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Molecular Basis of β-Lactamase Induction in Bacteria

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Inducible synthesis of β-lactamases was first described for the gram-positive organism Bacillus licheniformis (6) and was followed by similar reports for Bacillus cereus (1) and Staphylococcus aureus (7). Unlike the β-lactamases of gram-positive bacteria, the majority of β-lactamases of gram-negative bacteria are expressed constitutively (9). However, in 1967, Hennessey (10) described an inducible β-lactamase of Enterobacter cloacae that is now known to be encoded by the chromosomal ampC gene (23, 31). Inducible expression of ampC-like genes has subsequently been reported for Citrobacter freundii, Pseudomonas aeruginosa, and various other gram-negative bacteria (2, 15, 23, 31, 36, 40).

Staphyloccocal β-lactamases are predominantly plasmid encoded, extracellular enzymes, most of which are inducible (29). Early studies by Richmond (35) led to the proposal that inducible β-lactamase synthesis in S. aureus is under the negative control of a diffusible repressor (blaI or PenI) that bound to the operator site of the β-lactamase structural gene (blaZ or penP), thereby preventing its transcription. Broadly similar conclusions were made by Sherratt and Collins (38) regarding the inducible expression of the chromosomally encoded β-lactamase of B. licheniformis. In addition to the blaI gene, two further regulatory loci, designated blaR1 and blaR2, have now been identified (38).

A class of DNA-binding regulatory proteins (AmpR) that control the inducible expression of ampC genes has been identified for C. freundii, E. cloacae, and P. aeruginosa. In contrast to B1a1, which is a transcriptional inhibitor (repressor), AmpR is a transcriptional activator in the presence of certain β-lactam antibiotics in the culture medium and a repressor in their absence (23, 31). In addition, four other proteins have been implicated in ampC expression (see below).

Various models have been advanced to explain how the presence of a β-lactam antibiotic may lead to increased transcription of genes encoding β-lactamases (23, 31). Although β-lactams can cross some membrane bilayers (17), there is no evidence that uptake is a requirement for β-lactamase induction (8). Indeed, in gram-positive bacteria, it appears that the cell registers the presence of a β-lactam molecule at the external face of the cytoplasmic membrane and that this information is transmitted to the intracellular regulatory components that control the expression of β-lactamases. In contrast, a different mechanism may exist in gram-negative bacteria: perturbation of peptidoglycan synthesis by the β-lactam antibiotic may lead to the accumulation of cell wall precursors, within either the periplasm or the cytoplasm, thereby producing a signal for enzyme induction. This theory is discussed below.

CONTROL OF β-LACTAMASE GENE EXPRESSION IN B. LICHENIFORMIS AND S. AUREUS

As mentioned above, genetic analysis conducted in the 1970s established that B. licheniformis contains three regulatory genes (now designated blaI, blaR1, and blaR2) that influence the expression of chromosomal β-lactamase gene blaP (38). Genes blaI and blaR1, together with blaP, form a cluster (Fig. 1) that has been cloned and sequenced (12, 16, 18, 21). The position of blaR2 has not been determined, although this gene is known to be unlinked to the blaR1-blaI-blaP cluster (38).

Earlier genetic data indicated that blaI has a negative control function (38), and there is now little doubt that BlaI is a protein that negatively regulates the transcription of blaP, since it has now been established that BlaI is a typical repressor that binds to operator sites between blaI and blaP to repress the transcription of both genes (44). Mutations in blaR1 or blaR2 lead to the defective induction of blaP and therefore appear to be involved in the chain of events leading to the induction of the enzyme at a stage before the interaction of BlaI with blaP (38), but whereas the loss of BlaR1 function results in noninducible basal-level expression of blaP, the loss of BlaR2 function confers high-level constitutive expression, similar to that conferred by null mutations in blaI.

