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The fate of the BlaI repressor during the induction of the *Bacillus licheniformis* BlaP β-lactamase

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Summary

The induction of the *Staphylococcus aureus* BlaZ and *Bacillus licheniformis* 749/I BlaP β-lactamases by β-lactam antibiotics occurs according to similar processes. In both bacteria, the products of the *blaI* and *blaR1* genes share a high degree of sequence homology and act as repressors and penicillin-sensory transducers respectively. It has been shown in *S. aureus* that the BlaI repressor, which controls the expression of BlaZ negatively, is degraded after the addition of the inducer. In the present study, we followed the fate of BlaI during β-lactamase induction in *B. licheniformis* 749/I and in a recombinant *Bacillus subtilis* 168 strain harbouring the pDML995 plasmid, which carries the *B. licheniformis blaP*, *blaI* and *blaR1* genes. In contrast to the situation in *B. licheniformis* 749/I, β-lactamase induction in *B. subtilis* 168/pDML995 was not correlated with the proteolysis of BlaI. To exclude molecular variations undetectable by SDS–PAGE, two-dimensional gel electrophoresis was performed with cellular extracts from uninduced or induced *B. subtilis* 168/pDML995 cells. No variation in the BlaI isoelectric point was observed in induced cells, whereas the DNA-binding property was lost. Cross-linking experiments with dithiobis(succimidylpropionate) confirmed that, in uninduced recombinant *B. subtilis* cells, BlaI was present as a homodimer and that this situation was not altered in induced conditions. This latter result is incompatible with a mechanism of inactivation of BlaI by proteolysis and suggests that the inactivation of BlaI results from a non-covalent modification by a co-activator and that the subsequent proteolysis of BlaI might be a secondary phenomenon. In addition to the presence of this co-activator, our results show that the presence of penicillin stress is also required for full induction of β-lactamase biosynthesis.

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Introduction

The *Bacillus licheniformis* 749/I BlaP and *Staphylococcus aureus* BlaZ class A β-lactamases are inducible by β-lactam antibiotics (Joris *et al*., 1994; Philippon *et al*., 1998). In both strains, β-lactamase expression is under the control of two gene products, BlaI and BlaR1, which are a repressor and a penicillin-sensory transducer respectively (Kobayashi *et al*., 1987; Nicholls and Lampen, 1987; Rowland and Dyke, 1990) At the DNA level, *blaZ/blaP*, *blaR*1 and *blaI* are organized as a divergon (*bla* divergon) in which *blaR*1 and *blaI* form an operon. It has been shown that purified BlaI is present as a dimer in solution, that the DNA-binding domain is located near the N-terminal end and that the dimerization domain is in the C-terminal region (Wittman *et al*., 1993). In the absence of β-lactam, both β-lactamase synthesis and expression of the *blaI–blaR1* operon are maintained at a low level by the BlaI repressor (Salerno and Lampen, 1988; Clarke and Dyke, 2001). Binding of a β-lactam to BlaR1 causes derepression and a high level of β-lactamase synthesis (Joris *et al*., 1990; Zhu *et al*., 1992). The low-affinity penicillin-binding protein 2′ (MecA protein) of *S. aureus* is under the control of the MecI and MecR proteins, which are similar to the corresponding BlaI and BlaR1 β-lactamase regulators. The membrane topology of *B. licheniformis* BlaR1 has been determined and highlights the amino acid sequence signature of a neutral zinc metallopeptidase in the intracellular B4 loop (Hardt *et al*., 1997). This motif is conserved in MecR/BlaR1 and is essential for the induction process (Zhang *et al*., 2001; K. Benlafya and B. Joris, unpublished data). Earlier results obtained using classical genetic experiments suggested that, in both *S. aureus* and *B. licheniformis*, an additional *blaR*2 gene was involved in β-lactamase or MecA induction (Sherratt and Collins, 1973; Dyke, 1979). The transformation of *Bacillus subtilis* BD224, a strain devoid of *blaP*, *blaR*1 and *blaI*, by a *Bacillus* plasmid harbouring the *B. licheniformis bla* divergon gives rise to an inducible BlaP β-lactamase phenotype, indicating that all the genes needed for the induction are present in the recombinant *B. subtilis* strain (Kobayashi *et al*., 1987). Consequently, if BlaR2 is important for β-lactamase induction, an equivalent *blaR*2 gene must be present in the *B. subtilis*

genome. Moreover, the *Bacillus blaP* and *blaI–blaR*1 promoters are functional in a recombinant *Escherichia coli* strain, but the β-lactamase is not inducible in the presence of penicillin (Kobayashi *et al*., 1987).

Recently, Zhang *et al*. (2001) proposed a mechanism for the induction of β-lactam resistance in *Staphylococci*. According to these authors, the acylation of the penicillin receptor BlaR1 by the inducer generates a cleavage of the cytoplasmic loop B4, which would convert this putative metalloprotease to its active form. Next, the activated B4 loop would generate a secondary signal, which would result in the proteolysis of BlaI. The proteolytic cleavage of *S. aureus* BlaI takes place between the N101 and F102 residues, generates two fragments (an 11 kDa fragment containing the DNA-binding domain and a 3 kDa fragment corresponding to the dimerization domain) and inactivates BlaI, which no longer acts as a repressor. However, the induction process does not cleave BlaI completely, and it was suggested that a heterodimer containing the wild-type monomer and the free dimerization domain could be present in the cytoplasm of induced cells and that this heterodimer did not bind to DNA (Gregory *et al*., 1997; Lewis *et al*., 1999).

Two different *B. licheniformis* 749/C strains that constitutively produce β-lactamase have been described. In these two strains, sequence analysis revealed that the mutation responsible for this phenotype was located in *blaI*. In one strain, the mutation results in an early termination of BlaI translation (Grossman and Lampen, 1987) and, in the second, the mutated repressor differs from the wild-type BlaI by three mutations; S34 → P and M97 V98 → I L (Wittman and Wong, 1988). For the latter mutant, the two mutations have been separated, and the two derived mutants, BlaI S34 → P and BlaI M97 V98 → I L, have been tested for their ability to repress the expression of the β-galactosidase gene fused to the *blaP* promoter in *E. coli*. In this strain, the individual mutations are not sufficient to inactivate the repressor, and it was concluded that a combination of the mutations is necessary for the inactivation of the repressor. But surprisingly, in a recombinant *B. subtilis*, the BlaI M97 V98 → I L mutation (BlaI-GM2) resulted in magnoconstitutive β-lactamase expression (T. Kobayashi, unpublished). The difference between the two hosts remains unexplained.

