THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 19, Issue of May 9, pp. 16482–16487, 2003
Printed in U.S.A.

Dimerization and DNA Binding Properties of the *Bacillus licheniformis* 749/I BlaI Repressor*

Received for publication, October 24, 2002, and in revised form, February 24, 2003
Published, JBC Papers in Press, March 3, 2003, DOI 10.1074/jbc.M210887200

Patrice Filée‡§, Christelle Vreuls¶, Raphaël Herman‡, Iris Thamm‡, Tony Aerts‖, Peter P. De Deyn‖, Jean-Marie Frère‡, and Bernard Joris‡**

From the ‡Centre d'ingénierie des Protéines, Institut de Chimie B6a, Université de Liège, Sart-Tilman, B4000 Liège, Belgium, the ¶Laboratoire de Physique Biomédicale, Institut de Physique B5, Université de Liège, Sart-Tilman, B4000 Liège, Belgium, and the ‖Department of Biomedical Sciences, University of Antwerp, B-2610 Antwerp, Belgium

In the absence of penicillin, the β-lactamase encoding gene *blaP* of *Bacillus licheniformis* 749/I is negatively regulated by the transcriptional repressor BlaI. Three palindromic operator regions are recognized by BlaI: two in the *blaP* promoter (OP1 and OP2) and one (OP3) in the promoter of the *blaI-blaR1* operon. In this study, the dissociation constant of the purified BlaI dimer was estimated at 25 μM by equilibrium ultracentrifugation. Quantitative Western blot analysis indicates that the intracellular concentrations of BlaI in *B. licheniformis* 749/I and *Bacillus subtilis* transformed by a multicopy plasmid harboring the β-lactamase locus (*blaP-blaI-blaR1*) were lower than (1.9 μM) or in the same range as (75 μM) the dissociation constant, respectively. This suggests that BlaI is partially dimeric in the cytoplasm of these strains and interacts *in vivo* with its operators as a preformed dimer. This hypothesis is supported by band shift assays on an operator containing a randomized half-operator sequence. The global dissociation constants of the operator-BlaI dimer complexes were measured by band shift assays and estimated as K_{dOP1} = 1.7 ± 0.5 10^{-15} M^2, K_{dOP2} = 3.3 ± 0.9 10^{-15} M^2, and K_{dOP3} = 10.5 ± 2.5 10^{-15} M^2. The role of the DNA binding properties of BlaI on the β-lactamase regulation is discussed.

The *blaP* gene encodes the class A β-lactamase of *Bacillus licheniformis* 749/I. In the absence of β-lactams antibiotics, the BlaI repressor prevents the transcription of the *blaP* gene (1–3). Two additional genes, *blaR1* and *blaR2*, are also involved in the induction of the β-lactamase synthesis (4, 5). The *blaP*, *blaI*, and *blaR1* genes are clustered in a divergeon (*bla* divergeon) in which *blaI* and *blaR1* form an operon (Fig. 1). The *blaR1* gene encodes a transmembrane protein that acts as penicillin receptor (6–8), and *blaR2* is not identified yet and is not linked to the *bla* divergeon. The BlaR2 protein is essential for the inactivation of BlaI. In *Staphylococcus aureus*, the *blaZ* and *mecA* genes, encoding respectively a β-lactamase and the low affinity penicillin-binding protein 2′, are regulated by similar elements (9, 10).

* This work was supported by Grant P5/33 from the Belgian Program on Interuniversity Poles of Attraction initiated by the Federal Office for Scientific, Technical and Cultural Affaires and Fond National de la Recherche Scientifique Grant 1.5201.02, and Fonds de la Recherche Fondamentale Collective Grant 2.4530.03. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Fellow of the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture.

** Research Associate of the Fond National de la Recherche Scientifique. To whom correspondence should be addressed. Tel.: 32-4-366-2954; Fax: 32-4-366-3364; E-mail: bjoris@ulg.ac.be.

The *blaI* gene encodes a 128-residue protein characterized by two functional and separate domains (11). The DNA-binding domain, a helix-turn-helix recognition motif, is located in the N-terminal region, and the dimerization domain is in the C-terminal region. After deletion of the C-terminal domain, the repressor becomes unable to dimerize and to form a stable complex with its operators. DNase footprinting experiments and filter binding reactions revealed the presence of three regulatory regions that are recognized specifically by BlaI (11). These operators present a 23-bp-long dyad symmetry and show the deduced 5′-AAAGTATTACATATGTAACNTTT-3′ consensus sequence (Fig. 1).

