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Binding of the *Citrobacter freundii* AmpR Regulator to a Single DNA Site Provides Both Autoregulation and Activation of the Inducible *ampC* β-Lactamase Gene

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Citrobacter freundii **encodes an inducible chromosomal β-lactamase. Induction requires the product of the *ampR* gene, which is transcribed in the opposite orientation from the *ampC* β-lactamase gene. We show here that the AmpR protein acts as a transcriptional activator by binding to a DNA region immediately upstream of the *ampC* promoter. The DNase I footprint pattern was not affected by growth in the presence of β-lactam inducer or by the use of extracts prepared from cells carrying the *ampD2* allele leading to semiconstitutive production of β-lactamase. It is suggested that activation of AmpR facilitates binding or open complex formation for RNA polymerase at the *ampC* promoter. The AmpR-binding site overlaps the *ampR* promoter, and β-galactosidase activity was decreased from an *ampR-lacZ* transcriptional fusion when AmpR was expressed from a coresident plasmid, suggesting that *ampR* is autogenously controlled. The AmpR protein belongs to a family of highly homologous transcriptional activators that includes LysR, which regulates the *E. coli* lysine synthetase gene, and the NodD protein, which regulates expression of a number of genes involved in nodulation in *Rhizobium*. The lack of sequence homology to any known β-lactam-binding protein suggests that AmpR does not bind directly to the β-lactam inducer but interacts with a second messenger of unknown nature.**

In many gram-negative enterobacteria, including *Citrobacter freundii*, *Enterobacter cloacae*, *Serratia marcescens*, and indole-positive *Proteus* spp., expression of the chromosomal β-lactamase can be induced by β-lactam antibiotics (40). Induction in *C. freundii* is regulated by a *trans*-acting protein, AmpR, which is encoded immediately upstream of the *ampC* β-lactamase gene (21). In the absence of an inducer, AmpR represses the synthesis of β-lactamase by 2.5-fold, whereas expression is induced 10- to 200-fold in the presence of a β-lactam inducer (21). Mutations in a second regulatory gene, *ampD*, lead to an AmpR-dependent constitutive or semiconstitutive overproduction of β-lactamase (19). *ampD* is *trans*-active, suggesting that its gene product either directly or indirectly affects the expression of β-lactamase.

DNA-binding regulatory proteins of the lambda-repressor class have been extensively studied (31). Crystal structures of the proteins alone and in complex with their target DNA have been examined. A conserved feature of these proteins is a helix-turn-helix motif that binds to the major groove of the DNA helix. The *ampR* gene has been sequenced from *E. cloacae* MHN1 (13); however, no DNA-binding domain could be found in the deduced amino acid sequence of AmpR. DNA-binding studies which could assess its mode of action as a regulator have not been reported for the *E. cloacae* AmpR protein.

In this paper we present the sequence of the *ampR* gene from *C. freundii* OS60. We demonstrate that AmpR regulates the expression of *ampC* at the level of transcriptional initiation. Cell extracts containing the AmpR protein bind directly to the region encompassing both the *ampR* and *ampC* promoters, irrespective of the presence or absence of inducer and of the allelic status of *ampD*. The AmpR protein exhibits significant amino acid sequence similarity to the transcriptional activators LysR, which regulates the lysine synthetase gene *lysA* in *Escherichia coli* (39), and NodD, a DNA-binding regulator affecting the expression of inducible nodulation genes in *Rhizobium* spp. (29, 34, 37). Like *lysR* and *nodD*, the *ampR* gene was found to be autogenously controlled.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains used in this study were SN03 (*ampA1 ampC8 pyrB recA rpsL*) (30) and its *ampD2* derivative SN0302 (19). *E. coli* MC1029 [*araD139* Δ(*ara leu*)*7697* Δ(*lacZ*)M15 *galU galK rpsL recA56*] (4) was used in the transcriptional fusion assays, and JM103 (26) was used for phage M13 propagation. Plasmid pNU305 carries the *C. freundii ampR* and *ampC* genes, whereas pNU311 carries *ampR* only (21). The *ampR* gene on plasmid pNU312 is truncated and does not express an active AmpR protein (21). A derivative of pNU311, pNU316, was constructed by digesting pNU311 with *Cla*I, end-filling with the Klenow fragment of DNA polymerase I (24), and inserting an 8-mer *Xho*I linker (New England Biolabs). Similarly, an 8-mer *Eco*RI linker (New England Biolabs) was introduced into the *Apa*I site of pNU305 to result in pNU371 (Fig. 1). The *ampC-lacZ* transcriptional fusion plasmid, pNU330, was constructed by ligating the *Bam*HI-*Xho*I fragment of pNU316 into the vector pRZ5255 (a Kan^r derivative of pRZ5202 [32; L. Munson and W. S. Reznikoff, personal communication]), digested with *Bam*HI and *Sal*I (Fig. 1). The *ampR-lacZ* transcriptional fusion plasmid pNU372 was constructed by ligating the *Bam*HI-*Hpa*I fragment of pNU305 into the *Bam*HI-*Sma*I sites of vector pRZ5202 (Fig. 1).