In this study, we describe the expression of wild-type BlaI and the BlaI-GM2 mutant in *E. coli* and their purification. We used Western blot, two-dimensional electrophoresis, bandshift assays and intermolecular cross-linking to analyse the fate of *B. licheniformis* BlaI or BlaI-GM2 during BlaP β-lactamase induction in wild-type, *blaR*1$^-$ and *blaR*2$^-$ *B. licheniformis* strains or in a recombinant *B. subtilis* strain harbouring a plasmid carrying the *B. licheniformis bla* divergon. This work reveals that, in a recombinant *B. subtilis*, BlaI is not cleaved during the induction process. We provide experimental evidence that BlaI is always present as a homodimer in both uninduced and induced conditions. This result suggests that the inactivation of BlaI is mediated by a specific co-activator generated by the 'activated' BlaR1 and that BlaI proteolysis is a secondary phenomenon resulting from the activity of cytoplasmic proteases. Finally, the fate of BlaI-GM2 in the presence of wild type or inactivated BlaR1 receptor confirms this hypothesis and emphasizes that, in addition to the acylation of BlaR1, an intracellular factor generated by a penicillin stress is required for full derepression of β-lactamase biosynthesis.

Results

Production and purification of B. licheniformis *BlaI*

The purified BlaI-WT and BlaI-GM2 repressors were analysed by gel retardation using a fluorescent oligo and an ALFexpress sequencer. The results indicate that BlaI-GM2 retains its ability to bind the *blaP* operator but that its affinity for its DNA target is reduced compared with BlaI-WT. Indeed, in the same experimental conditions, the BlaI-GM2 concentration necessary to obtain the same bound:free DNA ratio is threefold higher than that of BlaI-WT (Fig. 1). To demonstrate that BlaI-GM2 has retained

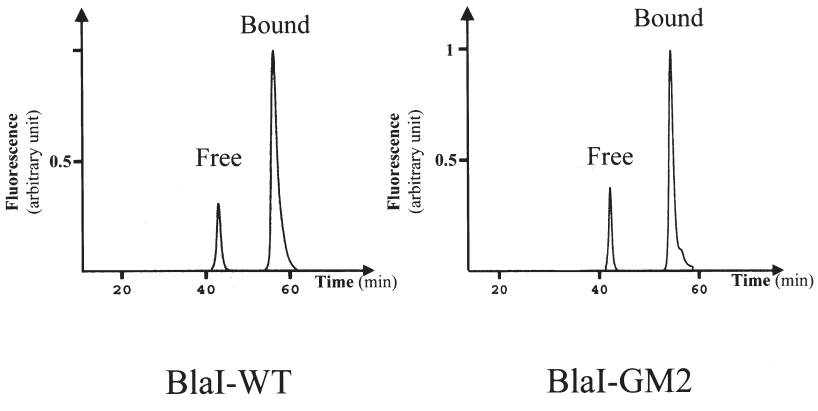


Fig. 1. DNA-binding assays with purified BlaI-WT and BlaI-GM2. The OP1 DNA fluorescent probe (0.5×10^{-8} M) was incubated with purified BlaI-WT (6.6×10^{-8} M) or BlaI-GM2 (1.9×10^{-7} M). Free and bound represent free OP1 and the DNA–protein complex respectively. On the basis of the peak areas, the ratios of bound/total DNA are equal (0.72) for the BlaI-WT and BlaI-GM2 DNA complexes.

its ability to dimerize, the mutant repressor was incubated with the homobifunctional reagent dithiobis succimidyl-propionate (DSP). As shown by SDS–PAGE in Fig. 2, purified BlaI-GM2 was present as a single 12 kDa band in the absence of DSP. After treatment with DSP, an additional band was detected with a molecular size corresponding to that of the dimer form of BlaI-WT (28 kDa). This result indicates that, in our experimental conditions, the BlaI-GM2 mutant retains its ability to dimerize. The limitations of the method do not allow us to detect modifications of the dimer association constant.

Construction of the Bacillus/E. coli *shuttle plasmid carrying* blaI*-GM2 in the* bla *divergon and β-lactamase induction in* B. subtilis *168*

The *blaI*-GM2 mutation was introduced in the *bla* divergon as described in *Experimental procedures*, and the resulting *Bacillus*/*E. coli* shuttle plasmid pCIP158 was transferred to *B subtilis* 168 and assayed for β-lactamase induction. The β-lactamase production in *B. subtilis*/pCIP158 was nearly 25-fold higher than in the wild type under uninduced conditions. This level of production is half that obtained for an induced *B. subtilis* strain harbouring the wild-type *bla* divergon (*B. subtilis*/pDML995). In addition, in the presence of inducer (2.5 μg ml^{-1} cephalosporin C), the mutant divergon did not confer the inducible phenotype to the host.

Bandshift assay and time course of blaI *expression during induction of* B. licheniformis *and recombinant* B. subtilis *strains*

Western blot analysis of induced and uninduced *B. licheniformis* 749/I cellular extracts showed that, in induced conditions, the band corresponding to BlaI-WT was not detected (Fig. 3A). In agreement, no DNA-binding activity of BlaI-WT was observed by bandshift assay in induced cellular extracts (Fig. 3E). Moreover, experiments carried out with cellular extracts of BlaR1$^-$ and BlaR2$^-$ non-inducible strains confirmed that, in both strains, the presence of the inducer did not inactivate BlaI-WT, which could be detected by Western blotting and bandshift assays. (Fig. 3B, C, F and G). Surprisingly, in the recombinant *B. subtilis*/pDML995, the apparent molecular mass of BlaI-WT under induced and uninduced conditions was identical to that of the purified BlaI-WT (Fig. 3D). In addition, in the induced culture, an increased quantity of BlaI-WT was observed, with a maximum 3 h after the addition of the inducer (Fig. 3D). To exclude undetectable (<1.5 kDa) proteolysis of BlaI-WT during the induction process, the BlaI-WT isoelectric point (pI) was probed by two-dimensional gel electrophoresis. Indeed, the N- and C-terminal BlaI-WT sequences are rich in charged residues, and a cleavage of 2–10 residues would give rise to a modification of its pI (predicted values for truncated BlaI-WT species range from 6.7 to 10.5 instead of 7.9 for intact BlaI-WT). No modification of BlaI-WT pI was observed even upon co-electrophoresis of BlaI-WT from induced and uninduced cells, the protein being detected as a single spot by anti-BlaI antibodies (Fig. 4). To demonstrate that dimerization of BlaI-WT is not modified during BlaP β-lactamase induction, intermolecular cross-linking experiments were carried out using DSP as cross-linking agent. Incubation of a dilute solution of purified BlaI (17 μM) with DSP (20 mM) resulted in the specific formation of a 28 kDa adduct as a result of the cross-linked BlaI-WT dimer (Fig. 2). In cellular extracts of uninduced and induced *B. subtilis*/pDML995 strains, the same result was obtained (Fig. 5).