BlaR1 contains 601 amino acid residues and appears to be a membrane-spanning, β-lactam-interactive protein (16, 18). An early model suggested that the protein has five potential transmembrane domains located in the first 60% of the protein and a C-terminal β-lactam receptor domain exposed on the surface of the cytoplasmic membrane (18). More recently, the model has been revised to highlight the structural similarity between BlaR1 and the gram-negative chemotactic transducers Tsr and Tar (16) (Fig. 2). These membrane proteins are responsible for the transmission of chemical signals (via protein methylation) to the interior of the cell in response to the presence, respectively, of serine and aspartate in the environment (16) (Fig. 2). Alignment of amino acid sequences demonstrates that the polypeptide segment between residues 115 and 143 of BlaR1 is similar, in primary structure, to transmembrane segment B of Tsr and Tar. The BlaR1 polypeptide segment between residues 5 and 21 is similar to transmembrane segment A of the Escherichia coli Tar and Tap transducers (the latter is involved in signalling the presence of dipeptides) (16) (Fig. 2). Joris and co-workers (16) have therefore predicted that the 325-amino-acid amino-terminal region of BlaR1 has the same membrane

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topology and function as those of the chemotactic transducers Tsr, Tar, and Tap (Fig. 2). In agreement with this view, site-specific mutagenesis of Gly-124 to Asp-124 in BlaR1 (affecting transmembrane segment B) renders the system noninducible by β-lactams (16).

Homology between BlaR1 and various penicillin-interactive proteins has also been examined (16). The region surrounding the active-site serine of β-lactamases was shown to have close homology to the region around serine 402 of BlaR1. In particular, the motif Phe-X₃-Ser-X₂-Lys conserved in β-lactamases was also found in BlaR1 (Phe-398 to Lys-405). Furthermore, penicillin-interactive proteins of known tertiary structure contain upstream of the active-site serine a tripeptide (KGT) that forms part of the enzyme active site (16). A similar motif is evident in BlaR1 at residues 539 to 541 (Fig. 2). The functional involvement of this region in the induction of one β-lactamase has been demonstrated by site-specific mutagenesis. The conversion of Gly-538 to Asp desensitizes the system to β-lactams, since B. licheniformis containing mutant BlaR1 exhibits a noninducible β-lactamase phenotype (16).

The intracellular domain of BlaR1 has neither candidate sites for ATP binding nor methylation-demethylation sites, which are characteristic of chemotactic transducers (16).

**FIG. 2.** Membrane topology of the chemotactic transducers Tsr, Tar, and Tap involved with the recognition, respectively, of serine, carbohydrates, and dipeptides in enteric gram-negative bacteria and the product of B. licheniformis gene blaR1. The motif STYK in BlaR1 may be contained in the beta-lactam receptor domain, and this region of the protein probably interacts at the tertiary level with the region containing residues GKTG at the carboxy terminus of the protein. For further details, see the text. This figure is reproduced, with permission, from reference 16.
Nevertheless, BlaR1 appears to play an essential role in the first stage of the induction process by serving as a β-lactam receptor.

Although the following stages by which the stimulus is transmitted to BlaI are unknown, β-lactam challenge eventually results in the release of BlaI from the blaP operator and the induction of β-lactamase synthesis. The product of the unregulated and blaR1 gene may be an intermediate in the process of signal transduction between BlaR1 and BlaI (but see below).

The nucleotide sequence of staphylococcal transposon Tn552, which encodes an inducible β-lactamase, has been determined (37). Loci corresponding to B. licheniformis genes blaI and blaR1 and encoding proteins homologous to B. licheniformis proteins BlaI and BlaR1 were identified. Although a gene corresponding to blaR2 was not detected within Tn552, the staphylococcal counterpart has been identified as a chromosomal gene (7). Therefore, the regulatory basis of β-lactamase induction in S. aureus is similar to that in B. licheniformis. Nevertheless, the organizations of blaI and blaR1 with respect to the β-lactamase structural gene are unique in the staphylococcal and B. licheniformis systems (Fig. 1). More recently, Wang et al. (43) showed that the organization of blaI and blaR1 in S. aureus penicillinase plasmid p1285 is identical to that in Tn552.

