(pBP131)(pGKS161-4) | 0.12 | 25 | 823 | 4 |

[a] *ampRampC* from *E. cloacae* 55(pGKS131-3) or 55M-L(pGKS132-3); *ampD* from *E. cloacae* 55(pGKS161-4) or 55M-L(pGKS162-4).
[b] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

To determine whether *E. cloacae* 55M-L could mutate back to the *E. cloacae* 55M phenotype, a mutation experiment was performed. When *E. cloacae* 55M-L was plated onto agar containing 32 μg of cefotaxime per ml, spontaneous revertants showing the *E. cloacae* 55M phenotype occurred at a frequency of $10^{-8}$. Results of tests with one such revertant, *E. cloacae* 55M-L-Mu13, are shown in Table 4. Another such revertant (*E. cloacae* 55M-L-M2) behaved identically to *E. cloacae* 55M-L-Mu13 (data not shown). Introduction of *ampD* (pBP141-1-2) by transformation into these revertants produced an inducible phenotype similar to that seen with *E. cloacae* 55M/pBP141-1-2 (Tables 2 and 4). These results suggested that the low-level constitutive phe-

TABLE 4. Cefotaxime susceptibility and β-lactamase expression of *E. cloacae* 55M-L and derivatives

| *E. cloacae* strain | Cefotaxime MIC (μg/ml) | Enzyme sp act[a] | | Inducer concn of cefoxitin (μg/ml) |
|---|---|---|---|---|
| | | Uninduced | Induced | |
| 55M-L-Mu13 | 64 | 10,562 | 9,691 | 32 |
| 55M-L-Mu13(pBP141-1-2) | 0.12 | 9 | 43 | 4 |
| 55M-L(pGKS171-3) | 128 | 9,432 | 8,624 | 32 |
| 55M-L(pGKS171-3)(pBP141-1-2) | 0.12 | 7 | 34 | 4 |

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

notype of *E. cloacae* 55M-L was due to a mutation in an as yet unrecognized gene, which we designated *ampG*. Thus, the genotype of *E. cloacae* 55M-L appeared to be *ampR*$^+$*ampC*$^+$*ampD ampG*.

**Cloning of *ampG*.** Based on the hypothesis that low-level constitutive expression of β-lactamase in *E. cloacae* 55M-L was due to a mutation in *ampG*, it was proposed that wild-type *ampG* should be dominant over the mutated allele. If this assumption were correct, introduction of *ampG*$^+$ should change *E. cloacae* 55M-L into a cefotaxime-resistant strain with high-level production of β-lactamase, since *E. cloacae* 55M-L, like *E. cloacae* 55M, is mutated in *ampD*. In a "shotgun" approach for cloning of *ampG*, chromosomal DNA of *E. cloacae* 55 cut with *Bam*HI was ligated into the vector pACYC184, which had been digested with the same enzyme and then treated with alkaline phosphatase. Since *E. cloacae* 55M-L is very difficult to transform, the ligation mixture was first transformed into *E. coli* FL01 to enrich recombinant plasmids. Then, in the second step for cloning *ampG*, plasmid DNA isolated from all chloramphenicol-resistant transformants was used to transform *E. cloacae* 55M-L. In this experiment, transformants were selected on chloramphenicol and cefotaxime (1 μg/ml). All colonies growing on this medium were tested on higher concentrations of cefotaxime and found to be highly resistant to this β-lactam antibiotic. To ensure that *ampG* had been cloned, plasmid DNA of the cefotaxime-resistant constructs of *E. cloacae* 55M-L was transformed into *E. coli* HB101 and from there back into *E. cloacae* 55M-L. This time, chloramphenicol alone was used for selection. Testing of the transformants revealed that the recombinant plasmid pGKS171-3, containing a 2.9-kb insertion of chromosomal DNA from *E. cloacae* 55, was able to transform *E. cloacae* 55M-L into a cefotaxime-resistant strain that produced β-lactamase at the same high level as *E. cloacae* 55M (Table 4). Transformation of *ampG* and *ampD* into *E. cloacae* 55M-L resulted in inducible expression of AmpC (Table 4) similar to that seen with *E. cloacae* 55 and *E. cloacae* 55M with the cloned *ampD* gene (Table 2).