Media and growth conditions. The rich medium used was LB of Bertani (2) supplemented with medium E (41), 0.2% glucose, and thiamine (1 mg/liter). Minimal medium was medium E supplemented with 0.2% glucose, thiamine (1 mg/liter), and uracil (50 mg/liter). When required, chloramphenicol (10 mg/liter) was added. M9CA medium was M9

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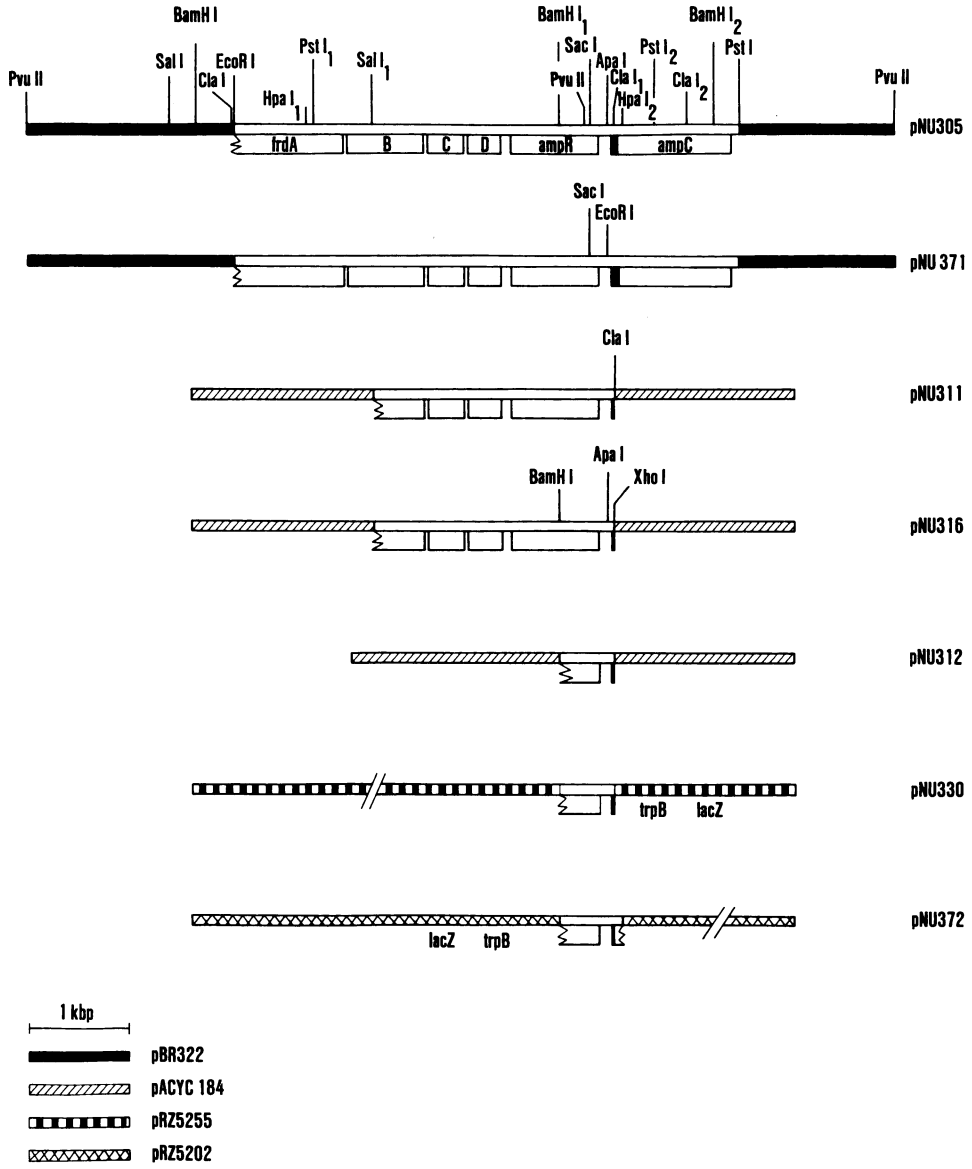

FIG. 1. Genetic and physical map of the plasmid pNU305 and its derivatives. The black area in the *ampC* box represents the region encoding the signal peptide. Only the restriction sites relevant to this paper are shown for plasmids pNU371, pNU311, and pNU316.

salt medium (28) supplemented with 0.2% glucose, thiamine (1 mg/liter), uracil (50 mg/liter), and Casamino Acids (0.2%; Difco Laboratories, Detroit, Mich.).

