Common Mechanism of \textit{ampC} β-Lactamase Induction in Enterobacteria: Regulation of the Cloned \textit{Enterobacter cloacae} P99 β-Lactamase Gene

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Expression of the chromosomal β-lactamase from the \textit{ampC} gene is inducible in both \textit{Enterobacter cloacae} and \textit{Citrobacter freundii}. Cloning of \textit{ampC} as well as its regulatory gene, \textit{ampR}, from \textit{E. cloacae} P99 revealed a gene organization identical to that of \textit{C. freundii} in the corresponding region. Although almost no similarities could be found between the restriction maps of \textit{ampC} and \textit{ampR} in the two species, the genes cross-hybridize. Also, both \textit{ampR} gene products have a size of about 31,000. The regulatory features of \textit{E. cloacae} β-lactamase induction are very similar to those in \textit{C. freundii}, i.e., β-lactamase synthesis is repressed by AmpR in the absence, and stimulated in the presence, of inducer. The AmpR function can be transcomplemented between the two species, but there are quantitative regulatory aberrations in such hybrids, in contrast to the total complementation obtained within each system. These results suggest that the mechanism of β-lactamase induction is the same in \textit{E. cloacae}, \textit{C. freundii}, and other gram-negative bacteria with inducible chromosomal β-lactamase expression.

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Purification and amino-terminal sequence determination of the P99 β-lactamase from SN0302/pNU346. β-Lactamase was purified from a periplasmic fraction by ammonium sulfate precipitation, CM-Sepharose chromatography, and fractionation on Sephacyr S-200 as described for the C. freundii β-lactamase from SN0302/pNU305 (19). The P99 β-lactamase eluted from the CM-Sepharose column as a single peak, centered at 12 mM potassium phosphate buffer (pH 6.8). The yield from a 15-liter culture was 40 mg. Twenty steps of automated Edman degradation (8) of the purified enzyme were performed (9). The sequence obtained was NH2-Thr-Pro-Val-Ser-Glu-Lys-Gln-Leu-Ala-Glu-Val-Ala-Ala-Asn-Thr-Ile-Thr-Pro-Leu-Met.

Determination of ampicillin resistance. Bacteria were grown to an optical density at 420 nm of 0.8 (Zeiss PM3 spectrophotometer) in M9CA medium, maintaining the selection for the plasmid mediating tetracycline or chloramphenicol resistance, and then diluted 10^-5-fold. A volume of 0.1 ml (200 to 500 CFU) was spread on each of a series of plates containing ampicillin at increasing concentrations. Resistance was defined as IC50, i.e., the concentration at which 50% of the bacteria were inhibited from forming colonies. Plates were read after 36 to 48 h at 37°C.

Determination of β-lactamase production. Bacteria were grown logarithmically for at least eight generations in M9CA medium selecting for plasmids that encode tetracycline and chloramphenicol resistance. At an optical density at 420 nm of 0.8, a 20-ml sample was chilled quickly on ice, and the cells were pelleted, washed twice in 50 mM phosphate buffer (pH 7.0), and resuspended in 0.8 ml of phosphate buffer. Extracts were prepared by adding 0.2 ml of 500-mg/liter lysozyme-50 mM EDTA, followed by a 30-min incubation at 37°C and sonication on ice with a Branson Sonifier B-12 equipped with a microtip (six cycles of 10-s sonication at set 3 with 30-s pauses). A suitable amount of extract (5 to 40 μl) was then added to 1 ml of cefalexin solution (170 μM in 0.1 M potassium phosphate buffer [pH 7.0]). Hydrolysis was assayed as the rate of decrease in A260. Specific activity was expressed as micromoles of cefalexin hydrolyzed at 30°C per minute per milligram of protein, as determined by the method of Lowry et al. (22), using bovine serum albumin as the standard. Induction was achieved by two-fold dilution of the culture at an optical density at 420 nm of 0.8 into prewarmed medium containing the inducer 6-aminopenicillanic acid (6-APA). The final 6-APA concentration was 2 g/liter. After 40 min of growth a 20-ml sample was processed as described above.