In the presence of β-lactam antibiotics, the C-terminal domain of BlaR1 is acylated (6, 7). This event triggers the activation of the putative cytoplasmic metalloprotease motif of BlaR1 by self-proteolysis (8, 12). In *S. aureus*, the β-lactamase induction correlates with BlaI proteolysis between residues Asn^{101} and Phe^{102}, and it is postulated that the activated form of BlaR1 proteolyses BlaI (12–15). The *blaR2* gene could be involved in this process or in the activation of BlaR1, but our recent studies in a recombinant *Bacillus subtilis* harboring a plasmid carrying the *bla* divergeon show contrasting results with those in *S. aureus* and suggest that the inactivation of BlaI in *Bacillus* is mediated by the binding of a coactivator (16). This coactivator could maintain the BlaI dimer in a conformation unable to bind to the DNA operators and sensitive to *cytoplasmic B. licheniformis* or *S. aureus* proteases. To determine the mechanism by which the coactivator interferes with the DNA binding properties of BlaI, a detailed analysis of the interaction pathway between BlaI and its operators was essential.

In the present study, we used purified BlaI to analyze the multimerization of the protein by equilibrium ultracentrifugation and its binding to the three operator sequences mentioned above by band shift assay. The implications of the DNA binding properties of BlaI on the β-lactamase regulation are also discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—*B. licheniformis* 749/I is a β-lactamase-inducible strain (7). *B. subtilis* 168 (ATCC 23857) was used as recipient of plasmid pDML995 (chloramphenicol resistance). *Escherichia coli* GI724 (Invitrogen) was used as host for plasmid pCIP152 for the overexpression of BlaI-WT.

Plasmid pDML995 is a derivative of pMK4 (17) in which the *B. licheniformis* 749/I divergeon (*blaP-blaI-blaR1*) is inserted. Plasmid pCIP152 is a pLEX derivative plasmid (Novagen) allowing the BlaI-WT overexpression (16).

Overexpression and Purification of BlaI-WT—The overexpression and the purification of BlaI-WT from *E. coli* GI724 transformed by the pCIP152 were performed as described previously (16).

Fluorescent Band Shift Assay—The fluorescent band shift assays were performed as described previously (16, 18). The fluorescent dou-

ble-stranded oligonucleotides used in this study are presented in Table I.

Quantitative Analysis by Western Blot—*B. licheniformis* 749/I and *B. subtilis*/pDML995 were grown in LB medium supplemented or not with chloramphenicol. When the absorbance of the cultures reached 0.8 at 600 nm, the cells were harvested, and the cell titer was determined by plating dilutions. This was repeated with several independent cultures. Cells from 10 ml of these cultures were successively pelleted, washed with 1 ml of AQ buffer (50 mM sodium phosphate, pH 6.8, 50 mM KCl, 1 mM EDTA, 1 mM Pefabloc), and suspended in 500 μl of AQ buffer before sonication (three times for 20 s). The supernatants were collected by centrifugation, added with SDS loading buffer, and heated for 5 min in a boiling water bath. Different amounts of these cytoplasmic extracts were subjected to SDS-PAGE (15%) before analysis by Western blotting. Western blots were performed using polyvinylidene difluoride membranes, anti-BlaI-WT rabbit polyclonal serum (18), goat alkaline phosphatase-conjugated anti-rabbit antibodies, and revelation with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Bio-Rad). To estimate the BlaI concentration in cytoplasmic extracts, known quantities of purified BlaI were loaded on the same gel. To take account of the potential loss of BlaI during the preparation of samples, the standards were prepared according to the following procedure: nontransformed *B. subtilis* cells were pelleted, washed, and suspended in AQ buffer containing purified BlaI. After sonication, the supernatant was collected by centrifugation, added with SDS loading buffer, and heated for 5 min in a boiling water bath. The blots were scanned and analyzed with the software Densito (Cybertech).

Analytical Ultracentrifugation—Analytical ultracentrifugation experiments were carried out in a Beckman Optima XLA instrument at 20 °C using optical absorption detection at a wavelength of 280 nm. The protein samples were transferred in 50 mM Hepes buffer, pH 7.6, containing 200 mM NaCl and 1 mM EDTA by dialysis, and the final BlaI concentrations were 0.447, 0.386, 0.33, 0.276, 0.218, 0.141, and 0.076 mg/ml. Sedimentation equilibrium experiments were carried out using an AN-60 Ti Analytical rotor with standard double-sector centerpieces and quartz windows at 11500 rpm. Equilibrium was reached after 65 h as determined by comparing scans taken at 5-h intervals. Equilibrated scans were used for determining equilibrium association constants for the self-association of BlaI-WT.

Sedimentation Equilibrium Data Analysis—Equilibrium data were analyzed by nonlinear least squares fitting of primary absorbance data using the Mixedfit program for Windows from L. Holladay (19, 20). To determine the best model for describing the BlaI multimerization, the data were fitted by using the following ideal equilibrium models: monomer-dimer, monomer-dimer-tetramer, or isodesmic models 1, 2, and 4. The best fit was determined by examining the distributions of residuals.