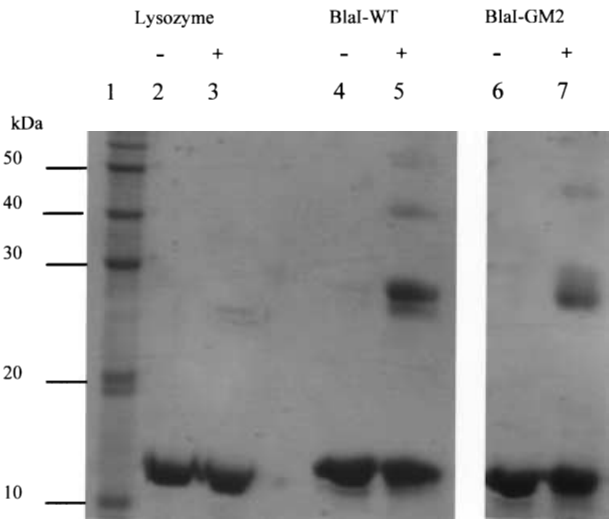


Fig. 2. BlaI-WT and BlaI-GM2 form dimers *in vitro*. SDS–PAGE analysis of purified BlaI-WT or BlaI-GM2 (17 μM) before (lanes 4 and 6) and after treatment with 20 mM DSP (lanes 5 and 7). To check the specificity of the experimental conditions for protein dimerization, hen egg white lysozyme (17 μM, lane 2), known to be a monomeric protein in solution, was treated with DSP (lane 3) and used as negative control. Mr standards were loaded in lane 1.

For *B. subtilis*/pCIP158, BlaI antibodies detect two BlaI-GM2 species in the cellular extract corresponding to 12 kDa and 10 kDa, respectively, a degradation pattern similar to that obtained for *S. aureus* BlaI during β-lactamase induction (Fig. 6). Thus, the behaviour of the *B. subtilis*/pCIP158 strain indicates that the proteolysis of BlaI does not necessarily rest on the presence of the inducer in the medium. To highlight that the hydrolysis of BlaI-GM2 is BlaR1 independent, the zinc-binding motif H212EXXH, present in the B4 cytoplasmic loop of BlaR1, has been mutated in AAXXA in pCIP158 to generate pCIP159. With wild-type BlaI, this mutation, as in *S. aureus*, gives rise to a non-inducible β-lactamase phenotype (data not shown). Surprisingly, the BlaR1 mutation