Restriction mapping and construction of plasmid derivatives. The restriction map of pNU346 was constructed by digesting the plasmid with various restriction endonucleases either alone or in combinations of two or three. These digests were electrophoresed on 0.7 to 2.0% agarose gels in Tris acetate-EDTA buffer as previously described (14). A HindIII digest of bacteriophage λ cI857 and a HaeIII digest of bacteriophage φX184 (new England BioLabs, Inc., Beverly, Mass.) were used as size markers. The order of the SaII fragments of pNU346 was determined by treating a BamHI digest of the plasmid with exonuclease Bal 31 (25). Samples were taken at different times and digested with SaII. By subsequent agarose gel electrophoresis, the SaII fragments could be ordered from their sequence of disappearance.

Plasmid pNU359 was constructed by ligating a SaII-BamHI double digest of pNU346 with plasmid pUC9 (37) digested with the same enzymes, followed by transformation (24) into FL01, selecting for cefotaxime resistance (0.2 mg/liter). Small-scale DNA preparations (4) were digested with BamHI and SaII, and one of the plasmids with the smallest insert was further characterized. Subsequently, a detailed restriction map of this plasmid was prepared.

To construct a plasmid carrying only ampR, but not ampC, pNU359 was digested with SaII and EcoRV and ligated to SaII-EcoRV-digested pACYC184, selecting for chloramphenicol resistance and screening for tetracycline sensitivity. The structure of the plasmid pNU362, which was verified by restriction analysis. To obtain pNU364, we subcloned the smaller Sacl-EcoRI fragment of pNU359 into the vector pUC78 digested with the same enzymes. In the construction of pNU363, a plasmid carrying the ampC gene, the vector pNU78 was digested with PstI followed by the removal of the 3' extension with T4 DNA polymerase (25) and digestion with EcoRI. Subsequently, this vector was ligated to the HindII-EcoRI fragment of pNU359 followed by transformation with FL01 selecting for tetracycline resistance and cefotaxime resistance (0.1 mg/liter).

Southern blot analysis. Double and triple digests of plasmids pNU346 and pNU359 were separated on 1.5% agarose gels. After denaturation, the DNA was transferred (34) to Zeta-probe membranes (BioRad Laboratories, Richmond, Calif.). Three probe fragments of C. freundii OS60 DNA were isolated from pNU302 and 32P labeled as previously described (2). These fragments covered parts of the coding regions for frdA (EcoRI-HindIII), ampR (BamHI-Sacl), and ampC (SacII-BamHI) (2, 21). The extent of the regions covered by the probes is shown in Fig. 1. Hybridization was done under conditions of both high and lower stringency as previously described (23). After exposure to Du Pont Cronex 4 X-ray film for 2 to 24 h, the filter was incubated for 30 min in 0.1 M NaOH at 40°C, followed by repeated washing in 0.1 M Tris hydrochloride (pH 7.5). After removal of the probe in this manner, the filter was reprobed with the other probes.

Analysis of protein expression in minicells. The different plasmids were transfected into the minicell producer ORN103, and minicells were isolated by sucrose gradient centrifugation as previously described (21). Minicell preparations were labeled with a 2-min pulse of [35S]methionine, either directly or after incubation for 10 min in ampicillin (1 g/liter) (21). Equal fractions of the induced and noninduced preparations were separated in parallel on sodium dodecyl sulfate-15% polyacrylamide gels (18).

Chemicals and enzymes. Restriction endonucleases were purchased from New England Biolabs, Inc., Boehringer GmbH (Mannheim, Federal Republic of Germany), Pharmacia Biotechnology International AB (Uppsala, Sweden), or Amersham Corp. (Arlington Heights, Ill.). Bacteriophage phage T4 DNA ligase and the Klone fragment of DNA polymerase I were purchased from Boehringer

FIG. 1. Restriction and gene map of the C. freundii insert in pNU305 (adapted from reference 21). The extent of the three different probes used in the hybridization experiments with the E. cloacae P99 frd and amp genes is indicated. The probes correspond to part of the frdA (A), ampR (R), and ampC (C) genes. bp, Base pairs.
GmbH. Phage T4 DNA polymerase and [α-32P]dGTP are products of Amersham Corp. Ampicillin and cefotaxime were gifts from Astra Läkemedel AB and Svenska Hoechst AB, respectively. All chemicals were of the highest grade commercially available.