To determine whether *ampG* was located close to *ampR*, *ampC*, *or ampD*, a Southern blot was performed. In this experiment, *ampG* did not hybridize with the 8.8-kb *Eco*RI fragment containing the *ampRampC* region or with the 5-kb *Hpa*I fragment containing *ampD*, *nadC*, and 2.8 kb of additional sequences distal to *ampD*. However, a homologous region of less than 300 base pairs between the probe and a positive control could be detected. From this, we concluded that *ampG* was not linked to one of the previously known genes involved in expression of β-lactamase in *E. cloacae*.

**Influence of *ampG* on β-lactamase production in an *E. coli* host.** Although *E. coli* expresses its own chromosomal enzyme constitutively (34), it produces the AmpC β-lactamase of *E. cloacae* inducibly when *ampR* is also present (16, 21, 26). Therefore, *E. coli*, like *E. cloacae*, appears to be *ampG*$^+$. To study the effect of additional *ampG* on AmpC production in a well-defined host, *E. coli* HB101 containing the *ampRampC* plasmid pBP131 was transformed with pGKS171-3. The results of induction experiments (Table 5) revealed that additional *ampG* has no positive or negative influence on induction. Similar results were observed when *E. cloacae* 55 was transformed with pGKS171-3 (data not shown). Thus, in contrast to *ampD*, *ampG* does not show a gene dosage effect.

TABLE 5. Cefotaxime susceptibility and β-lactamase expression of *E. coli* HB101 containing *amp* genes from *E. cloacae*[a]

| *E. coli* strain | Cefotaxime MIC (μg/ml) | Enzyme sp act[b] | |
|---|---|---|---|
| | | Uninduced | Induced[c] |
| HB101(pBP131) | 0.12 | 48 | 211 |
| HB101(pBP131)(pGKS171-3) | 0.12 | 47 | 216 |
| HB101(pBP131)(pBP141-1-2) | 0.12 | 31 | 50 |
| HB101(pBP131)(pBP141-1-2)(pGKS171-3) | 0.12 | 33 | 59 |

[a] *ampRampC* on pBP131; *ampG* on pGKS171-3; *ampD* on pBP141-1-2.
[b] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.
[c] Inducer concentration of cefoxitin, 4 μg/ml.

## DISCUSSION

Inducible expression of AmpC β-lactamase in genera like *Enterobacter* and *Citrobacter* has been shown in previous studies to be regulated by two genes: *ampR*, coding for an activator of *ampC* expression (13, 16, 20–22), and *ampD*, the gene product of which has a negative influence on *ampC* expression (16, 17, 26, 28). In this study, we have now shown that a third gene, *ampG*, is essential for expression of high levels of AmpC β-lactamase. Whether these levels result from the effects of an inducer upon an *ampD*$^+$ strain or from the loss of a functional AmpD protein due to a mutation in *ampD*, *ampG* appears to be necessary for high level expression of AmpC. *E. cloacae* 55M-L, a derivative of the cefotaxime-resistant strain *E. cloacae* 55M, was found to be mutated in this gene since its low-level constitutive phenotype could be converted to high-level production only by the introduction of an *ampG*-containing plasmid. From these results, it appears that *ampG* is responsible for the activation of *ampC* expression by AmpR.