Given the known characteristics of BlaR1, it would seem unlikely that the induction of β-lactamase in B. licheniformis or S. aureus depends on the accumulation of cell wall precursors, the entry of free antibiotic into the cytoplasm, or the interaction of a β-lactam with the classical membrane-bound penicillin-binding proteins (PBPs) involved in the final stages of peptidoglycan biosynthesis (39). Rather, a highly specialized and specific mechanism for induction has evolved, comprising a signal pathway from BlaR1 to membrane to BlaI in the cytoplasm, and this pathway involves at least one other component, the product of blaR2.

CONTROL OF β-LACTAMASE GENE EXPRESSION IN C. FREUNDII, E. CLOACEAE, AND E. COLI

The inducible β-lactamase genes of gram-negative bacteria are exclusively chromosomal genes. The structural genes, collectively termed ampC, appear to belong to an extended, phylogenetically related family (5, 24), some members of which are no longer inducible, e.g., the ampC genes of E. coli and Shigella flexneri.

The ampC genes of both C. freundii and E. cloacae have been cloned in E. coli, in which each retains its inducible characteristic (13, 25, 26). In both cases, induction depends on a second, adjacent gene, designated ampR. Both genes are transcribed divergently from the common intracistronic region. Deletion mutations of ampR generate a noninducible phenotype, with ampC being expressed at a level two- to threefold higher than the normal, uninduced basal level, indicating that AmpR acts both as a repressor, under noninducing conditions, and as an activator, during induction (28). The ampR genes of C. freundii and E. cloacae have been sequenced (13, 28), and the predicted amino acid sequences indicate that the proteins are highly related and belong to the family of transcriptional regulators typified by LysR (11). These regulators act primarily as transcriptional activators, and several are activated by a diffusible ligand (11). However, it is unlikely that these proteins interact directly with β-lactams, since it has been demonstrated that an intracellular β-lactamase fails to prevent the induction of ampC (8), while the same enzyme located in the periplasm completely blocks induction. This result is consistent with a β-lactam triggering induction by interacting with a periplasmic component. Footprinting analysis has revealed that AmpR binds to the intercistronic region between ampR and ampC, as expected for an activator of ampC (28).

As stated above, the cloned ampC genes from C. freundii and E. cloacae are inducible in E. coli, provided that they are accompanied by the functional, specific ampR gene. Mutants of these hybrid systems that are derepressed for expression of the heterospecific ampC gene can then be isolated (22). The mutations map to a locus designated ampD and located at 2.5 min on the E. coli chromosome (22). The ampD gene has been cloned and sequenced (14, 27). The predicted protein contains 183 amino acids and has a hydrophathy profile indicative of a soluble protein, consistent with the reported location of AmpD in the cytoplasm. Further analysis has revealed a weak helix-turn-helix motif in the middle of the protein, indicative of a DNA-binding protein. However, if AmpD is a DNA-binding protein, it does not influence the expression of ampC directly, because DNA protection studies have failed to show binding to the regulatory region upstream of ampC (31).

Extended sequence analysis has revealed a second gene, ampE, downstream from and coordinately expressed with ampD; the start codon of this gene overlaps the last four nucleotides of ampD (including the terminator codon). The predicted protein of 284 amino acids possesses the Ser-X2-Lys motif common to all PBPs and has the hydrophathy profile of a membrane protein with four membrane-spanning domains. Minicell experiments involving cell fractionation have confirmed that AmpE is a membrane protein. It also has two motifs that resemble ATP-binding domains and are predicted to be on the inside surface of the cytoplasmic membrane between the second and third membrane-spanning domains (14, 27). It has been suggested that AmpE functions as a β-lactam sensor to trigger the induction of β-lactamase, analogous to the proposed role for the BlaR1 proteins of B. licheniformis and S. aureus, but more recent results are not fully consistent with this suggestion. Furthermore, AmpE does not bind benzylpenicillin (27), consistent with the absence of the Lys-Thr-Gly motif upstream from the active-site Ser. This tripeptide motif has been found on all but one PBP, including the BlaR1 elements from B. licheniformis and S. aureus (18, 37).

The suggestion that AmpE acts as a sensor for the system stems from findings that mutations that damage or delete the carboxyl terminus of the protein result in a noninducible or poorly inducible phenotype (14). However, it has been shown that induction can occur normally in the absence of AmpE (31). Hence, if AmpE is a sensor for the system, it cannot be the only or indeed the main one, since it is dispensable.