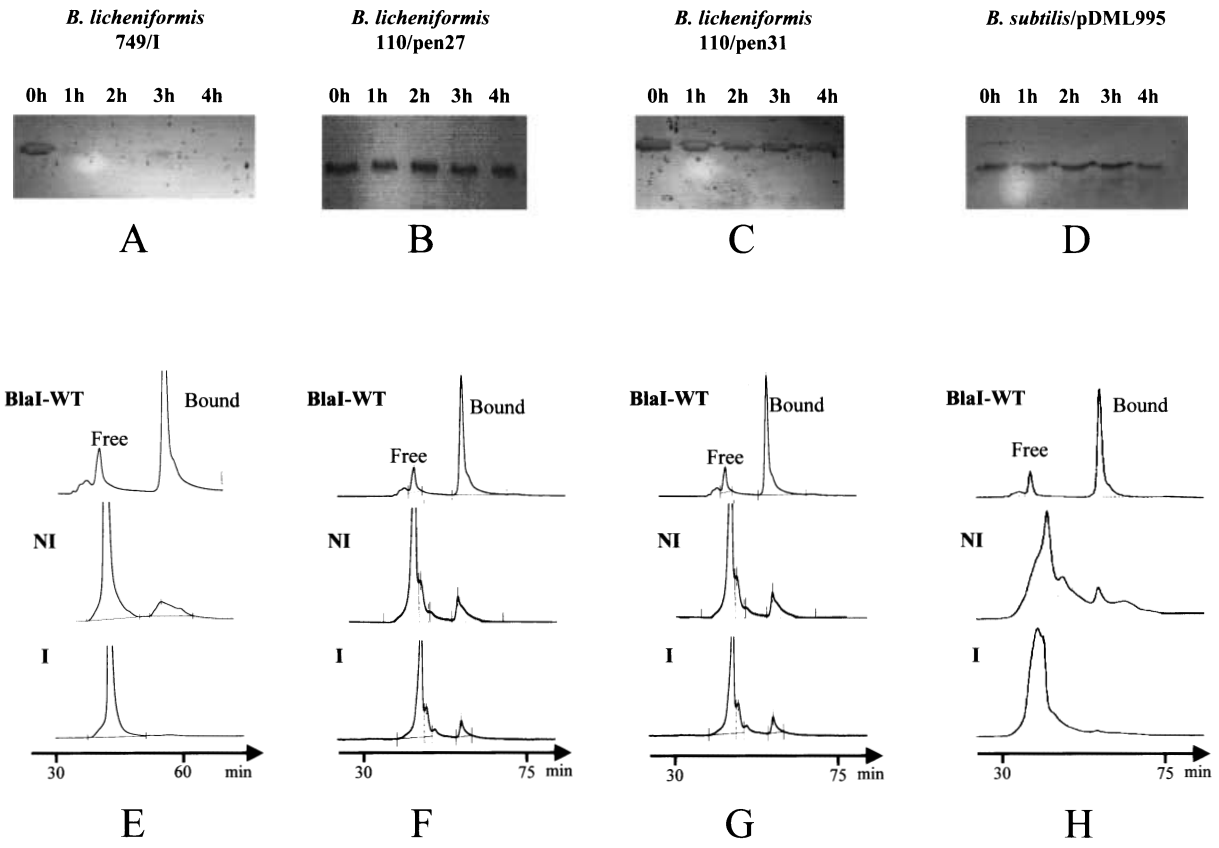


Fig. 3. Fate of BlaI during an induction time course. Western blot analyses of cellular extracts prepared from induced *B. licheniformis* 749/I (50 μg of proteins, A), *B. licheniformis* 110/*pen*-27 (*bla*R1$^-$) (50 μg of proteins, B), *B. licheniformis* 110/pen31 (*bla*R2$^-$) (50 μg of proteins, C) and *B. subtilis*/pDML995 (25 μg of proteins, D) were performed as described (*Experimental procedures*) using anti-BlaI antibodies. Lane numbers correspond to the number of hours elapsed after the initiation of induction by the addition of cephalosporin C (2.5 μg ml^{-1}). For each strain, the ability of BlaI-WT to bind DNA was probed by bandshift assay as described in Fig. 1 (E, *B. licheniformis* 749/I; F, *B. licheniformis* 110/*pen*-27; G, *B. licheniformis* 110/pen31; H, *B. subtilis*/pDML995; NI, non-induced; I, induced; Free, free fluorescent Op1; Bound, BlaI-WT and Op1 complex).

complements the BlaI-GM2 mutant and restores the inducibility of the β-lactamase in *B. subtilis*/pCIP159. In the double mutant, for uninduced cells, the level of β-lactamase expression is identical to that obtained with the wild-type divergon. On the contrary, in the presence of inducer, β-lactamase expression is lower than that obtained with the wild-type operon, and the induction factor is 12 instead of 53 for the wild-type operon (Fig. 7A). The analysis of the fate of BlaI-GM2 during the induction process shows that the mutant repressor is not proteolysed during the induction phenomenon (Fig. 7B).

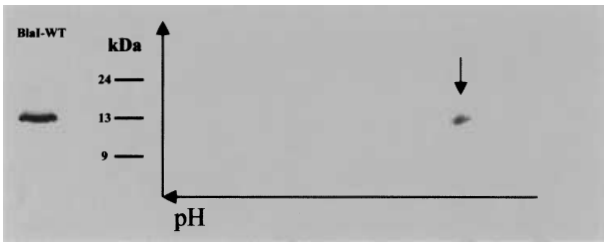


Fig. 4. Two-dimensional gel electrophoresis of cellular extracts of a mixture of uninduced (50 μg ml^{-1}) and induced (50 μg ml^{-1}) *B. subtilis*/pDML995. Proteins were extracted from cells induced for 2 h with 2.5 μg ml^{-1} cephalosporin C; they were analysed by isoelectric focusing (first dimension) using a pH range from 6 to 11 followed by SDS–PAGE (8–18% gradient; second dimension). Proteins were transferred to a PVDF membrane before revelation by anti-BlaI antibodies.

Discussion

The BlaI repressor has two functional domains. The amino-terminal domain is responsible for operator recognition, and the carboxy-terminal domain is involved in subunit dimerization. The formation of the BlaI dimer is necessary for repressor activity, and C-terminal truncated BlaI is unable to both dimerize and bind DNA (Wittman *et al*., 1993). Many studies have reported the proteolysis of the BlaI repressor during the induction of the BlaZ β-lactamase in *S. aureus*. The cleavage site has been localized between residues N101 and F102. It was postulated

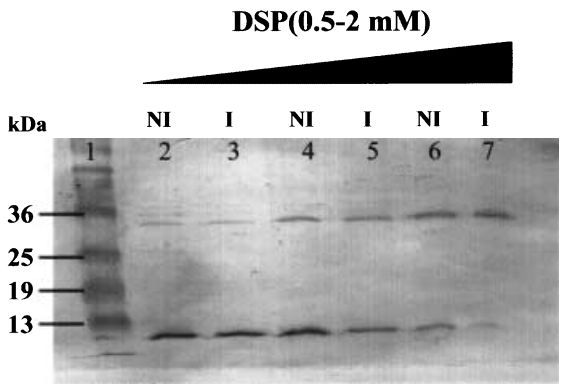


Fig. 5. In induced conditions, BlaI-WT retains its ability to form dimers. Western blotting of cellular extracts (50 μg of total proteins) of uninduced and induced *B. subtilis*/pDML995 strain were treated with increasing concentrations of DSP ranging from 0.5 to 2 mM. Lane 1, Mr standards; lanes 2, 4 and 6 and lanes 3, 5 and 7 correspond to non-induced (NI) and induced (I) cellular extracts respectively.

that this cleavage, directly or indirectly mediated by BlaR1, inhibits BlaI dimerization and reduces the affinity of BlaI for its DNA target so that it can no longer act as a repressor (Gregory *et al*., 1997; Zhang *et al*., 2001). In all these studies, however, it is clear that induction does not completely hydrolyse the repressor, and 40–50% of the intact form is always present. Two hypotheses have been proposed to explain these results: (i) some of the bacteria in the population are no longer inducible; and (ii) a heterodimer consisting of the dimerization domain of BlaI (102–128) and the intact BlaI is unable to act as a repressor.

Based on amino acid sequence alignments (data not shown), the *B. licheniformis* and *S. aureus* BlaI repressors are 37% identical. The site of staphylococcal cleavage (N101▼F102) is also present in the *Bacillus* sequence, and the four upstream residues are highly conserved in both primary structures [S97(M/L)VLNF102 in the *S. aureus* BlaI numbering, *B. licheniformis* BlaI being one residue shorter at its N-terminal end]. In this study,

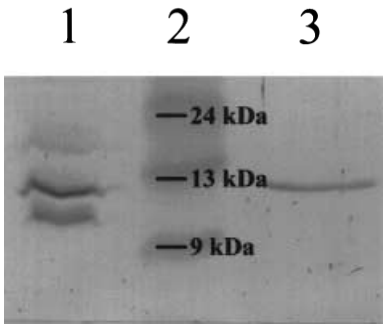
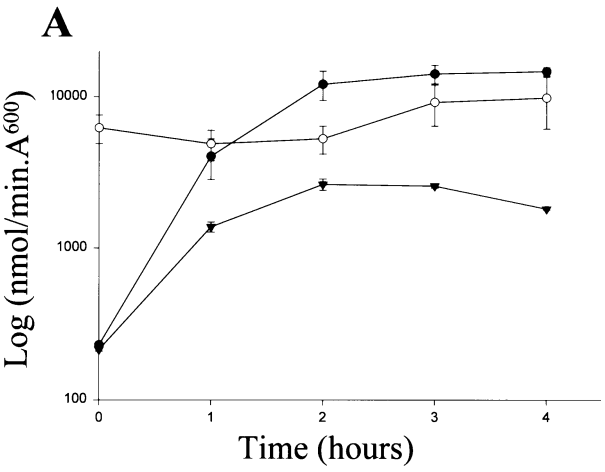