To explain the mechanism of elevated β-lactamase production in *E. cloacae* and other species with inducible cephalosporinase, several models have been proposed (6, 18). However, in light of the discovery of *ampG* and new information concerning AmpR (22), a revision of these models is indicated. Recent studies have demonstrated a great deal of homology between *ampR* and activator genes unrelated to β-lactamase production (4, 12, 22). These homologous regions were found to be located in the sequence encoding the N terminus of the protein that is thought to be responsible for the DNA binding of the activator protein. The diversity in the regions encoding the C terminus was suggested to reflect the specificity of the inducer-binding site. Furthermore, the promoters of *ampR* and *ampC* are divergently organized and overlap, similar to other positively regulated operons (35). Considering the function of an activator, it appears that this regulatory protein usually exists in two forms: inactive and active. Very recently, Lindquist et al. showed that these two forms of AmpR can bind to a DNA region immediately upstream of the *ampC* promoter in either the presence or absence of an inducer (22). Two studies dealing with the activator proteins AraC and IlvY strongly suggest that the conversion from the inactive to the active conformation of an activator protein occurs by the binding of a ligand which is identical or similar to a substrate in the regulated pathway (24, 35).

Projection of these findings onto the regulation of *ampC* expression resulted in the model depicted in Fig. 1. The assumption that AmpR has to be converted into an activator to generate high levels of AmpC is supported by data from *ampR* mutants (13, 16, 21, 26). The expression of AmpC

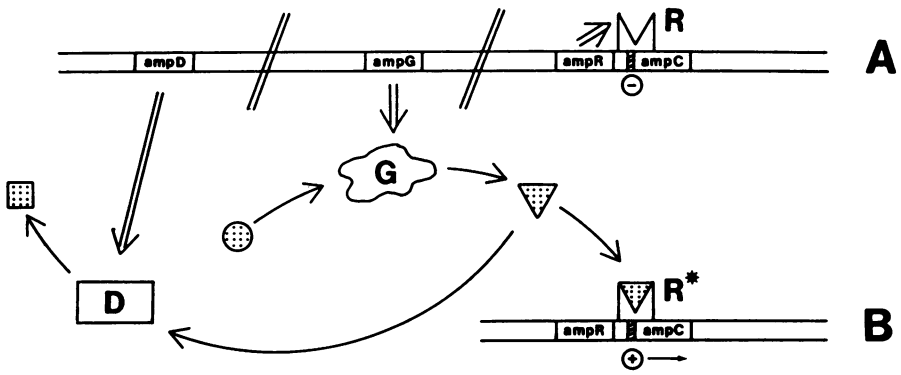


FIG. 1. Regulation model for the expression of AmpC in *E. cloacae*. A, Basal levels of β-lactamase; B, elevated levels; ⊕, substrate for AmpG; ▽, product of AmpG, substrate for AmpD, ligand for AmpR; ▣, product of ampD; R*, activated AmpR.

β-lactamase in *E. coli* constructs containing only *ampC* is usually 2 to 5 times higher than the basal levels expressed by similar constructs containing *ampR* and *ampC*. The most likely explanation for this is that the binding of an inactive AmpR protein interferes with the activity of the DNA-dependent RNA polymerase. In induced or *ampD* mutant *E. coli* or *E. cloacae* constructs containing an intact *ampRampC* region, transcription of *ampC* occurs with much higher efficiency than in *ampR* mutant constructs due to the effects of an activated AmpR protein. As suggested by others (22), the conversion of AmpR into an activator of transcription is presumably due to the binding of a ligand. However, this ligand is probably not the inducing β-lactam molecule, since there is no evidence that β-lactam antibiotics cross the cytoplasmic membrane (6, 18). Furthermore, Lindquist et al. could not find any sequence homology between AmpR and any known β-lactam-binding protein (22). Therefore, these authors suggest an interaction of AmpR with a second messenger of unknown nature. From the data presented in this study, it appears most likely that this ligand is provided enzymatically by the AmpG protein. Although possible, it is highly unlikely that the AmpG protein binds to or activates AmpR directly, since substrates related to the regulated pathway are usually involved in such activation (35). In the absence of *ampG*, AmpR remains bound to DNA in its inactive form; thus no elevated expression of *ampC* is possible either via induction or via mutation of *ampD*. Conversely, in the absence of *ampD*, AmpR can be maximally activated by the ligand provided by the AmpG protein. No data were generated in our study that proved how *ampD* exerts its negative influence. However, the presence of a gene dosage effect with *ampD* but not *ampG* suggests that the AmpD protein may inactivate the ligand produced by AmpG. This observation also makes it unlikely that AmpG is directly inactivated by AmpD, although this remains a possibility. The absence of a gene dosage effect for *ampG* is probably due to the fact that the small amount of AmpR produced by the cell and its rapid turnover (19, 21) limits the positive impact of this gene on AmpC expression. Exactly how the negative influence of *ampD* is removed or diminished by exposure of a strain to a β-lactam inducer is unknown. However, as hypothesized by others (6, 14, 18), this must certainly involve a transmembrane protein capable of binding the β-lactam antibiotic. One or more penicillin-binding proteins may ultimately be found to be the transmembrane sensors that initiate the events leading to a decrease in the negative effect of *ampD*. In a recent study, Oliva et al. presented evidence that in *E. coli*, PBP 2 might be one of the sensors involved in induction of the cloned *C. freundii* class I chromosomal β-lactamase (27).