Mutation of ampD results in two forms of β-lactamase expression. Null mutations cause derepression, while other mutations generate a hyperinducible phenotype, whereby lower levels of inducer are required to promote ampC expression and the maximum induced level of β-lactamase is increased three- to fourfold (20, 22, 27). In addition, certain β-lactams that fail to induce ampC in the parental strain do induce ampC in these mutants (42). One such mutant, SNO301, is something of an enigma. Sequence analysis has shown that the mutant allele is a 52-bp deletion that starts after codon 23 (27). Such a deletion would also be expected to generate a frameshift, so that the product of the mutant gene would be homologous to AmpD only over the first 23 amino acids. Furthermore, the switch in reading frames...
would bring into frame a premature termination codon, resulting in a peptide of only 59 amino acids. It seems very unlikely that such a product could substitute for whatever function AmpD fulfills. Nonetheless, despite the damage to ampD in SN0301, given the phenotype, it must be able to produce a functional AmpD derivative. One possible explanation is that translation of the mutant mRNA is subject to a degree of ribosome frameshifting (31), presumably in the vicinity of the deletion, to restore the reading frame after the deletion and so produce an altered but partly functional form of AmpD. This explanation, although having no reported experimental basis, cannot be discounted. However, an analysis of the mutant sequence suggests another explanation. Eighteen nucleotides into the ampD mRNA sequence, there is a triplet, GUG, which could be an alternative translational start codon. Initiation of translation at this point would result in the normal ampD reading frame being restored immediately after the 52-bp deletion. Interestingly, the change in reading frame would also move the apparent deletion three codons towards the N terminus, so that in the putative mutant protein, the first 37 amino acids of AmpD would be replaced by a 14-amino-acid sequence encoded by an alternative reading frame. Such a mutation, which would leave 80% of the protein intact, could conceivably alter the activity of the protein without destroying the protein completely.

Interestingly, it has been reported that when the mutant ampD allele from SN0301 is used to complement the ampD null mutation, which is also polar with respect to the expression of ampE carried by E. coli SN0302, ampC expression remains derepressed, rather than becoming hyperinducible (27). This result is an unexpected one were AmpD to act independently, as indicated by other results. However, two pieces of information point to AmpD and AmpE being normally interactive. First, as stated above, mutations that remove the normal carboxy terminus of AmpE create noninducible phenotypes; these phenotypes are dominant over the normal inducible phenotype conferred by AmpD (14). Second, deletion of ampE results in a raised basal level of ampC expression, without a major effect on induction (31). These observations indicate that in E. coli, AmpE enhances the repressive effect of AmpD. Although AmpD can function independently of AmpE with respect to ampC expression, it may normally interact physically with AmpE, causing a modification of its activity. The mutant AmpD protein produced by strain SN0301 may retain this ability to interact with AmpE. Such an interaction may serve partially to suppress the ampD mutation in SN0301 and to stabilize the mutant AmpD protein. Only in the absence of AmpE would the conditional null mutation be fully expressed.

A β-lactam-sensitive, noninducible mutation was isolated from an ampD mutant of E. cloacae that hyperproduces β-lactamase (19). The mutation was not complemented by a wild-type ampR gene but was complemented to β-lactamase hyperproduction by a novel cloned DNA fragment from E. cloacae. The mutation defined a new locus, ampG, that is necessary for both induced and derepressed high-level expression of ampC. Similar mutations have been isolated from strains of E. coli showing high-level constitutive expression of β-lactamase and are complemented by the cloned E. cloacae ampG gene (31).

Since the phenotype of ampG mutants is similar to that of blaR1 mutants of B. licheniformis, AmpG may function as a signal transducer, permitting communication between the periplasmic and cytoplasmic cellular compartments. This function could be achieved in a manner similar to those of membrane transducers Tsr, Tap, and Tar (chemotaxis proteins) in gram-negative bacteria (Fig. 2). However, there is no evidence that covalent modification, let alone methylation of proteins is involved. Alternatively, AmpG may form part of a membrane transport system for the uptake of a small molecule, perhaps a peptidoglycan fragment released as the result of peptidoglycan breakdown triggered by β-lactam inhibition of PBP activities, so making it available to combine with AmpR to form the activator (31). In both models, the loss of AmpG would inhibit induction.