Fig. 6. BlaI-GM2 is proteolysed in uninduced *B. subtilis*/pCIP158. Western blotting analysis of cellular extracts (lane 1, 25 μg of total proteins), purified BlaI-WT (lane 3) and Mr standards (lane 2).

we have shown that the BlaI repressor is completely degraded during β-lactamase induction in wild-type *B. licheniformis* 749/I but remains intact when induction is carried out in a recombinant *B. subtilis* strain in which the *B. licheniformis* divergon has been cloned (*B. subtilis*/pDML995). Our results appear to rule out the hypothesis that inactivation of BlaI during the induction process results from a proteolytic cleavage. Two-dimensional electrophoresis of *B. subtilis*/pDML995 cellular extracts revealed that, in the presence of inducer, the pI of BlaI is not modified. This result excludes N- and C-ragged ends of BlaI (see *Results*) and is consistent with the observation that BlaI is not cleaved during induction. Interestingly, cross-linking experiments with DSP highlighted the fact that BlaI retains its ability to form homodimers during



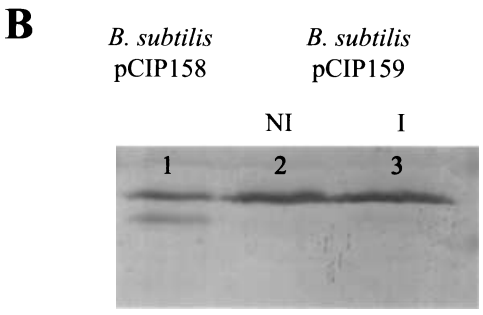


Fig. 7. A. Induction of β-lactamase synthesis in *B. subtilis*/pDML995 (closed circles, wild-type *bla* divergon), *B. subtilis*/pCIP158 (open circles, *bla* divergon carrying *blaI*-GM2 mutant) and *B. subtilis*/pCIP159 (filled inverted triangles, *bla* divergon carrying *blaI*-GM2 and inactivated *blaR1*). Cephalosporin C (2.5 μg ml^{-1}) was added as inducer at time zero. The β-lactamase activity is expressed as nmol of chromogenic substrate hydrolysed min^{-1} per unit of cell density. Cell densities were estimated by measuring the A_{600} of the cultures.
B. Induction is not correlated to BlaI-GM2 proteolysis. Western blotting analysis of cellular extracts (25 μg of total proteins); lane 1, *B. subtilis*/pCIP158; lanes 2 and 3, uninduced and induced *B. subtilis*/pCIP159 respectively.