DNA sequencing. Digests of pNU305 plasmid DNA were separated on low-melting-point agarose gels to purify the appropriate suitable fragments (24). These were subsequently cloned into the phage vectors M13mp8, M13mp9, M13mp18, or M13mp19 (27, 43) and transformed into *E. coli* JM103. Single-stranded DNA was prepared as described by Sanger et al. (35) and sequenced using the dideoxy chain-termination method of Sanger et al. (36).

β-Galactosidase assay. The specific activity of β-galactosidase was assayed as described by Miller (28).

Isolation of total RNA and Northern blot hybridization. Cells were grown at 37°C in M9CA medium to an optical density at 420 nm of 0.8, and total RNA was extracted from lysed cells by the hot-phenol method (42). The RNA was separated on a 1% agarose–2.2 M formaldehyde gel and transferred to nitrocellulose paper (Schleicher and Schüll, BA85) as described (1). To detect *ampC* transcripts, a *Pst*I_2-*Cla*I_2 fragment from pNU305 (Fig. 1) was cloned into M13mp18 and subsequently used to prepare a specific *ampC* probe labeled with [α-^{32}P]dATP (14, 33). Similarly, a *Bam*HI_1-*Pvu*II fragment was cloned from pNU305 to obtain an *ampR*-specific probe.

Preparation of protein extracts. Protein extracts were prepared essentially as described by Keegan et al. (17). Cells were grown in 250 ml of minimal medium to an A_{420} of 1.2. The culture was harvested, washed once in NaCl (150 mM), and suspended in 2 ml of buffer A (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 0.6 μM leupeptin). This mixture was sonicated on ice by 10 cycles of sonication (15 s with a Branson sonifier at setting 3 and 30 s of cooling on ice), and cellular debris was

FIG. 2. The DNA sequence together with the deduced amino acid sequence of the *C. freundii* AmpR protein. The proposed ribosomal binding site (RBS) and some relevant restriction sites are underlined. Arrows indicate the proposed *ampR* transcriptional terminator. The *frdD* stop codon (TAA) is also marked.

removed by centrifugation at 48,000 × *g* for 30 min. Protein concentration was determined according to Lowry et al. (23).

Labeling of DNA. Plasmid pNU316 was digested with either *ApaI-BamHI* or *ApaI-XhoI* and labeled with [α-^{32}P]dATP by filling in recessed 3′ ends using the Klenow fragment of DNA polymerase I (24). Plasmid pNU371 was cleaved with *Eco*RI and similarly endlabeled with [α-^{32}P] dATP. Labeled DNA was digested with *Sac*I, and the 179-base-pair (bp) *Eco*RI-*Sac*I fragment was purified from a 5% polyacrylamide gel. This small fragment carries the intercistronic region between *ampR* and *ampC*, including their respective promoters, and was used both in gel mobility-shift assays and in DNase I footprinting.

Gel mobility-shift assay. The method of gel mobility-shift assay has been described by Fried and Crothers (9). Binding reactions were carried out in 10 μl of buffer B (25 mM HEPES, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, 50 mM KCl) with 9 fmol of ^{32}P-labeled DNA fragment, 0.5 μg of poly(dI-dC), 0.5 μg of poly(dA-dT), and various amounts of protein extract. The mixture was incubated at 25°C for 15 min and analyzed on a 5% polyacrylamide gel (30:0.8, acrylamide-bisacrylamide) in Tris-glycine buffer (40 mM Tris, 0.4 M glycine, pH 8.5).

DNase I footprinting. The technique of DNase I footprinting has been described by Galas and Schmitz (10). Reaction mixtures were prepared as described for the gel mobility-shift assay but in a total volume of 50 μl. After 10 min of incubation at 25°C, 2 μl of a freshly prepared solution of DNase I (100 μg/ml) was added to each reaction. The DNase I digestion was initiated by the addition of $MgCl_2$ to 5 mM and was stopped after 120 s by the addition of 10 μl of stop mix (250 mM EDTA, 1.5 M NaCl, and 1.5 mg of oyster glycogen per ml). Reaction mixtures were extracted with phenol-chloroform (1:1) and ethanol precipitated, and the DNA pellet was dissolved in 3 μl of formamide-dye mix and analyzed on a 7% polyacrylamide-urea gel (25). To localize the footprint, the DNA fragment was sequenced as described by Maxam and Gilbert (G+A reaction) (25). The sequence reaction was loaded adjacent to the footprint reactions on the gel.