A recent report has described an ampR mutation that suppresses certain null, missense mutations in ampG (3). The simplest explanation for these findings is that AmpG and AmpR normally interact and that while the ampG mutations inhibit a productive interaction with wild-type AmpR, the ampR mutation compensates for and relieves the inhibition, thereby restoring ampC expression. From this explanation it can be postulated that AmpG would normally respond, directly or indirectly, to the presence of a β-lactam inducer by communicating with AmpR, causing it to convert from the repressor to the activator form.

As stated above, AmpR belongs to the LysR family of transcriptional regulators (11, 31) and, in general, these respond to small diffusible ligands. If AmpR functions in a similar manner, then a model in which a small molecule is the functional inducer, the so-called autoinducer of earlier models, is favored. However, in this scenario, it is difficult to explain how a mutation in ampR suppresses mutations in ampG, because AmpG is only expected to be responsible for capturing and transporting peptidoglycan fragments that then interact independently with AmpR. There would be no need for AmpG and AmpR to interact directly.

PBP2, encoded by pbpA, is another component implicated in ampC induction in E. coli because a temperature-sensitive pbpA mutation of E. coli is unable to induce β-lactamase at the nonpermissive temperature (32). In contrast, mutations in other PBP genes were found to have little or no effect on β-lactamase induction. These findings indicate that β-lactamase production depends on PBP2 remaining functional. Similar experiments with a temperature-sensitive allele of ftsZ have also implicated FtsZ in the induction of the ampC β-lactamase (34). FtsZ is a protein intimately involved in the cascade of reactions that mediates the septation-division process (4). FtsA and FtsQ are thought not to be involved in ampC β-lactamase induction (34). Interestingly, PBP2, in conjunction with RodA, is thought to act consecutively with FtsZ during cell growth. This idea suggests that PBP2 and FtsZ may also participate consecutively in the β-lactamase induction process. However, the mechanisms by which these proteins operate in the induction pathway are unknown.

In E. coli, PBP2 is the only PBP that has been implicated in β-lactamase induction (32), but the contributions of PBP7 and PBP8, if any, have not, to our knowledge, been examined. For both P. aeruginosa and E. cloacae, a role for PBP5 has been ascribed on the basis that the induced level of β-lactamase in both organisms is found to correlate with the degree of inducer binding to PBP5 but not to other PBPs (30). These findings cannot easily be extrapolated to E. coli, however, since a double-deletion mutant lacking the genes encoding PBP5 and PBP6 and carrying the ampC and ampR genes of C. freundii displays normal β-lactamase induction kinetics (33). These findings raise the possibility that in different genetic backgrounds, different PBPs are necessary for β-lactamase induction. In no case has the mechanistic
FIG. 3. Hypothetical model for control of the expression of inducible ampC genes in gram-negative bacteria. The inducer, a β-lactam (BL), interacts with the PBPs of the cell to disrupt the peptide-cross-linking step of peptidoglycan (PG) synthesis. The disruption is “sensed” by AmpG (G), possibly as an increased periplasmic concentration of a normal PG turnover product. This peptidoglycan fragment stimulates AmpG interaction with AmpR, promoting the conversion of the repressor (R) form of AmpR to the activator form (A), possibly by protein modification. In its activator form, AmpR stimulates the expression of ampC. Under steady-state, noninducing conditions, AmpD (D), possibly in association with AmpE (E), maintains AmpR in the repressor form by inhibiting or reversing the AmpG-mediated conversion step (AmpE interacts with AmpD to amplify the effect but is dispensable; see the text). When an inducer is added, the balance between the activities of AmpG and AmpD is shifted in favor of the former, so that a proportion of AmpR molecules is converted to the activator form and ampC is expressed. When the inducer is removed, the stimulation of AmpG declines and the balance between the AmpG and AmpD activities once more is shifted in favor of AmpD. The activator form of AmpR is reconverted to the repressor form, and the expression of ampC is largely curtailed. A loss of AmpD because of a mutation shifts the balance between AmpG and AmpD decisively in favor of the former, which then maintains AmpR permanently in the activator form. High-level constitutive expression, i.e., derepression, of ampC is the consequence. CM, cytoplasmic membrane. Arrows indicate a possible influence on a particular component, positive (+) or negative (−).

role of a PBP has been elucidated, although it has been suggested that DD-carboxypeptidase activity is necessary for ampC gene expression (31, 41).