RESULTS

Cloning of E. cloacae P99 β-lactamase gene. Labeled DNA representing the C. freundii OS60 β-lactamase gene (2, 21) was used to probe Southern blots of chromosomal DNA of E. cloacae P99 DNA cut with various enzymes. It was thereby established that no EcoRI sites are present within the β-lactamase gene in strain P99 (data not shown). To clone the E. cloacae P99 ampC gene, we therefore digested chromosomal DNA with EcoRI and ligated it into pACYC184. The ligated DNA was transformed into E. coli FL01, selecting on plates containing tetracycline and ampicillin (10 mg/liter). Five colonies were obtained, and all five mediated resistance to ampicillin (10 mg/liter) and cefotaxime (0.2 mg/liter) and expressed a β-lactamase-celphalosporin C or cephalaxin as substrates. Plasmid DNA from a clone was EcoRI digested, and its insert was cloned into pNU78. The resulting plasmid, pNU346, consists of three EcoRI fragments, the vector and two insert fragments. Repeated experiments to subclone separately the larger EcoRI fragment failed. Restriction maps of this plasmid and the Sacl-BamHI subclone pNU359 are shown in Fig. 2.

The β-lactamase encoded by pNU346 was purified to homogeneity and sequenced by Edman degradation from the amino terminus. The first 20 amino acids were unambiguously identified, and the sequence agrees exactly with the sequence published for the enzyme purified from E. cloacae P99 (16).

Three fragments of C. freundii OS60 DNA from plasmid pNU305 covering, respectively, a part of the C. freundii frdA gene (Fig. 1, A), a part of ampR (Fig. 1, R), and a segment of ampC (Fig. 1, C) were labeled and used to probe various digests of pNU346 and pNU359 in Southern hybridization experiments. The smallest fragments of pNU346 hybridizing with the three probes are shown in Fig. 2. A subclone carrying Sacl-BamHI mediated β-lactam resistance, whereas a similar construct carrying Sacl-XhoI did not (Fig. 2) (data not shown), which is in agreement with the placement of the ampC gene by hybridization experiments. These results indicate that the gene organization within this region of the C. freundii OS60 and E. cloacae P99 chromosomes is similar. The only similarity between the restriction maps of the frd-amp regions in these two strains are the Apal and Ball sites which are similarly placed with identical distances between the sites.
TABLE 1. Relative β-lactamase expression from the cloned ampC genes of C. freundii and E. cloacae in E. coli SN03 (ampD') and SN0302 (ampD2)

| Plasmid(s) | Origin of genes | IC50b of ampicillin (mg/liter) | Relative β-lactamase expressionc | Noninduced | Induced | Noninduced | Induced | Noninduced | Induced | Ratio of noninduced | SN03/ noninduced SN03 |
|------------|----------------|-------------------------------|---------------------------------|-----------|---------|-----------|---------|-----------|---------|---------------------|-------------------|
| pNU346     | EC             | <1                            | <0.02                           | 8.0       | 48      | 170       | 21      | 1.0       | 1.0      | ND                  |                   |
| pNU364     | EC             | <1                            | <0.02                           | 1.00      | 53      | 190       | 190     | 1.0       | 1.0      | ND                  |                   |
| pNU363     | EC             | <1                            | <0.02                           | 1.9       | 1.9     | 1.8       | 1.0     | 1.0       | 1.0      | ND                  |                   |
| pNU362, pNU363 | EC            | <1                            | <0.02                           | 1.06      | 44      | 139       | 131     | 1.0       | 1.0      | ND                  |                   |
| pNU305     | CF             | 4                             | 113                             | 13       | 12      | 12        | 1.0     | 1.0       | 1.0      | ND                  |                   |
| pNU314     | CF             | 14                            | 113                             | 13       | 12      | 12        | 1.0     | 1.0       | 1.0      | ND                  |                   |
| pNU311     | CF             | 1                             | 1                              | 0.38      | 15      | 20        | 53      | 2         | 2        | ND                  |                   |
| pNU314, pNU311 | CF          | 13                             | 111                             | 5.0       | 98      | 111       | 22      | 2.0       | 2.0      | ND                  |                   |
| pNU363, pNU311 | CF            | 2.5                           | 20                              | 3.8       | 56      | 410       | 7.4     | 3         | 3        | ND                  |                   |

a The origin of the genes is E. cloacae P99 (EC) or C. freundii OS60 (CF). When the gene resides on a pACYC184 derivative it is put within parentheses, otherwise it is located on a pBR322 derivative. If both genes have identical origin codes, they are present on the same plasmid. When two plasmids are present in the same cell, the first is always a pBR322 derivative carrying the ampC gene, and the second is a pACYC184 derivative carrying ampR.

b IC50, Concentration at which 50% of the bacteria are inhibited from forming colonies.

c Specific β-lactamase expression is given relative to SN03(pNU364) grown under noninducing conditions. The ratio given is that between specific β-lactamase expression in SN03 under noninducing conditions and that in SN03 under the same conditions.