RESULTS

Sequence analysis of the *C. freundii* OS60 *ampR* gene. The DNA region between *frdD* and *ampC* in *C. freundii* OS60 (Fig. 1) encodes a 31.5-kilodalton *trans*-acting regulatory protein designated AmpR (21). The nucleotide sequence of this region was determined using the dideoxy chain-termination method (Fig. 2). The genetically determined position of *ampR* encompasses one large open reading frame beginning at position 97 and encodes a polypeptide with a calculated molecular weight of 32,537 (Fig. 2). A ribosome-binding site precedes the open reading frame.

The deduced amino acid sequence of AmpR from *C. freundii* OS60 shows extensive homology to the corresponding sequence determined from the *ampR* gene of *E. cloacae* MHN1 (13). These two proteins differ at only 55 amino acid positions. The intercistronic region between *ampR* and *ampC* is also very conserved between these two species (Fig. 3). We propose that the locations of the *ampR* and *ampC* promoters in *C. freundii* are the same as has been experimentally determined for *E. cloacae* (13).

The amino acid sequence of *C. freundii* AmpR was compared with other sequences in the National Biomedical Research Foundation protein sequence data bank by using the FASTP program described by Lipman and Pearson (22). Significant homologies were found with both LysR and NodD (Fig. 4), two proteins known to act as transcriptional regulators (29, 34, 37, 39).

AmpR affects *ampC* transcription. To monitor the effect of AmpR on *ampC* transcription, the latter gene was transcrip-

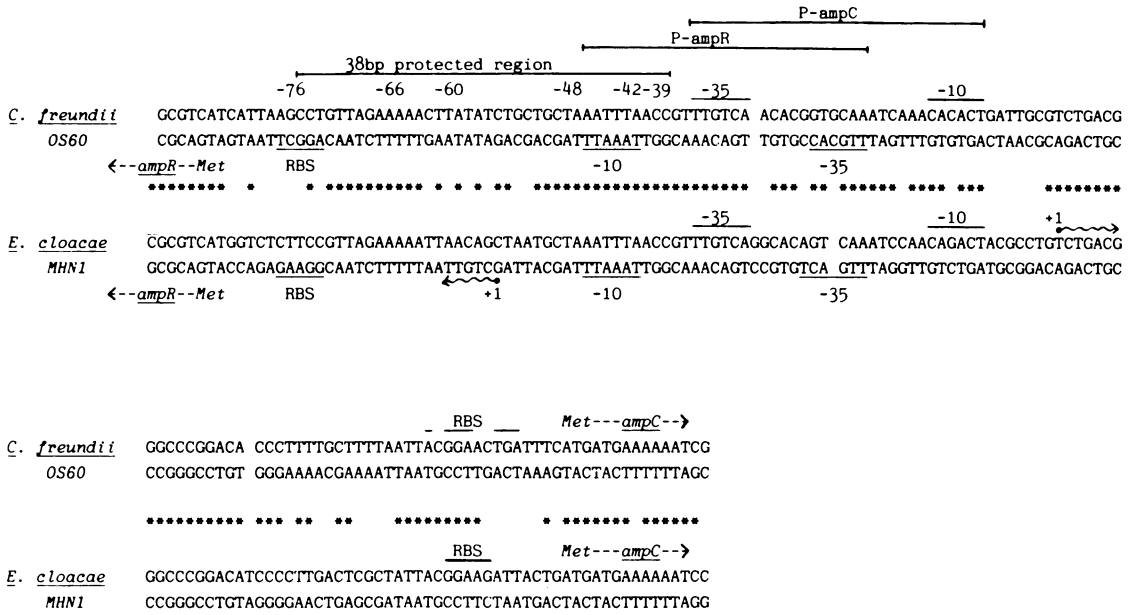

FIG. 3. Nucleotide sequence of the *C. freundii* OS60 and *E. cloacae* MHN1 *ampR-ampC* intercistronic region. The transcriptional start points (+1) for the *E. cloacae* MHN1 *ampR* and *ampC* genes are shown. Based on sequence homology, it is likely that *C. freundii* OS60 initiates transcription at the same sites. The translational (Met) start points are shown. Also indicated are the proposed ribosomal binding sites (RBS), the promoters (P-*ampR* and P-*ampC*) with their respective −10 and −35 regions, and the 38-bp region which is protected from DNase I cleavage by an AmpR-containing extract. An asterisk (*) marks nucleotides that are identical in *C. freundii* and *E. cloacae*. The data from *E. cloacae* are from Honoré et al. (13).

tionally fused to *lacZ* to form plasmid pNU330. This plasmid contains the 5′ end of *ampC* and the intercistronic region between *ampC* and *ampR* but lacks the *ampR* gene (Fig. 1). Addition of the inducer 6-aminopenicillanic acid (6-APA; 2 g/liter) to MC1029(pNU330) had no effect on the β-galactosidase activity. However, when *ampR* was introduced separately on plasmid pNU311, β-galactosidase expression was inducible (Fig. 5). Thus, *ampR* encodes a *trans*-acting regulator which activates *ampC* transcription in the presence of an inducer.