Further investigations on all known regulatory genes, *ampR*, *ampD*, and *ampG* and their corresponding gene products will be necessary to show whether the proposed model is correct or has to be refined again. It is clear that many aspects remain to be proven directly. To elucidate the induction process, it is necessary to include penicillin-binding proteins or other transmembrane proteins that could serve as sensors for induction into the investigations as well as the products of all the now known regulatory genes.

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## LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
4. **Chang, M., A. Hadero, and I. P. Crawford.** 1989. Sequence of the *Pseudomonas aeruginosa trpI* activator gene and relatedness of *trpI* to other procaryotic regulatory genes. J. Bacteriol. **171**:172–183.
5. **Clewell, D. B. , and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
6. **Cole, S. T., N. Honore, and M.-H. Nicolas.** 1988. Regulation de la cephalosporinase inducible chez *Enterobacter*: induction et mutation, autres hypotheses. Med. Mal. Infect. Hors Ser. 18–21.
7. **Edlund, T., T. Grundstroem, and S. Normark.** 1979. Isolation and characterization of DNA repetition carrying the chromosomal β-lactamase gene of *Escherichia coli* K-12. Mol. Gen. Genet. **173**:115–125.
8. **Goering, R. V., C. C. Sanders, W. E. Sanders, Jr., R. Guay, and S. Guerin.** 1988. Heterogeneity in *ampR-ampC* gene interaction in *Enterobacter cloacae*. Rev. Infect. Dis. **10**:786–792.
9. **Gootz, T. D., C. C. Sanders, and R. V. Goering.** 1982. Resistance to cefamandole: derepression of β-lactamase by cefoxitin