— No plasmid.

Regulation of E. cloacae ampR β-lactamase. Expression of β-lactamase from the E. cloacae clone pNU346 was inducible by 6-APA in E. coli (Table 1). When the plasmid was transformed into the ampD2 mutant E. coli SN0302, the E. cloacae β-lactamase from pNU346 was overproduced. These results closely resemble those obtained with the cloned C. freundii β-lactamase gene. Three subclones of pNU359 were constructed to further investigate the role of the postulated E. cloacae ampR gene (see Materials and Methods). The first of these, pNU364, extends from SacII through BamHI-2 and should carry frdD, ampR, and ampC (Fig. 2). The second subclone, pNU363, extends from HindII to BamHI and carries only ampC, whereas pNU362, a subclone from SacII to EcoRV, carries the frdC, frdD, and ampR genes, but lacks ampC.

The ampicillin resistances mediated by the different clones in E. coli SN03 and E. coli SN0302 are shown in Table 1, together with the specific β-lactamase activities expressed relative to pNU364 in SN03. As expected, pNU362 did not mediate any increase in ampicillin resistance or enzyme production. Plasmid pNU364 mediated a 38-fold-higher ampicillin resistance in SN302 (150 mg/liter) as compared with the resistance mediated in SN03 (4 mg/liter). The β-lactamase activity produced was about 190 times higher in the former strain than in the latter. The elimination of ampR, such as in pNU363, resulted in a slightly higher resistance and an approximately twofold increase in specific β-lactamase activity in SN03. In this case both the level of resistance and β-lactamase production were equal in SN03 and SN0302. Both of these effects could be complemented in trans by pNU362 (Fig. 2) carrying the E. cloacae ampR gene (Table 1). Expression of β-lactamase activity in SN03 was higher from pNU346 than from the subclone pNU364. This could be due to an external promoter reading into ampR or ampC or possibly to an additional positive regulatory factor present on pNU346 to the left of frdA or to the right of ampC in Fig. 2.

Expression of β-lactamase activity was induced by growing bacteria in 6-APA (2 g/liter). The relative amounts of specific β-lactamase activity were compared between extracts prepared from bacteria before and after 40 min of induction (Table 1). In agreement with the data obtained with the C. freundii ampR and ampC genes (21), β-lactamase expression was inducible in SN03 if ampR was present in cis or in trans, and synthesis was low and constitutive if ampR was not present. In SN0302 the cloned β-lactamase was overexpressed or constitutively expressed at a low level, depending on the presence or absence of ampR.

Identification of E. cloacae ampR and ampC gene products in minicells. Minicells were purified from cultures of E. coli ORN103 carrying the different subclones. These were pulse-labeled with [35S]methionine before and after induction with ampicillin. Identical portions of noninduced and induced preparations were electrophoresed on a sodium dodecyl sulfate–15% polyacrylamide gel (Fig. 3). A polypeptide with apparent molecular weight (Mr) of 39,000 and comigrating exactly with the purified E. cloacae β-lactamase was expressed from pNU363 and pNU364. This protein was not expressed from pNU362. Plasmids pNU362 and pNU364 expressed an Mr-31,500 polypeptide which is very similar in size to the C. freundii ampR gene product. Plasmid pNU363, which does not carry the ampR gene, did not express the Mr-31,500 protein. Also, an Mr-13,000 polypeptide, probably the frdD gene product, was expressed from pNU364 and pNU363. As is apparent from Fig. 3, the rate of β-lactamase synthesis increased in minicells induced with ampicillin if the ampR gene was present in cis (Fig. 3, lanes B1 and B2) or in trans (lanes D1 and D2).