To further investigate this point, RNA was prepared from *E. coli* SN03(pNU305) before and 10, 30, and 50 min after the addition of inducer (6-APA at 2 g/liter). Northern (RNA) blot analysis detected a 1,300-nucleotide-long transcript when an internal *ampC* fragment (*ClaI*$_1$-*PstI*$_2$) was used as a probe. There was a marked increase in the abundance of this transcript after the addition of inducer (Fig. 6). These RNA preparations were also analyzed with an internal *ampR* probe (*BamHI*$_1$-*PvuII*) (data not shown); however, no specific transcript was detected, suggesting that *ampR* is poorly transcribed.

Effect of AmpR on *ampR* transcription. An *ampR-lacZ* transcriptional fusion was constructed to monitor the effect of AmpR on *ampR* transcription. This plasmid, pNU372, has the *Bam*HI-*Cla*I fragment of pNU305 carrying the 5′ half of *ampR* and the *ampR-ampC* intercistronic region inserted into pRZ5202. The β-galactosidase activity of *E. coli* MC1029(pNU372) was quite low but significantly higher than that of the vector control (Table 1). Plasmids pNU311 (*ampR*$^+$) and pNU312 (*ampR*) (Fig. 1) were transformed into *E. coli* MC1029(pNU372). Expression of AmpR from pNU311 (*ampR*$^+$) resulted in a threefold decrease in expression of β-galactosidase (14.7 to 5.1 U) from the coresident plasmid pNU372. No effect on β-galactosidase was observed when pNU372 (*ampR-lacZ*) and pNU312 (*ampR*) were present in the same strain. Thus, *ampR* appears to be autoregulated since AmpR has a repressor function on its own transcription.

We have previously observed that the level of AmpR (as determined in [^{35}S]methionine-labeled minicells) is not affected by the addition of inducer (21), suggesting that the β-lactam induction of *ampC* is not due to an increased production of AmpR. Accordingly, the expression of β-galactosidase was not affected by the addition of 6-APA (2 g/liter) to MC1029(pNU372), MC1029(pNU372, pNU311), or MC1029(pNU372, pNU312) (Table 1).

Binding of AmpR to DNA in the *ampR-ampC* intercistronic region. Gel mobility-shift assays were used to examine the binding of the AmpR protein to the *ampR-ampC* intercistronic region. Cellular extracts were prepared from SN03(pNU311) expressing the AmpR protein, from the *ampD2* mutant SN0302 harboring the same plasmid, from SN03(pNU311) grown in the presence of inducer (6-APA at 2 g/liter) for 40 min, and from SN03 harboring the vector pACYC184 as a negative control. Plasmid pNU316 (Fig. 1) was subjected to double digestion with either *Apa*I-*Bam*HI or *Apa*I-*Xho*I. These digests were subsequently end labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I (24). The 496-bp large *Bam*HI-*Apa*I fragment was retarded after incubation with extracts from AmpR-expressing cells, but not after incubation with control preparations. The 53-bp *Apa*I-*Xho*I fragment was not retarded by the same cellular extracts (data not shown).

To define the binding region more precisely, the 179-bp *Eco*RI-*Sac*I fragment from plasmid pNU371 (encompassing the intercistronic region between *ampR* and *ampC*) was used in binding assays with the same cellular extracts described above. This 179-bp fragment was retarded when mixed with extracts from AmpR-expressing cells but not with extracts from cells lacking *ampR*. No significant difference in mobility shift was detected between extracts prepared from the wild type or the *ampD2* mutant. Furthermore, the presence

FIG. 4. Alignment of the deduced amino acid sequences of AmpR of *C. freundii*, LysR from *E. coli* (38), and NodD from *R. meliloti* (7). The amino acids are given according to the one-letter code. Gaps have been inserted into the sequences to improve alignment. Identical residues or conservative replacements of amino acids are boxed. Comparisons of AmpR from *C. freundii* and *E. cloacae* (13) and the NodD genes from *R. meliloti* and *R. leguminosarum* (37) are also presented. Asterisks indicate identical amino acids. The broken line indicates the DNA-binding region for NodD (*R. leguminosarum*) as suggested by Shearman and co-workers (37).

of the inducer did not affect fragment retardation of the wild-type strain (Fig. 7). These data indicate that AmpR is a DNA-binding protein interacting with the *ampR-ampC* intercistronic region.