and mutation in *Enterobacter cloacae*. J. Infect. Dis.**146:** 34–42.
10. **Guest, J. R., and P. E. Stephens.** 1980. Molecular cloning of the pyruvate dehydrogenase complex genes of *Escherichia coli*. J. Gen. Microbiol. **121:**277–292.
11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
12. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
13. **Honore, N., M.-H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. EMBO J. **5:**3709–3714.
14. **Kobayashi, T., Y. F. Zhu, N. J. Nicholls, and J. O. Lampen.** 1987. A second regulatory gene, *blaRI*, encoding a potential penicillin-binding protein required for induction of β-lactamase in *Bacillus licheniformis*. J. Bacteriol. **169:**3873-3878.
15. **Korfmann, G., C. Kliebe, and B. Wiedemann.** 1986. β-Lactamase antibiotics and selection of resistance: speculation on the evolution of R-plasmids. J. Antimicrob. Chemother. **18**(Suppl. C):113–121.
16. **Korfmann, G., and B. Wiedemann.** 1988. Genetic control of β-lactamase production in *Enterobacter cloacae*. Rev. Infect. Dis. **10:**793–799.
17. **Lindberg, F., S. Lindquist, and S. Normark.** 1987. Inactivation of *ampD* causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. J. Bacteriol. **169:**1923–1928.
18. **Lindberg, F., S. Lindquist, and S. Normark.** 1988. Genetic basis of induction and overproduction of chromosomal class I β-lactamase in nonfastidious gram-negative bacilli. Rev. Infect. Dis. **10:**782–785.
19. **Lindberg, F., and S. Normark.** 1986. Contribution of chromosomal β-lactamases to β-lactam resistance in enterobacteria. Rev. Infect. Dis. **8:**S292–S304.
20. **Lindberg, F., and S. Normark.** 1987. Common mechanism of AmpC β-lactamase induction in enterobacteria: regulation of the cloned *Enterobacter cloacae* P99 β-lactamase gene. J. Bacteriol. **169:**758–763.
21. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii* AmpC β-lactamase induction. Proc. Natl. Acad. Sci. USA **82:**4620–4624.
22. **Lindquist, S., F. Lindberg, and S. Normark.** 1989. Binding of the *Citrobacter freundii* AmpR regulator to a single DNA binding site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. J. Bacteriol. **171:**3746–3753.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Martin, K., L. Huo, and R. R. Schleif.** 1986. The DNA loop model for *ara* repression: AraC protein occupies the proposed loop sites *in vivo* and repression-negative mutations lie in these same sites. Proc. Natl. Acad. Sci. USA **83:**3654–3658.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Nicolas, M.-H., N. Honore, V. Jarlier, A. Philippon, and S. T. Cole.** 1987. Molecular genetic analysis of cephalosporinase production and its role in β-lactam resistance in clinical isolates of *Enterobacter cloacae*. Antimicrob. Agents Chemother. **31:** 295–299.
27. **Oliva, B., P. M. Bennett, and I. Chopra.** 1989. Penicillin-binding protein 2 is required for induction of the *Citrobacter freundii* class I chromosomal β-lactamase in *Escherichia coli*. Antimicrob. Agents Chemother. **33:**1116–1117.
28. **Peter, K., G. Korfmann, and B. Wiedemann.** 1988. Impact of the *ampD* gene and its product on β-lactamase production in *Enterobacter cloacae*. Rev. Infect. Dis. **10:**800–805.
29. **Richmond, M. H., and R. B. Sykes.** 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. **9:**31–88.
30. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.
31. **Sanders, C. C., and W. E. Sanders, Jr.** 1983. Emergence of resistance during therapy with the newer β-lactam antibiotics: role of inducible β-lactamases and implications to the future. Rev. Infect. Dis. **5:**639–648.
32. **Sanders, C. C., and W. E. Sanders, Jr.** 1986. Type I β-lactamases of gram-negative bacteria: interactions with β-lactam antibiotics. J. Infect. Dis. **154:**792-800.
33. **Sanders, C. C., W. E. Sanders, Jr., and E. S. Moland.** 1986. Characterization of β-lactamases in situ on polyacrylamide gels. Antimicrob. Agents Chemother. **30:**951–952.
34. **Sykes, R. B., and M. Matthew.** 1976. β-Lactamases from gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. **2:**115–157.
35. **Wek, R. C., and G. W. Hatfield.** 1988. Transcriptional activation at adjacent operators in the divergent-overlapping *ilvY* and *ilvC* promoters of *Escherichia coli*. J. Mol. Biol. **203:**643–663.
36. **Werner, V., C. C. Sanders, W. E. Sanders, Jr., and R. V. Goering.** 1985. Role of β-lactamases and outer membrane proteins in multiple β-lactam resistance of *Enterobacter cloacae*. Antimicrob. Agents Chemother. **27:**455–459.