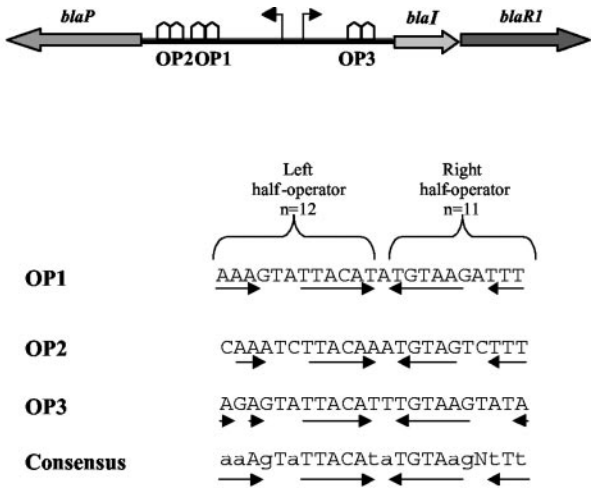

FIG. 1. **Organization of the *bla* divergeon involved in the regulation of β-lactamase synthesis in *B. licheniformis* 749/I and nucleotide sequence alignment of the OP1, OP2, and OP3 operators recognized by the BlaI repressor.** OP1 and OP2 are located in the *blaP* promoter and OP3 in that of the *blaI-blaR1* operon. ⌓, operator recognized by BlaI; → ←, inverted repeat sequences. The *bent arrows* indicate the position of the promoter and the direction of the transcription.

RESULTS

Determination of the Dissociation Constant of BlaI Dimer by Analytical Ultracentrifugation—To determine the mode of multimerization and the dissociation constant of the BlaI-WT dimer-monomer equilibrium, sedimentation equilibrium ultracentrifugation experiments were conducted over a large concentration range, and experimental data were collected at 280 nm. Typical data sets are displayed in Fig. 2. For the analysis of these data, the measured absorbance at 280 nm *versus* the radial position were fitted to mathematical functions describing various self-association systems (monomer-dimer equilibrium, monomer-dimer-tetramer equilibrium, and isodesmic models 1, 2, and 4). The best fit for BlaI-WT multimerization was obtained for isodesmic model 2 shown in Fig. 3. By using this model, the distributions of residuals (Fig. 2*B*) were in good agreement with the experimental data obtained at each protein concentration. Moreover, the calculated quantity of protein was equal to that introduced in the ultracentrifuge cell. Analysis of the experimental data revealed that there were monomeric, dimeric, tetrameric, and hexameric species in solution. Following isodesmic model 2, the monomeric, dimeric, tetrameric, and hexameric concentrations were calculated and are listed in Table II. Equilibrium sedimentation experiments showed that BlaI-WT can be present in solution as monomer, dimer, tetramer, and hexamer in the 10–30 μM range of protein concentration with a dissociation constant K_1 estimated to 25 μM, which is the same for each equilibrium.

Determination of the Intracellular Concentration of BlaI—In prelude to this work, the cell titers of *B. licheniformis* 749/I and *B. subtilis*/pDML995 were correlated to the $OD_{600\ nm}$ values of bacterial culture. Cytoplasmic extracts from several midlog phase cultures of *B. licheniformis* 749/I and *B. subtilis*/pDML995 were analyzed by Western blotting. The concentration of BlaI in cytoplasmic extracts was estimated with a standard curve prepared with purified BlaI. To take account of possible BlaI proteolysis during sample preparation, the standard curve was obtained by the addition of purified BlaI to pDML995-free *B. subtilis* cells from a midlog phase culture and by identical preparations of cytoplasmic extracts. According to the *B. subtilis* cell volume (10^{-15} liters) determined by Abril *et al.* (21), the cytoplasmic concentrations of BlaI in *B. licheniformis* 749/I and *B. subtilis*/pDML995 were estimated at about 1.9 μM (1,100 BlaI monomers/cell) and 75 μM (45,000

TABLE I
Oligonucleotides used in this study

Oligonucleotides	Sequences	Lengths
		bp
OP1_upper	5′-GAAAGTATTACATATGTAAGATTTAAATGC-3′	30
OP1_lower	Cy5-GCATTTAAATCTTACATATGTAATACTTTC-3′	30
OP2_upper	5′-GCAAATCTTACAAATGTAGTCTTTAAATGC-3′	30
OP2_lower	Cy5-GCATTTAAAGACTACATTTGTAAGATTTGC-3′	30
OP3_upper	5′-GAGAGTATTACATTTGTAAGTATAAAATGC-3′	30
OP3_lower	Cy5-GCATTTTATACTTACAAATGTAATACTCTC-3′	30
HalfOP1_upper	5′-GAAAGTATTACATCACATTCGAAACGTGGG-3′	30
HalfOP1_lower	Cy5-CCCACGTTTCGAATGTGATGTAATACTTTC-3′	30

BlaI monomers/cell), respectively (Fig. 4). By comparison with the dissociation constants estimated by equilibrium centrifugation, these results indicate that BlaI is partially present as a dimer in *B. licheniformis* 749/I (21% dimer) and mainly in *B. subtilis*/pDML995 (75% dimer). Dithiobis(succinimidylpropionate) cross-linking experiments with cytoplasmic extracts of *B. subtilis*/pDML995 yielded a similar conclusion (16).