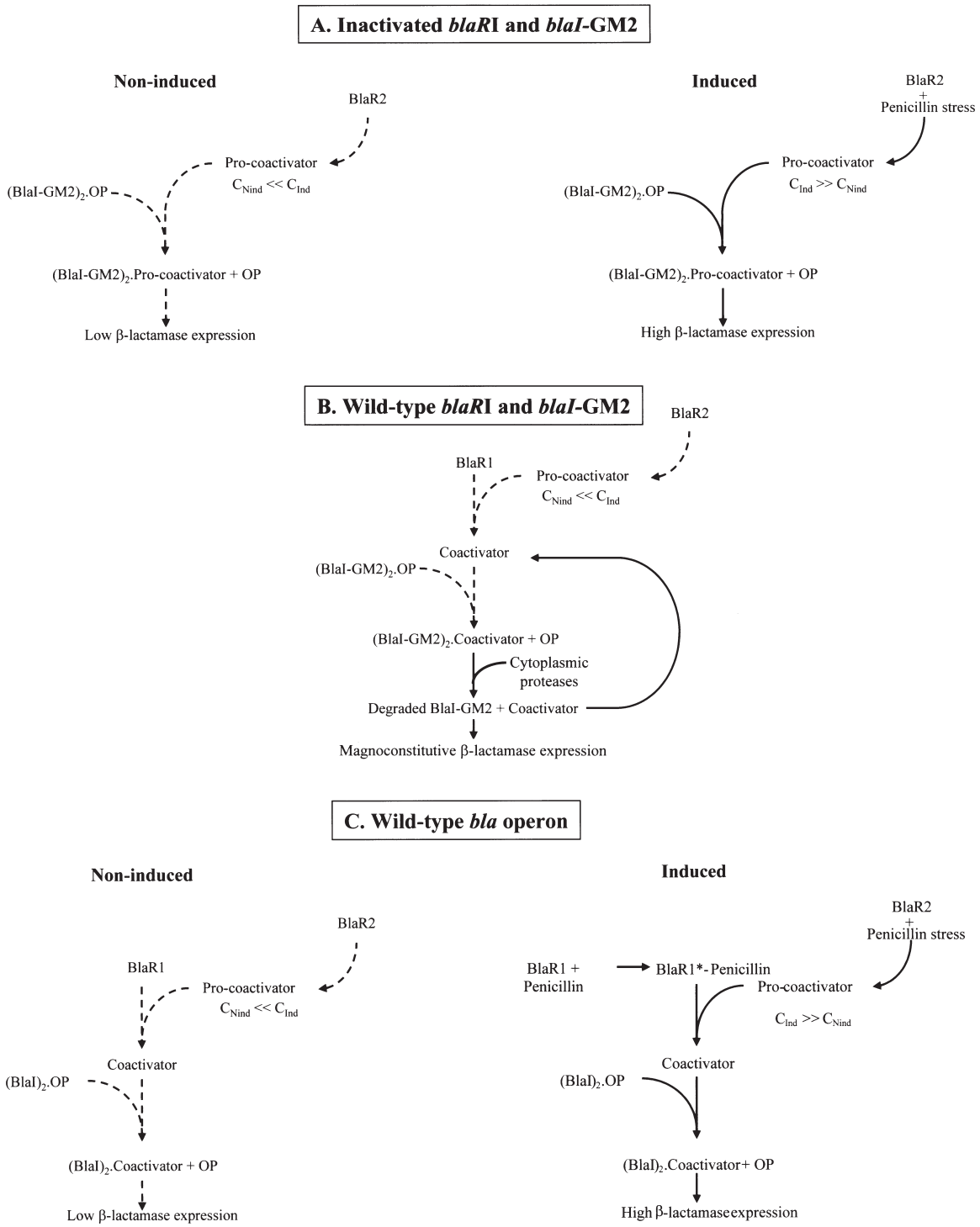


Fig. 8. Tentative models explaining the behaviour of cells producing mutant and wild-type BlaI and BlaR1.
A. Inactivated BlaR1 and BlaI-GM2; in the presence of penicillin stress, the concentration of pro-co-activator increases in the cell, resulting in a displacement of BlaI-GM2 from its operator (Op) sequence.
B. Wild-type BlaR1 and BlaI-GM2; the residual activity of BlaR1 produces a small amount of co-activator and, because of the sensitivity of BlaI-GM2–co-activator complex to proteases, a cyclic degradation of BlaI-GM2 is initiated resulting in magnoconstitutive β-lactamase production.
C. Wild-type *bla* operon; in uninduced conditions, the situation is as in (B), but the BlaI–co-activator complex is not protease sensitive, so that no BlaI degradation cycle occurs under induced conditions; BlaR1 is activated and penicillin stress increases the concentration of pro-co-activator, resulting in high production of co-activator and high β-lactamase expression.

β-lactamase induction. These results and the absence of BlaI DNA-binding activity in cell lysates derived from induced cells suggest the presence of a ligand acting as a co-activator that could displace the BlaI dimer from its DNA operator target, as described for the TetR repressor involved in the regulation of tetracycline resistance in Gram-negative bacteria (Orth *et al*., 2000). Indeed, binding of tetracycline to the C-terminal domain of the homodimer repressor induces conformational changes that increase the distance between the two N-terminal binding domains of the dimer by 3 Å, abolishing the affinity of TetR for its operator DNA. Similarly, the binding of the putative ligand by BlaI could induce a conformational change in the repressor, leading to a decrease in its affinity for its DNA target and, in the case of *S. aureus* and *B. licheniformis*, an increase in its susceptibility to the action of the cytoplasmic proteases.

The BlaI-GM2 mutation results in high-level constitutive production of β-lactamase in *B. subtilis*/pCIP158, whereas the mutant repressor is functional in *E. coli*. Compared with BlaI-WT, the affinity of BlaI-GM2 for the operator appears to be decreased by a factor of about three (Fig. 1) and, within the limits of the method, the DSP cross-linking experiment seems to show that its ability to dimerize is slightly altered (Fig. 2). Indeed, under the same experimental conditions, the intensity of the BlaI-GM2 cross-linked band is lower than that of the wild-type band. Western blotting experiments indicate that the mutated repressor is partially degraded in *B. subtilis*/pCIP158 (Fig. 2). But the most striking features are: (i) the *blaI*-GM2 proteolysis is linked to the presence of a functional BlaR1 receptor; (ii) an inactivated BlaR1 receptor restores the inducibility of the β-lactamase (Fig. 7A); and (iii) in this case, induction of the β-lactamase is not accompanied by proteolysis of BlaI-GM2. If the last result appears to confirm the hypothesis that induction is not a consequence of the degradation of the repressor, the constitutive production of β-lactamase in the presence of the wild-type BlaR1 and BlaI-GM2 repressor, which is accompanied by partial proteolysis of the latter, seems to contradict this hypothesis and is more difficult to rationalize. Taken together, the results indicate that the presence of penicillin outside the cell generates a signal into the cytoplasm that can be sensed by the mutant BlaI-GM2 repressor but not by the wild-type BlaI, because a non-functional BlaR1 associated with a wild-type BlaI gives rise to a non-inducible phenotype (Zhu *et al*., 1992). A possible explanation is given below, but we are perfectly conscious of the fact that it involves several assumptions. The main one is that a precursor of the co-activator (the pro-co-activator) is normally present in the cells at a very low concentration and that it is the substrate of BlaR1. The behaviour of the cells producing BlaI-GM2 but devoid of a functional BlaR1 is examined first. In the absence of penicillin stress, the intracellular concentration of pro-co-activator remains low, and it cannot displace BlaI-GM2 from its operator sequence, resulting in very low β-lactamase expression. Penicillin stress increases the pro-co-activator concentration, a process in which BlaR2 is involved. In contrast to the wild-type BlaI, BlaI-GM2 exhibits a non-negligible affinity for the pro-co-activator, sufficient to induce β-lactamase expression (Fig. 8A). To explain the constitutive expression in cells containing BlaI-GM2 and wild-type BlaR1, it is assumed that the latter exhibits a residual activity on the pro-co-activator, as observed in the case of many proenzymes that are not completely devoid of activity, and that the co-activator displaces BlaI-GM2 from its operator sequence (Fig. 8B). But the BlaI-GM2–co-activator complex is sensitive to the *B. subtilis* cytoplasmic proteases, regenerating the free co-activator and initiating a binding/proteolysis cycle in which a small amount of co-activator can be responsible for the degradation of an important proportion of BlaI-GM2. In contrast, the wild-type BlaI–co-activator complex is protease stable, so that, in uninduced conditions, the co-activator concentration is not sufficient significantly to displace BlaI from its operator sequence. Full induction then results from two consequences of the presence of penicillin: the activation of BlaR1 thanks to the acylation of its C-terminal domain and the increase in the intracellular pro-co-activator concentration as a result of penicillin stress.

Experimental procedures

Bacterial strains, plasmids and DNA manipulations

Bacillus licheniformis 749/I, 110/*pen*-27 and 110/*pen*-31 were wild-type, *blaR*1$^-$ and *blaR*2$^-$ strains respectively (Zhu *et al*., 1992). *E. coli* DH5α (Invitrogen) or *B. subtilis* 168 (ATCC 23857) were used as recipients of recombinant plasmids. *E. coli* GI724 (Invitrogen), carrying the *cI* λ repressor gene under the control of the *trp* promoter, and *E. coli* BL21 (DE3) (Novagen) were used as hosts for the overexpression of BlaI-WT and BlaI GM2 respectively.