Transcomplementation of ampR between C. freundii and E. cloacae. It was possible to complement ampR between the E. cloacae and C. freundii clones in E. coli minicells (Fig. 3, lanes E1 through F2). To investigate the quantitative aspects of these ampR complementations, E. coli SN03 and SN0302 were transformed with pNU78-based clones carrying ampC of C. freundii (pNU314) or E. cloacae (pNU363) but not the corresponding ampR gene. In addition, derivatives of pACYC184 carrying only the ampR gene of the respective species were transformed into the strains. These were plasmids pNU311, carrying the C. freundii ampR gene, and pNU362, which carries that of E. cloacae. In SN03, expres-
sion of the *E. cloacae* β-lactamase was slightly repressed by the addition in trans of *ampR* from either *C. freundii* or *E. cloacae*. In all cases β-lactamase production could be induced with 6-APA, showing that *ampR* could indeed be trans complemented between the species. This was also true in the *ampD* mutant SN0302. In this strain expression from the *C. freundii* *ampC* gene was significantly higher in the presence of the *E. cloacae* *ampR* gene than with the homologous *ampR* gene. In fact, with this combination β-lactamase production was so high (~5% of total protein) that the doubling time in M9CA medium increased to 70 min from the 60 min normal for SN0302.

**DISCUSSION**

We cloned the region carrying the β-lactamase gene from *E. cloacae* P99 into *E. coli*. The sequence for the 20 amino-terminal residues of the β-lactamase produced from this recombinant plasmid was identical to that published for the *E. cloacae* P99 enzyme (16). From molecular subcloning experiments and from hybridization studies with *C. freundii* probes, we conclude that the overall gene organization of the *ampC* region is identical in these two species, i.e., the genes encoding the fumarate reductase (frdA through frdB) are followed by the *ampR* regulatory gene and the *ampC* β-lactamase gene (Fig. 2). The chromosomal β-lactamases of different isolates of *E. cloacae* can be divided into two groups on the basis of their isoelectric points (32). The gene cloned from *E. cloacae* 208 by Seeberg and Wiedemann (33) expresses an enzyme belonging to one group, whereas the P99 enzyme belongs to the other. No evident similarities could be found when comparing the *E. cloacae* P99 restriction map with that published for *E. cloacae* 208, except for the similar size of the EcoRI fragments.

Inducibility of the cloned *E. cloacae* β-lactamase is dependent on the presence of the regulatory gene *ampR*. A deletion of the *E. cloacae* *ampR* gene resulted in a 1.9-fold higher β-lactamase expression which was not increased by the addition of inducer to the medium. Both this slight increase of expression in the absence of inducer and the loss of inducibility could be complemented by *ampR* in trans. The same is true for the cloned *C. freundii* β-lactamase gene, except that the deletion of *ampR* decreases expression 2.5-fold in the absence of inducer. Although the restriction maps for the two *ampR* genes differ, the genes hybridize to one another and express similarly sized AmpR proteins (Fig. 3).

Earlier, we described an *E. coli* mutation, *ampD2*, which results in constitutive or semiconstitutive *ampR*-dependent overproduction of the cloned *C. freundii* β-lactamase (21). The *E. cloacae* clone was affected in a similar manner when introduced into such a mutant, i.e., the *E. cloacae* β-lactamase was overproduced in an *ampR*-dependent manner.

The synthesis of *C. freundii* β-lactamase is 11-fold lower in the presence of the homologous *ampR* gene than when complementing with *ampR* from *E. cloacae*. Also, synthesis of *E. cloacae* β-lactamase in the presence of inducer or the *ampD2* mutation is much higher with the *E. cloacae* *ampR* gene than with that of *C. freundii*. These quantitative differences between homologous and heterologous complementations suggest a physical interaction between the AmpR protein and *ampC* DNA, or a product thereof. Since the β-lactamase is located in the periplasm, we find it unlikely that the AmpR protein would interact with this enzyme. Also, we have previously shown that a functional β-lactamase is not required for induction (20). Thus, we favor the view that AmpR interacts directly with either the *ampC* control region or the *ampC* transcript.

In conclusion, both gene organization and the regulatory features of the *ampC* region are almost identical in the two enterobacterial species with inducible β-lactamase production that have been studied. The regulatory function, AmpR, can be partially complemented between the species, in a manner suggesting direct interaction between this protein and the *ampC* control region or transcript.

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