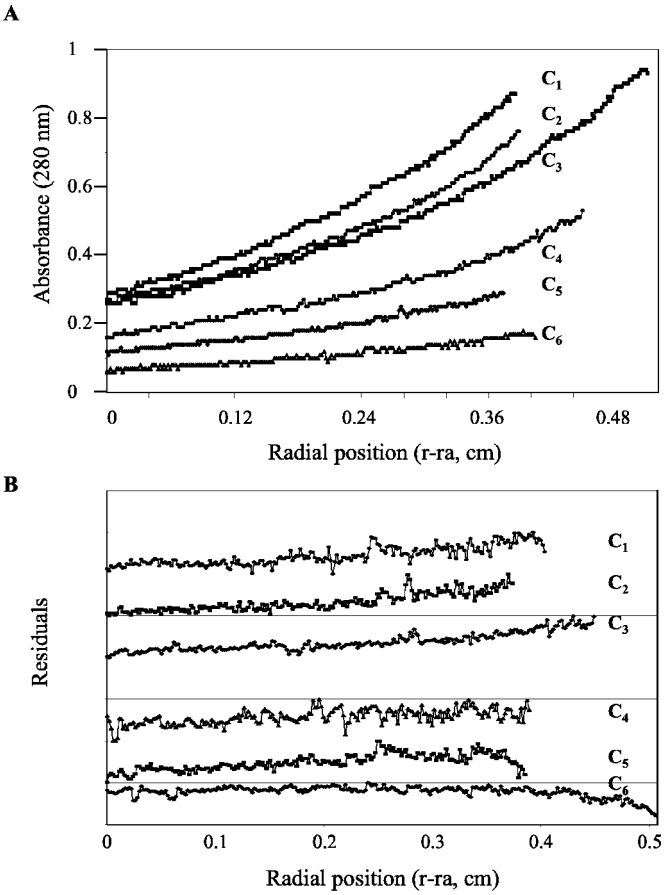

FIG. 2. **Equilibrium sedimentation data.** Centrifugation was carried out at 11,500 rpm and at 20 °C for 65 h as described under "Experimental Procedures." In *A*, the measured absorbance at 280 nm *versus* the radial position (distance from the center of the rotor in cm) is shown at various concentration of protein C_1 = 0.447 mg/ml, C_2 = 0.86 mg/ml, C_3 = 0.330 mg/ml, C_4 = 0.276 mg/ml, C_5 = 0.218 mg/ml, and C_6 = 0.141 mg/ml. *B* gives the residuals to the fit expressed as the differences between the experimental data and fitted values.

$$\text{BlaI} + \text{BlaI} \rightleftharpoons (\text{BlaI})_2 \qquad K_1 = [\text{BlaI}]^2 / [(\text{BlaI})_2]$$

$$\text{BlaI} + \text{BlaI} + (\text{BlaI})_2 \rightleftharpoons (\text{BlaI})_4 \qquad K_1 = [\text{BlaI}]^2 . [(\text{BlaI})_2] / [(\text{BlaI})_4]$$

$$\text{BlaI} + \text{BlaI} + (\text{BlaI})_4 \rightleftharpoons (\text{BlaI})_6 \qquad K_1 = [\text{BlaI}]^2 . [(\text{BlaI})_4] / [(\text{BlaI})_6]$$

FIG. 3. **Isodesmic model 2.**

Study of the Interaction Pathway between BlaI and Its Operators—Firstly, we examined the binding curve of BlaI to its three operators. A fixed concentration of labeled operator (5 nM) was incubated with increasing concentrations of purified BlaI (3.3 nM to 0.33 μM) for 2 h before loading onto the gels. Analysis of the electropherograms revealed the presence of two peaks. The faster and the slower peaks correspond to the unbound operator and the BlaI dimer-operator complex, respectively (16). No peak corresponding to BlaI monomer-operator was observed under these conditions. As presented in Fig. 5, a sigmoidal binding curve was observed for each operator. For OP1, the Scatchard plot (Fig. 6) indicated a 2/1 stoichiometry for the BlaI-operator complex, which confirms that the retarded DNA observed on the electropherograms corresponds to the BlaI dimer-operator complex. A Hill plot of the data obtained between 10 and 90% of OP1 saturation (Fig. 7) yielded a Hill coefficient of 2. This value corresponds to a pathway in which two BlaI monomers simultaneously bind the operator. This situation does not allow us to distinguish between the dimer pathway and a highly cooperative monomer pathway (18) (Fig. 8) characterized by a sequential binding of monomers to each half-operator. The dimer is formed when the second monomer interacts with the free half-operator.

Fig. 9 shows band shift assays performed by incubating BlaI with the semi-conserved OP1 operator (half-OP1; Table I). The half-OP1 is a 30-bp-long oligonucleotide in which the right half-binding site of OP1 is completely degenerated. In the presence or absence of nonspecific DNA, no complex corresponding to BlaI monomer-half-OP1 was detected. In these conditions, only the BlaI dimer-half-OP1 complex could be visualized, but the affinity of the BlaI dimer for the half-OP1 was considerably reduced compared with OP1.