The plasmids used in this study are listed in Table 1. Plasmid pRTW8 (Kobayashi *et al*., 1987) was the source of the wild-type *B. licheniformis* 749/I *blaP*, *blaI* and *blaR*1 genes. Plasmid pDML995 (A. Brans, unpublished data) is a derivative of pMK4 (Sullivan *et al*., 1984), in which a 3.5 kb fragment of pRTW8 containing the *B. licheniformis* 749/I *bla* divergon is inserted in the *Sal*I–*Sma*I site.

pLex (Invitrogen), containing the λ p_L promoter under the control of the CI λ repressor, was used as a vector for the overexpression of the *blaI*-WT product. A 400 bp fragment covering the *blaI* gene was amplified by polymerase chain reaction (PCR) using pRTW8 as template and the following oligonucleotides as primers: 5′-ATACATATGAAAAAAATAC CTCAAATCTCTG-3′ (BlaINdeI) and 5′-ATAGAATTCATT TCATTCCTTCTTTCTGTTCTTATG-3′ (BlaIEcoRI). These created *Nde*I and *Eco*RI restriction sites in the ATG start codon and after the stop codon of *blaI* respectively. The

Table 1. Plasmids used in this study.

Plasmids		Source or reference
pUC18	Cloning plasmid	Amersham Pharmacia
PCRScript	Plasmid allowing cloning of PCR fragments	Stratagene
pET22b	Plasmid allowing gene expression from inducible T7 promoter	Novagen
pLex	Plasmid allowing gene expression from inducible P-lambda promoter	Invitrogen
pDML995	A derivative of the *Bacillus*/*E. coli* shuttle vector pMK4 carrying the wild-type *B. licheniformis* 749 *bla* divergon	A. Brans (unpublished)
pCIP151	pCRScript derivative carrying the *blaI*-WT coding sequence	This study
pCIP152	pLex derivative allowing *blaI*-WT expression	This study
pCIP153	A derivative of pCIP151 encoding the *blaI*-GM2 gene	This study
pCIP154	pET22b derivative allowing *blaI*-GM2 expression	This study
pCIP155	A derivative of pDML995 with an unique *Sna*BI site in *bla* divergon	This study
pCIP156	pUC18 derivative carrying the *Pst*I–*Sac*I fragment of the pDML995 *bla* divergon	This study
pCIP157	A derivative of pCIP156 in which the *blaI*-GM2 mutation has been introduced	This study
pCIP158	A derivative of *Bacillus*/*E. coli* shuttle vector pCIP155 carrying a *bla* divergon containing the *blaI*-GM2 mutation	This study
pDML1268	A derivative of *Bacillus/E. coli* shuttle vector pMK4 carrying a *bla* divergon containing the *blaR*1 mutation H212EXXH → AAXXA (*blaR*1$^-$)	K. Benlafya (unpublished)
pCIP159	A derivative of *Bacillus*/*E. coli* shuttle vector pCIP155 carrying a *bla* divergon containing the *blaI*-GM2 and *blaR*1-A212AxxA mutations	This study

amplified fragment was ligated to the pCRScript plasmid to give pCIP151. The identity of the amplified DNA segment was confirmed by determination of its nucleotide sequence. pCIP151 was digested with *Nde*I and *Eco*RI, and the fragment corresponding to *blaI* was purified by agarose gel electrophoresis and ligated to the pLex vector digested with the same restriction endonucleases to give pCIP152.

Plasmid pCIP153 is a derivative of plasmid pCIP151, which contains the BlaI M97 V98 → I L mutation. The mutation was introduced using the QuickChange kit (Stratagene) and the following two primers: 5′-GGAACTCTTAATTCGATATTAT TAAAC-3′ (BlaIGM2+) and 5′-GTTTAATAATATCGAATTAA GAGTTCC-3′ (BlaIGM2–). The *Nde*I–*Eco*RI fragment of pCIP153 containing *blaI*-GM2 was inserted into pET22b+ (Novagen) to produce the *Bla*I-GM2 overexpression plasmid pCIP154.

Plasmid pCIP155 is a derivative of pDML995, in which the *Sna*BI site in the *bla* divergon is unique. To construct this plasmid, pDML995 was digested with *Bsp*EI and *Sma*I to remove the second *Sna*BI site present in pDML995, treated with Klenow polymerase and self-ligated. To substitute the wild-type *blaI* gene for *blaI*-GM2 in pCIP155, a fragment carrying the wild-type *blaI* gene was obtained by digestion of pDML995 with *Sac*I–*Pst*I and cloned into the *Sac*I–*Pst*I site of pUC18 to construct pCIP156. The BlaI-GM2 mutation was introduced in pCIP156 as described above to generate pCIP157, and the mutated *Sna*BI–*Sac*I fragment was cloned in pCIP155 to produce pCIP158. To construct the double mutant divergon, *blaI-GM2* and *blaR1* H212EXXH → AAXXA, the *Sna*BI–*Sac*I fragment carrying the BlaI-GM2 mutation was cloned in pDML1268, carrying the BlaR1 mutation, to generate pCIP159.

Restriction endonucleases, sequencing enzymes and *Tth* or *Pwo* DNA polymerases were purchased from Amersham Pharmacia and Gibco BRL. Oligonucleotides and primers for DNA sequencing were obtained from Amersham Pharmacia. Routine DNA manipulations were carried out as described by Sambrook *et al*., and *B. subtilis* was transformed according to the method of Msadek *et al.* (1998). DNA sequencing of the mutagenized fragment was performed by the dideoxy chain termination method using an ALFexpress DNA sequencer (Amersham Pharmacia).

Luria–Bertani (LB) medium was used as a rich liquid for both *E. coli* and *Bacillus* and as a solid medium (1.5% agar). Recombinant *E. coli* and *Bacillus* strains were selected with 100 μg ml^{-1} ampicillin and 7 μg ml^{-1} chloramphenicol respectively.

Expression and purification of BlaI-WT and BlaI-GM2

The purification of the repressors was adapted from the method of Grossman and Lampen (1987). The *E. coli* GI724/pCIP152 strain was grown in a tryptophan-defective medium in the presence of ampicillin (100 μg ml^{-1}) at 30°C. At an absorbance of 0.8 at 600 nm (A_{600}), the expression of BlaI-WT was induced by the addition of 100 μg ml^{-1} tryptophan. After 3 h of induction at 37°C, the cells were harvested, washed and resuspended in lysis buffer [15 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 100 mM KCl and 1 mM Pefabloc (Boehringer)]. Cells were disrupted by passage through an Inceltech disintegrator (basic Z model). After the addition of benzonase (500 U l^{-1} culture; Eurogentec), the soluble cell fraction was obtained as the supernatant after 45 min of centrifugation at 9000 *g* and 4°C. After the addition of glycerol (10% final concentration), the solution was diluted with lysis buffer supplemented with glycerol to reach a protein concentration of 12 mg ml^{-1}. The diluted solution was 45% saturated in ammonium sulphate. The pH was adjusted to 7.5, and proteins were precipitated overnight at 4°C. After centrifugation, the resulting supernatant containing BlaI was dialysed against the loading buffer (50 mM HEPES, pH 7.6, 1 mM EDTA and 5% glycerol). The dialysed solution was submitted to ion-exchange chromatography on an S-sepharose fast flow column (2.6 × 40 cm; Amersham Pharmacia), and the adsorbed BlaI-WT was eluted by a step gradient (200 ml of loading buffer supplemented with 0.5 and 0.58 M NaCl respectively). To obtain a purity > 95%, an additional chromatography was performed on a HiTrap

heparin column (5 ml; Amersham Pharmacia) equilibrated with 50 mM HEPES, pH 7.6, 1 mM EDTA, 5% glycerol and 0.2 M NaCl. BlaI was eluted with a linear NaCl gradient ranging from 0.2 to 2 M.