What is known about β-lactamase induction in gram-negative bacteria has been assembled into a tentative model, in which AmpG is part of a membrane transport system for the uptake of a peptidoglycan fragment arising from wall degradation or turnover (31, 41). This fragment serves as the inducing ligand, which combines with AmpR in its repressor form to create the activator form. AmpD acts to divert the inducing ligand away from AmpR, perhaps as part of a normal, but nonessential, recycling process, given that ampD can be deleted with no obvious effect on cell growth. Only when AmpD activity is saturated does induction occur, and in the absence of AmpD, the inducing ligand, which is postulated to be a normal product of peptidoglycan turnover, is free to accumulate and promote high-level expression of ampC by maintaining the activator form of AmpR at a maximum level.

An alternative model (Fig. 3) is that AmpG is a membrane sensor that responds to peptidoglycan turnover and that can interact directly with AmpR and probably other control elements involved in normal cell wall metabolism to generate the transcriptional activator for ampC. Activation of AmpR may well be effected by some form of protein modification. This modification would occur maximally in ampD mutants. In the wild-type state, induction is inhibited by AmpD, which acts either to reverse AmpR modification or to inhibit it. In the normal system, induction occurs when AmpD activity is overcome by superior AmpG activity as the result of increased peptidoglycan breakdown brought about by β-lactam inhibition of PBP activities.

In both models (31; Fig. 3), there is a link between AmpG activity and AmpR activation, and AmpD acts to modulate the strength of the signal between the two components of the induction pathway. (In addition, in E. coli, AmpD activity may be enhanced by AmpE [Fig. 3].)

### GENERAL THOUGHTS REGARDING β-LACTAMASE INDUCTION

The basic scenario outlined above for β-lactamase induction in gram-negative bacteria may also describe induction in gram-positive bacteria (Table 1). For example, for B. licheniformis it is plausible that the β-lactam sensor, BlaR1, interacts directly with the repressor, BlaI, to relieve the repression of blaZ. As suggested for AmpR, protein modification may occur to inactivate the transcriptional regulator, BlaI. In such a system, BlaR2 would be analogous to AmpD (Table 1) and would function to antagonize BlaR1 activity. In the normal, uninduced state, BlaR2 activity would dominate and the expression of blaZ would be repressed. In this hypothetical system, when blaZ is induced, BlaR1 activity would be greatly stimulated and BlaR2 activity would be titrated out, resulting in the net inactivation of BlaI and the expression of blaZ.

Therefore, although the mechanisms for β-lactamase induction in gram-positive and gram-negative bacteria differ somewhat in biochemical details, the overall mechanisms operating in the two systems may be quite similar, involving membrane sensors that communicate with the transcriptional regulator and antagonists that interfere with this communication to prevent induction in the absence of a β-lactam inducer.

### REFERENCES


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**TABLE 1. Elements involved in the expression of inducible β-lactamases**

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<thead>
<tr>
<th>Gram positive</th>
<th>Gram negative</th>
<th>Role or description</th>
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<tbody>
<tr>
<td>BlaI</td>
<td>AmpR</td>
<td>Transcriptional regulation</td>
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<tr>
<td>BlaR1</td>
<td>AmpG</td>
<td>Necessary for induction; integral membrane protein responsible for transmission of induction signal</td>
</tr>
<tr>
<td>BlaR2</td>
<td>AmpD</td>
<td>Down-regulation of β-lactamase expression; maintenance of repressor form of transcriptional regulator?</td>
</tr>
<tr>
<td>BlaR1</td>
<td>AmpE</td>
<td>Enhancement of repression; dispensable β-Lactam-interactive protein</td>
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