In this study, all of the band shift assays were done with BlaI concentrations well below the dissociation constant of the BlaI dimer estimated by equilibrium ultracentrifugation. This implicates that BlaI dimer formation could be the rate-limiting step in the BlaI-DNA binding reaction and could be responsible for the sigmoidal binding curves. This hypothesis is reinforced

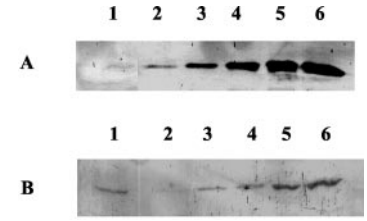

FIG. 4. **Quantification of the cytoplasmic BlaI concentration in *B. licheniformis* 749/I and *B. subtilis*/pDML995 by Western blotting.** *A*, *lane 1*, cytoplasmic extract *from B. licheniformis* 749/I; *lanes 2–6*, 1.5, 5, 10, 20, and 40 ng, respectively, of purified BlaI added with cytoplasmic extracts from nontransformed *B. subtilis*. *B*, *lane 1*, cytoplasmic extract *from B. subtilis*/pDML995; *lanes 2–6*, 1.5, 5, 10, 20, and 40 ng, respectively, of purified BlaI added with cytoplasmic extracts from nontransformed *B. subtilis*. *A* was overexposed to allow detection of the very low quantity of BlaI in *lane 1*.

TABLE II
Concentration of monomer, dimer, tetramer, and hexamer calculated by isodesmic model 2 starting from equilibrium ultracentrifugation data (K_1 = 25 μM)

Initial BlaI-WT concentration	Calculated BlaI-WT concentration by using isodesmic model 2						Calculated total BlaI-WT concentration
	[Monomer]		[Dimer]		[Tetramer]	[Hexamer]	
mg/ml	*mol/liter*	*mg/ml*	*mol/liter*	*mg/ml*	*mol/liter*	*mol/liter*	*mg/ml*
0.447	1.56×10^{-5}	0.234	7.97×10^{-6}	0.239	3.22×10^{-11}	7.96×10^{-16}	0.473
0.386	1.41×10^{-5}	0.212	6.52×10^{-6}	0.196	3.28×10^{-11}	4.75×10^{-16}	0.407
0.330	1.31×10^{-5}	0.198	5.67×10^{-6}	0.170	2.38×10^{-11}	2.46×10^{-16}	0.368
0.276	1.22×10^{-5}	0.184	4.93×10^{-6}	0.148	1.87×10^{-11}	8.11×10^{-17}	0.332
0.218	8.63×10^{-6}	0.13	2.45×10^{-6}	0.073	1.05×10^{-11}	3.46×10^{-17}	0.203
0.141	6.08×10^{-6}	0.091	1.22×10^{-6}	0.036	3.71×10^{-12}	7.15×10^{-18}	0.128

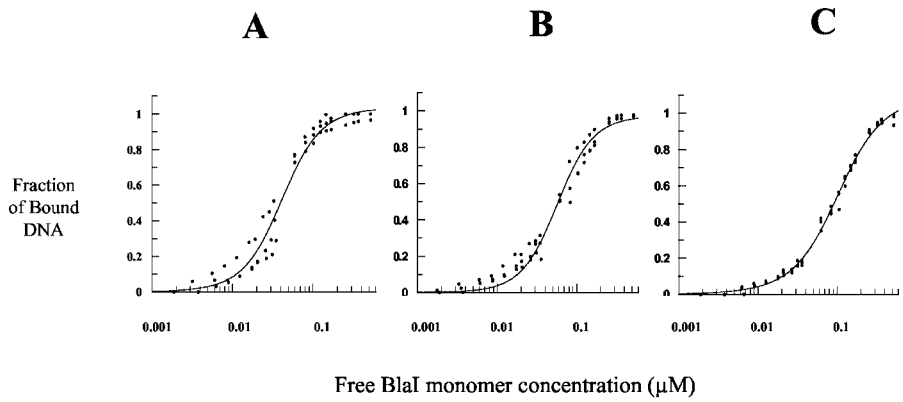

FIG. 5. **Binding curves of BlaI to its operators.** A constant concentration of fluorescent double-stranded operator (5 nM) was titrated with increasing concentrations of BlaI. *A*, *B*, and *C* correspond to the binding curves of BlaI to OP1, OP2, and OP3, respectively. The data were fitted to: Fraction of bound DNA = $[BlaI]^2/([BlaI]^2 + K)$, where $K = K_2' \cdot K_3'$ in the monomer pathway and $K_1 \cdot K_1'$ in the dimer pathway. [BlaI] is the free BlaI expressed as the monomer concentration.