Localization of the AmpR-binding site. To define the site of AmpR binding in the *ampRC* control region, DNase I footprinting was performed on the 179-bp *Eco*RI-*Sac*I fragment from plasmid pNU371 (Fig. 1). Protein extract from SN03(pNU311) (*ampR*$^+$) protected DNA from positions −39 through −76 (Fig. 3 and Fig. 8, lanes C). No protection was observed with a protein extract from the control strain SN03(pACYC184) (Fig. 8, lanes A). The addition of inducer (6-APA at 2 g/liter) 40 min before preparation of the extract did not affect protection of this region (Fig. 8, lanes D). Cellular extracts prepared in the absence of inducer from the *ampD2* mutant SN0302(pNU311) (*ampR*$^+$) gave results identical to those obtained with the wild type carrying the same plasmid (Fig. 8, lanes B). In all cases an increase in the amount of cellular extract from 0.1 to 3.8 μg did not affect the binding pattern, suggesting that the intercistronic region between *ampR* and *ampC* contains only one binding site for AmpR.

The protected 38-bp region is positioned immediately upstream of the *ampC* promoter and does not contain any perfect direct or inverted repeats longer than 5 bp. However, positions −42 to −48 and −60 to −66 show a 6-of-7-bp homology. The first sequence is part of an 8-bp palindromic sequence (5′-TAAATTTA-3′).

DISCUSSION

The *ampR* gene and the *ampRC* control region are the only regulatory factors unique to gram-negative enterobacteria with inducible β-lactamase production as opposed to those with constitutive expression of the enzyme (21). Here we have demonstrated that regulation of β-lactamase operates at a transcriptional level and have defined the site to which AmpR binds in the *ampRC* control region. The AmpR proteins from *C. freundii* and *E. cloacae* MHN1 are identical at 236 of 291 (81%) amino acid positions. The region protected by AmpR in *C. freundii* has a 30-of-38-base homology to the corresponding sequence of *E. cloacae*. Although the *ampR* genes from these two species are functionally interchangeable, there are marked quantitative differences in the induction levels achieved in the various complementation pairs (20). Thus, the sequence differences between these two species in AmpR and in their respective binding sites must, at least partially, be complementary.

Honoré et al. (13) have determined the 5′ ends of each of the *ampR* and *ampC* transcripts from *E. cloacae*, which has led to the identification of the putative promoter regions. Due to the extensive homology between the *ampR* genes of *E. cloacae* and *C. freundii*, these observations can be extrapolated to *C. freundii* (Fig. 3). The AmpR-binding site covers the proposed *ampR* promoter and is located immediately upstream of the inferred *ampC* promoter. Thus, both

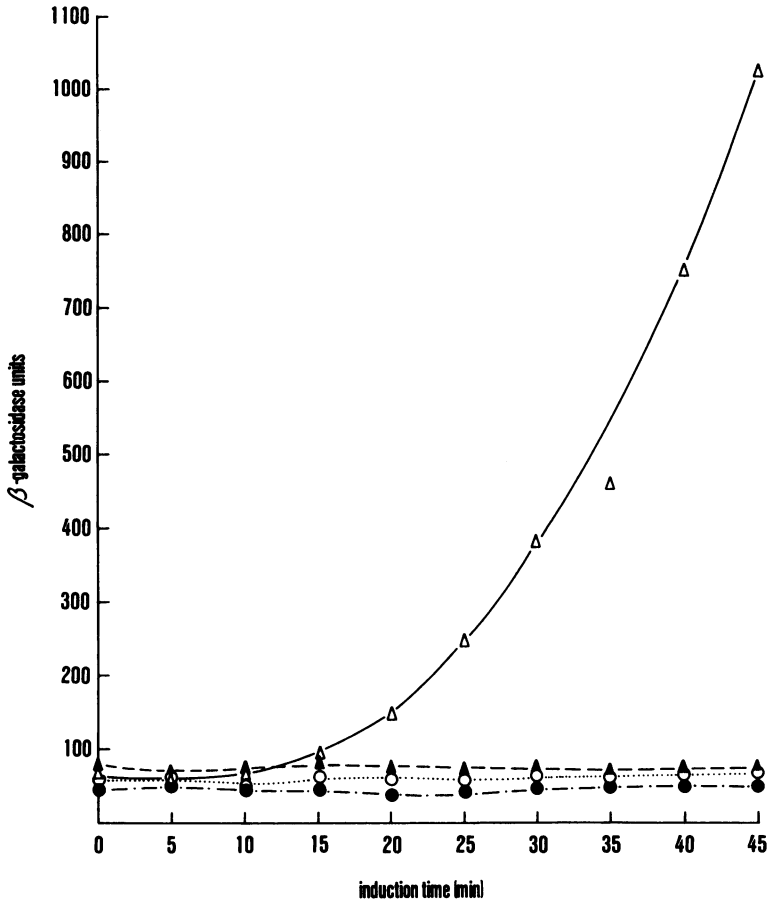

FIG. 5. Induction of β-galactosidase expression from the *ampC-lacZ* fusion plasmid pNU330 in *E. coli* MC1029. The inducer (6-APA at 2 g/liter) was added, and samples were then taken at 5-min intervals and assayed for β-galactosidase activity (28). Symbols: ○, MC1029(pNU330) (induced; ●, MC1029(pNU330) (noninduced); △, MC1029(pNU330, pNU311) (induced); ▲, MC1029(pNU330, pNU311) (noninduced).

negative autoregulation and stimulation of *ampC* transcription can occur by AmpR binding to this site.