Overexpression of BlaI-GM2 was achieved using *E. coli* BL21 (DE3) transformed by pCIP154. The recombinant strain was grown in LB. When the culture reached an A_{600} of 0.8, expression of BlaI-GM2 was induced by 1 mM IPTG, and the culture was grown for an additional 3 h. The purification of BlaI-GM2 was performed as described for BlaI, except that the ammonium sulphate precipitation was carried out at 30% saturation.

β-Lactamase induction

Bacillus licheniformis and recombinant *Bacillus* strains were grown in LB at 37°C until A_{600} reached 0.8. Cephalosporin C was added at a final concentration of 2.5 μg ml^{-1}, and the incubation was continued at 37°C for 4 h.

β-Lactamase activity was measured spectrophotometrically with nitrocefin (Becton Dickinson) and expressed as nmol of substrate hydrolysed min^{-1} per unit of cell density. Cell densities were determined by measuring the A_{600}.

Anti-BlaI antibodies and Western blot (immunoblot) analysis

A polyclonal anti-BlaI antiserum was generated by immunizing New Zealand white rabbits with purified BlaI-WT (Centre d'Economie Rurale et d'Hormonologie de Marloie). The crude serum was used in immunoblotting at a final dilution of 1:200.

Ten millilitres of induced or uninduced *B. licheniformis* or recombinant *B. subtilis* strains was sedimented by centrifugation and resuspended in 200 μl of 50 mM Tris-HCl, pH 7, 1 mM EDTA and 1 mM Pefabloc. Bacterial cells were disrupted by sonication in a Branson ultrasonic disintegrator at an amplitude of 6 μm for three 30 s bursts. Soluble cell fractions were obtained as supernatants after centrifugation of the lysates. Protein concentrations in cellular extracts were determined by the 2-bicinchoninic acid assay (BCA protein assay; Pierce).

Proteins (25–50 μg) were separated by SDS–PAGE (15%) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Immunoblot analysis using polyclonal anti-BlaI rabbit antibodies and the detection of rabbit antibodies on blots were carried out using goat alkaline phosphatase-conjugated anti-rabbit antibodies and a colour reaction with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium, as instructed by the manufacturer (Bio-Rad; immunoblot alkaline phosphatase assay system). The Benchmark prestained protein ladder (Gibco BRL) was used as molecular ratio (Mr) standard.

Dithiobis(succimidylpropionate) (DSP) cross-linking

The cross-linking of pure BlaI-WT or BlaI-GM2 was carried out as recommended by the supplier (Pierce). Cell pellets from 100 ml of induced or uninduced cultures were suspended in 500 μl of ice-cold cross-linking buffer (50 mM sodium phosphate, pH 6.5, 50 mM KCl and 1 mM EDTA) and disrupted by sonication as described above. The soluble cellular extracts were recovered by centrifugation, and the protein concentrations were determined using the BCA assay. Total proteins (50 μg) were incubated for 2 h at room temperature with DSP concentrations ranging from 0.2 to 2 mM. The cross-linking reaction was stopped by the addition of Tris base at a final concentration of 20 mM. Before SDS–PAGE, the samples were denatured by the addition of Laemmli denaturing buffer without mercaptoethanol. After electrophoresis, BlaI was detected by the immunoblotting procedure described above.

DNA-binding assay

For DNA-binding assays, induced or uninduced cells from 100 ml of culture were collected by centrifugation, and the pellet was resuspended in 500 μl of ice-cold DNA-binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 5% glycerol and 50 μg ml^{-1} bovine serum albumin (BSA, protease and nuclease free)]. After sonication, the soluble cellular extract was recovered by centrifugation, and the DNA-binding assay was carried out using an ALFexpress DNA sequencer and the fluorescent double-stranded oligonucleotide OP1 (5′-Cy5-GCATTTAAATCTTACATATGTAATAC TTTC-3′) as described previously (Filée *et al.*, 2001).

Two-dimensional electrophoresis

Cells from 10 ml of induced or uninduced cultures were harvested by centrifugation, and the pellets were resuspended in 250 μl of Milli-Q water and sonicated as described above. The soluble extracts were recovered by centrifugation, and the protein concentration was adjusted to 5 μg μl^{-1} by the addition of Milli-Q water. Eighty microlitres of denaturing buffer [7 M urea, 2 M thiourea, 4% CHAPS, 6.5 mM dithiothreitol (DTT) and 0.8% pharmalytes (Amersham Pharmacia)] was added to a 20 μl sample. The mixture was incubated for 2 h on ice and centrifuged at 25 000 *g* for 30 min to eliminate insoluble material. Cellular extracts (100 μl) from uninduced and induced cells were pooled and loaded onto an Immobiline Drystrip (pH 6–11, 18 cm; Amersham Pharmacia). The isoelectric focusing run was performed at 20°C on a Multiphor II (Amersham Pharmacia) according to the following programme: step 1, the voltage was increased from 1 to 300 V over 1 min; step 2, 300 V for 4.5 h; step 3, the voltage was increased from 300 to 3500 V over 30 min; step 4, 3500 V for 23 h. Thereafter, the gel was incubated for 15 min in the denaturing buffer (50 mM Tris-HCl, pH 7.8, 6 M urea, 2% SDS and 30% glycerol), followed by a second incubation in fresh denaturing buffer supplemented with iodoacetamide (140 mM final concentration). The SDS–PAGE run was performed at 15°C with an acrylamide gel gradient from 8% to 18% (Amersham Pharmacia). BlaI was detected by the Western blotting procedure described above.

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