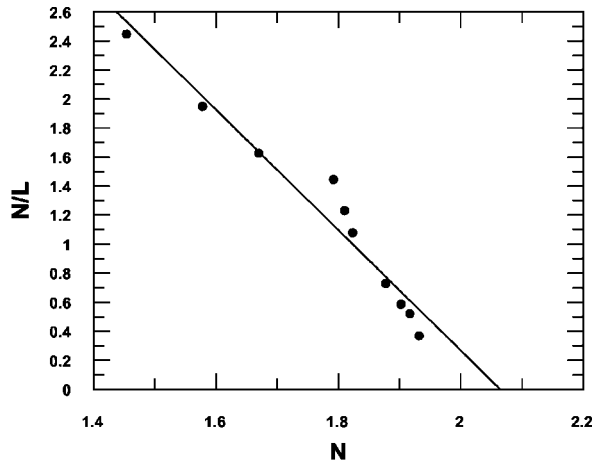

FIG. 6. **Scatchard plot of the data obtained with the OP1 operator.** *N* is the ratio between the bound BlaI monomer concentration and the total operator concentration. *L* corresponds to the free BlaI monomer concentration.

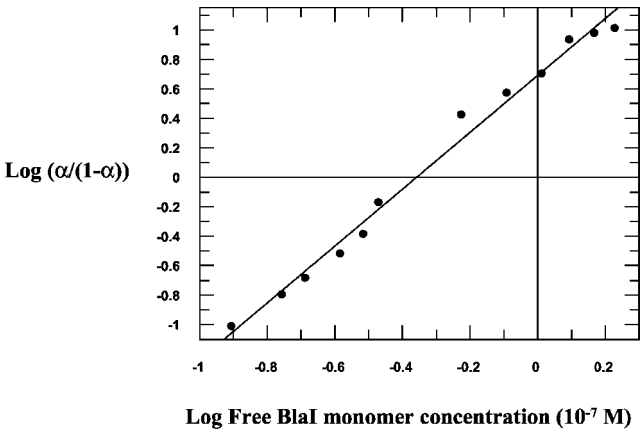

FIG. 7. **Hill plot of the data obtained for 10–90% saturation of OP1.** α corresponds to the fraction of Bound operator.

by binding time courses of BlaI to OP1. In this assay, 16 nM BlaI and 5 nM OP1 were incubated at 30 °C. The samples were taken after different incubation times and submitted to the electrophoresis. Fig. 10 shows that the fraction of bound OP1 stabilizes after 2 h.

Analysis of the DNA Binding Activity of BlaI—Because no BlaI monomer-DNA complex was observed in band shift assays, it can be concluded that a high degree of cooperativity prevails in the monomer pathway or that the dimer pathway is valid. Under these conditions, the fraction of bound DNA is $[BlaI]^2/([BlaI]^2 + K_1 \cdot K_1')$ according to the dimer pathway and $[BlaI]^2/([BlaI]^2 + K_2' \cdot K_3')$ according to the highly cooperative

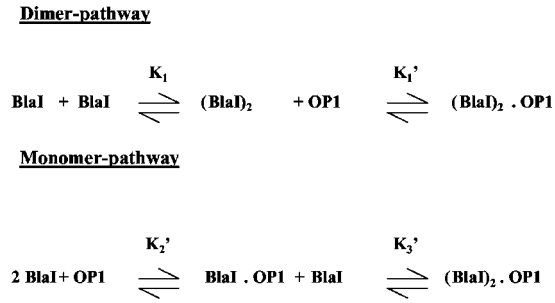

FIG. 8. **DNA-binding pathways of BlaI to its operators.**

monomer pathway. Only the global dissociation constant *K* ($K = K_1 \cdot K_1'$) can be estimated on the basis of the data of Fig. 5. The values are shown in Table III together with the K_1' values calculated on the basis of a K_1 (dimer/monomer) dissociation constant of 25 μM (see above). These results reveal that the affinity of BlaI for the operator sequences increase in the order OP3 < OP2 < OP1.

DISCUSSION

Previously, we reported that the β-lactamase induction in *B. licheniformis* 749/I first requires both an acylation of BlaR1 by a β-lactam and a β-lactam stress of the cell (6, 16). Our studies also indicated that in *B. licheniformis* 749/I, the hydrolysis of BlaI during β-lactamase induction was a consequence of its inactivation. This hypothesis was supported by the fact that in an induced *B. subtilis*/pDML995 strain, BlaI lost its DNA binding capacity but retained its ability to dimerize. In our working model, the inactivation of BlaI occurs by interaction with a coactivator that induces an inactive conformation of the BlaI dimer. As a result of this conformational change, the susceptibility of the BlaI dimer to the cytoplasmic proteases would be increased in *B. licheniformis* 749/I. The mechanism by which this conformational change prevents the binding of BlaI to its operators is not clarified yet.

The interaction between DNA-binding proteins and operators with a dyad symmetry can follow two distinct mechanisms (22): the monomer pathway and the dimer pathway (Fig. 8). The former is often cooperative and is observed for the LexA bacterial transcriptional repressor (23, 24). Many regulators bind DNA according to the dimer pathway (25). Because a helix-turn-helix recognition motif interacts only with five or six base pairs, the ability of this class of DNA-binding proteins to form dimers and higher order oligomers is fundamental to stabilize its binding to target DNA.