A search through the National Biomedical Research Foundation protein data base has revealed striking homologies between AmpR and the LysR and NodD proteins. The three proteins could be aligned over their entire lengths (Fig. 4).

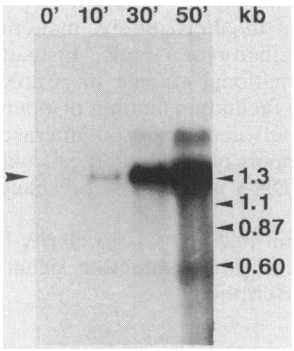

FIG. 6. Northern (RNA) blot analysis of the transcription of the *C. freundii ampC* gene. Total RNA was extracted from *E. coli* SN03(pNU305) before and 10, 30, and 50 min after induction with 6-APA (2 g/liter). The RNA sample was hybridized to an internal *ampC* probe (see text). The arrow to the left indicates the *ampC* transcript; arrows to the right indicate molecular weight standards.

LysR is a positive activator of the *lysA* gene in *E. coli*, whereas NodD transcriptionally regulates several nodulation genes in *Rhizobium* spp. We have shown by the β-galactosidase transcriptional fusion experiments that *ampR*, like *lysR* and *nodD* (34, 38), is autoregulated. A number of regulators have recently been shown to belong to the LysR family of activators (5, 11).

It has not been demonstrated that LysR is a DNA-binding protein. However, cell extracts containing NodD bind to DNA positioned upstream of the inducible nodulation genes. It is likely that NodD binds to the conserved "*nod* box" sequence (8).

TABLE 1. Effect of AmpR expressed in *trans* on *ampR-lacZ* transcriptional fusions

Plasmids (genotype)	β-Galactosidase sp act (U)	
	Noninduced	Induced[a]
pNU372 Φ(*ampR-lacZ*)	14.7	14.0
pNU372 Φ(*ampR-lacZ*) + pNU311 (*ampR*$^+$)	5.1	4.4
pNU372 Φ(*ampR-lacZ*) + pNU312 (*ampR*)	12.8	14.7
pRZ5202 (vector control)	4.6	4.0

[a] Inducer (6-APA at 2 g/liter) was added 40 min before samples were withdrawn.

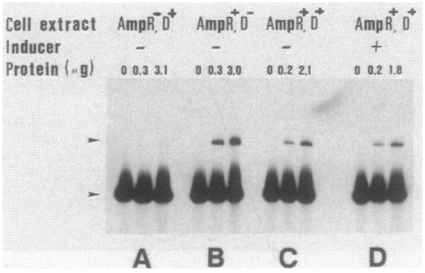

FIG. 7. Gel mobility shift. A ^{32}P-labeled, 179-bp *Eco*RI-*Sac*I fragment from plasmid pNU371, carrying the *ampR-ampC* intercistronic region, was mixed with various amounts of protein extract prepared from (A) *E. coli* SN03 (*ampD*$^+$) harboring plasmid pACYC184 (vector control); (B) *E. coli* SN0302 (*ampD2*) harboring plasmid pNU311 (*ampR*$^+$); (C) *E. coli* SN03 (*ampD*$^+$) with pNU311 (*ampR*$^+$); and (D) SN03 (*ampD*$^+$) with pNU311 (*ampR*$^+$) after induction with 6-APA (2 g/liter) for 40 min. Upper arrow marks a mobility shift which was observed when extracts containing the AmpR protein were used.

On the basis of comparisons with the proposed DNA-binding region of AraC and other DNA-binding proteins, it has been suggested that the region from residues 36 to 49 in the NodD protein of *Rhizobium leguminosarum* constitutes a DNA-binding domain (37). The program PCOMPARE with the Dayhoff MDM-78 matrix (6) identified the regions from residues 23 to 42 in AmpR, 21 to 40 in LysR, and 26 to 45 in NodD from *Rhizobium meliloti* to have the highest probability of constituting DNA-binding domains. These regions all overlap the proposed DNA-binding domain of NodD (*R. leguminosarum*). Interestingly, the two AmpR proteins from *E. cloacae* and *C. freundii* differ from each other at only two positions in the proposed DNA-binding region.