In this study, equilibrium ultracentrifugation experiments showed that at concentrations in the micromolar range, BlaI monomers, dimers, and tetramers were present in solution and that the BlaI multimerization follows a pathway described by isodesmic model 2 (Fig. 3). By fitting the experimental data, a

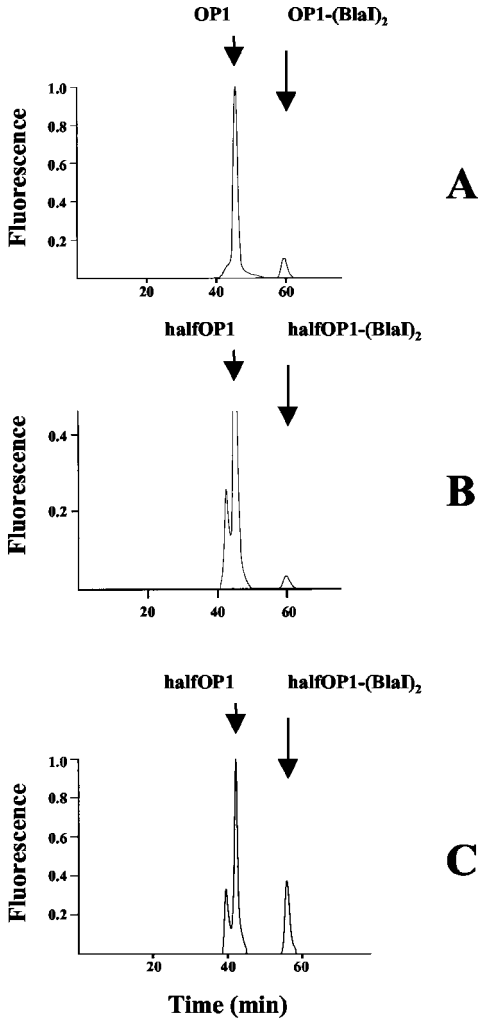

FIG. 9. **Band shift assays with the semi-conserved OP1 operator (*halfOP1*).** *A*, fluorogram obtained by incubation of BlaI with the OP1 operator in the presence of nonspecific DNA (400-fold excess). The final concentrations of BlaI and OP1 were 3.3 and 5 nM, respectively. The reaction mixture was incubated at 30 °C for 2 h. *B*, obtained by incubation of BlaI with the semi-conserved OP1 operator (*halfOP1*) in the presence of nonspecific DNA (400-fold excess). The final concentrations of BlaI and halfOP1 were 5.3 μM and 5 nM, respectively. *C*, obtained by incubation of BlaI with the semi-conserved OP1 operator (*halfOP1*) in the absence of nonspecific DNA. The final concentrations of BlaI and halfOP1 were 88 and 5 nM, respectively.

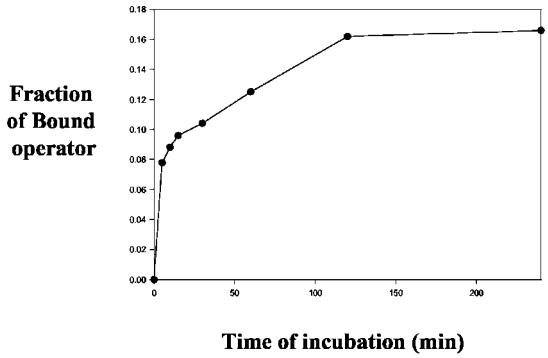

FIG. 10. **Binding time course of BlaI to the OP1 operator.** The concentrations of BlaI and OP1 were 16 and 5 nM, respectively. Nonspecific DNA (400-fold excess) was added to the reaction. The mixture was incubated at 30 °C, and the samples were taken after 5, 10, 15, 30, 60, 120, and 240 min.

TABLE III
Equilibrium parameters of the BlaI-operator interactions

K ($=K_1 \times K_1'$) was derived from the data shown in Fig. 5 on the basis of the equation given in the text for the dimer pathway. The values of K_1' were estimated at 25 μM by equilibrium ultracentrifugation.

	K	K_1'
	10^{-15} M^2	M
OP1	1.7 ± 0.5	4×10^{-11}
OP2	3.3 ± 0.9	7.76×10^{-11}
OP3	10.5 ± 2.5	2.5×10^{-10}

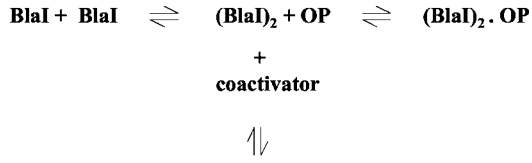

FIG. 11. **Model representing the inactivation pathway of BlaI upon β-lactamase induction in *B. licheniformis* 749/I and *B. subtilis*/pDML995.**

dissociation constant of 25 μM was found for the dissociation of the BlaI dimer, tetramer, or hexamer. To determine the *in vivo* concentration of BlaI, quantitative analysis of cytoplasmic extracts were performed by Western blotting. According to these results, it appeared that the intracellular concentration of BlaI in *B. licheniformis* 749/I (1.9 μM) is significantly lower than the dissociation constant of the BlaI dimer so that BlaI should be only partially dimeric in the cell. In *B. subtilis* transformed by the pDML995, a high copy number plasmid, the intracellular concentration of BlaI (75 μM), is 3-fold higher that the dissociation constant so that multimeric forms of BlaI are predominant. This result confirms previous cross-linking experiments in which the addition of increasing concentrations of dithiobis(succinimidylpropionate) to cytoplasmic extracts permitted the recovery of most of the BlaI signal as a dimer both in the absence and presence of β-lactams antibiotics (16). In consequence, it appears that the preformed BlaI dimer is present in both *B. licheniformis* 749/I and *B. subtilis*/pDML995 although in very different proportions.

The fluorescent band shift assays presented in this study revealed that the binding curves of BlaI to its operators are sigmoidal, suggesting that the binding parameters of BlaI include two equilibria. For each operator, the fluorograms were characterized by slower and faster bands corresponding to free operators and BlaI dimer-operator complexes, respectively (Fig. 9). In Scatchard plots, saturation of the operator by BlaI was reached at a molar ratio of 2 (Fig. 6). In these conditions, no BlaI monomer-operator complex was detected. In the monomer pathway, this would indicate a very strong cooperativity. Indeed, the Hill coefficient was 2, a value indicating a situation where the binding of the two monomers to the operator is simultaneous. Consequently, in this case, the monomer pathway and the dimer pathway cannot be distinguished on the basis of the Hill coefficient. By using the semi-conserved operator OP1 (half-OP1), additional arguments were found that favor the dimer pathway. In these experiments, we observed the formation of a complex between the BlaI dimer and half-OP1 both in the absence and the presence of nonspecific DNA. The absence of BlaI monomer-half-OP1 complex seems to exclude the possibility that the binding of BlaI occurs according to the monomer pathway. Previously, we obtained similar results by mass spectrometry that only the BlaI dimer could bind to the OP1 operator (26) and the semi-conserved OP1 operator.[1]

To substantiate the dimer pathway model, a time course of

[1] C. Vreuls and V. Gabelica, unpublished data.

the binding reaction was performed in conditions where BlaI was predominantly present as a monomer ([BlaI] = 0.016 μM). The determination of the fraction of bound DNA revealed that the equilibrium of the DNA binding reaction was only reached after 2 h. These results underline the presence of a rate-limiting step that could be the dimerization of BlaI.

Finally, it was also shown that BlaI recognized the three specific operators with distinct global dissociation constants ranging from 10^{-15} to 10^{-14} M^2. Because the dissociation of the BlaI dimer was 25 μM, the dissociation constants of the BlaI dimer-operator complexes are between 10^{-11} and 10^{-10} M. These values are typical of prokaryotic repressors with helix-turn-helix recognition motifs (25, 27). Comparison of the global dissociation constants indicates that BlaI presents increasing affinities for OP3 < OP2 < OP1. In regard to the nucleotide alignments of the operators presented in Fig. 1. It appears that the OP1 operator, which has the highest affinity for BlaI, is similar to the nucleotidic consensus sequence of the three operators of the *bla* divergeon. Consequently, these results suggest that in the absence of β-lactam antibiotics, transcription from the *blaP* promoter, containing the OP1 and OP2 operators, is more repressed than that from the *blaI-blaR1* promoter, which includes the OP3 operator. It has not been verified yet whether cooperative binding occurs between the three operators of the *bla* divergeon. Nevertheless, this hypothesis is supported by equilibrium ultracentrifugation and chemical cross-linking experiments (16) that reveal that BlaI is able to form higher order multimers. In addition, the analysis of the intergenic region of the *bla* divergeon reveals that the distance between OP1 and OP3 operators is 168 bp or 17 helical turns, indicating that these operators are on the same side of the DNA and could be involved in a looping of the DNA.

In conclusion, it is highly probable that the repression of β-lactamase synthesis in *B. licheniformis* 749/I requires preformed BlaI dimers (Fig. 11). In the presence of inducer, activation of the metalloprotease domain of the BlaR penicillin receptor and β-lactam stress suffered by the cell generate a coactivator in the cytoplasm. This coactivator induces a conformational change of the BlaI dimer that yields an inactive form of the repressor by a mechanism similar to that described for the inactivation of the TetR repressor involved in the tetracycline resistance in Gram-negative bacteria (28). Indeed, the binding of tetracycline to the C-terminal domain of the TetR repressor homodimer induces a conformational change that increases the distance between the two N-terminal binding domains of the dimer by 3 Å, abolishing the affinity of TetR for its operator without destabilizing the dimer.

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