All penicillin-binding proteins and serine β-lactamases contain the sequence Ser-X-X-Lys, in which the serine is known to be acylated by the β-lactam (16). AmpR from *C. freundii* and *E. cloacae* does not contain this or any other sequences conserved among β-lactam-binding proteins. Furthermore, β-lactam antibiotics do not appear to be able to penetrate the cytoplasmic membrane, since cytoplasmically located β-lactamase does not provide protection against β-lactams in *E. coli* (3). Hence, we believe that the conversion of AmpR to an activator is not by β-lactam acylation.

Unlike *C. freundii* and *E. cloacae*, *Bacillus licheniformis* encodes a penicillinase whose induction requires the expression of at least two regulatory genes, *blaI/penI* and *blaR1/penJ*, present in the same operon. In this species, *blaI/penI* encodes a 15-kilodalton repressor protein, while the gene product of *blaR1/penJ* behaves as an antirepressor (12, 15, 18). The 68-kilodalton BlaR1/PenJ protein contains the Ser-X-X-Lys as well as several hydrophobic regions and has been suggested to be a transmembrane protein capable of binding the β-lactam. The mechanism by which the signal from the sensor is transmitted to the repressor remains unknown. Neither the BlaI/PenI or BlaR1/PenJ protein has any significant sequence similarity to AmpR.

In the AmpD/AmpR system of *C. freundii*, the sensor protein is unknown. An IS*1* insertion in *ampD* (*ampD2*) leads to semiconstitutive overproduction of β-lactamase, whereas inactivation of *blaR1/penI* results in low-level constitutive penicillinase expression. This latter response would be expected to arise from inactivation of a gene encoding a sensor protein. One possibility is that the *ampD2* mutation affects the level of a β-lactam sensor or affects the signal transmission from a sensor to the AmpR regulator.

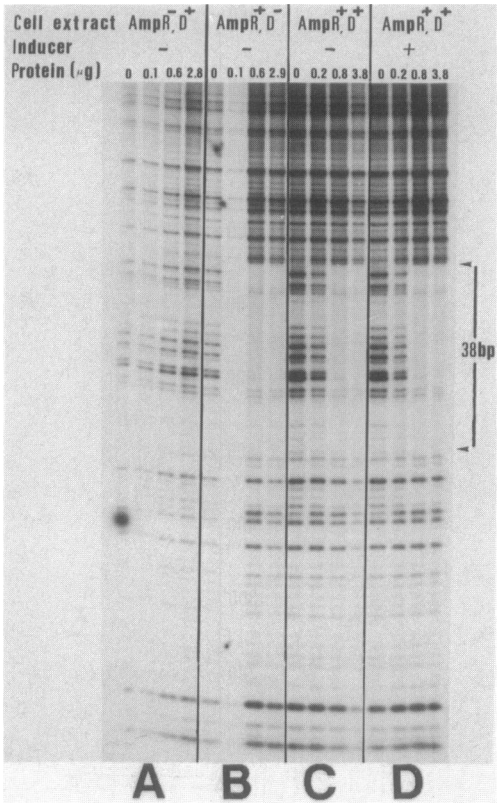

FIG. 8. DNase I footprint analysis of the *C. freundii ampR-ampC* intercistronic region. Protein extracts were analyzed for their ability to protect a 179-bp *Eco*RI-*Sac*I fragment from plasmid pNU371 carrying the *ampR-ampC* intercistronic region from DNase I cleavage. Various amounts of protein extracts were prepared from (A) *E. coli* SN03 (*ampD*$^+$) with plasmid pACYC184 (vector control); (B) *E. coli* SN0302 (*ampD2*) with pNU311 (*ampR*$^+$); (C) *E. coli* SN03 (*ampD*$^+$) with pNU311 (*ampR*$^+$); and (D) *E. coli* SN03 (*ampD*$^+$) with pNU311 (*ampR*$^+$) after induction with 6-APA (2 g/liter) for 40 min. AmpR-containing extracts protected a large 38-bp region (indicated at the right).

DNase footprinting revealed that there were no differences in AmpR binding when cellular extracts were prepared from noninduced or induced cells or from an *ampD2* mutant. This result may have been due to the loss of low-molecular-weight effector molecules during the preparation of the cellular extracts, or, alternatively, induction may not alter the binding specificity of AmpR. Instead, induction may evoke a conformational change in AmpR to an activator state which then facilitates binding or open-complex formation, or both, between RNA polymerase and the *ampC* promoter. In support of this hypothesis was the finding that NodD binds to DNA in both the presence and absence of inducer (8).

The most urgent need now is to clarify how, and in what form, AmpR receives the induction signal and how it activates *ampC* transcription.

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