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NOTES

Overproduction, Isolation, and DNA-Binding Characteristics of Xre, the Repressor Protein from the *Bacillus subtilis* Defective Prophage PBSX

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PBSX is a phage-like bacteriocin (phibacin) of *Bacillus subtilis* 168. Lysogeny is maintained by the PBSX-encoded repressor, Xre. The Xre protein was overproduced in *Escherichia coli* and isolated by affinity chromatography. Gel retardation and DNase I footprinting studies indicated that Xre binds to four sites close to its own gene. These sites overlap putative promoters for *xre* and a divergent transcriptional unit, containing the middle genes.

PBSX is a bacteriophage-like bacteriocin, or phibacin, of *Bacillus subtilis* 168 (4). PBSX DNA is maintained in the repressed state on the chromosome by the product of the *xre* gene (3, 5, 12, 13). Wood et al. described the isolation and sequence analysis of *xre* (13). Its putative product showed some similarity to the C2 repressor of phage P22, the P1/P2 C repressor, and the φ105 repressor, particularly in the vicinity of proposed helix-turn-helix motifs within the N-terminal domains of these proteins (13). Four 15-bp repeated palindromic sequences were identified upstream from the *xre* gene. These repeated sequences overlap putative SigA-type promoters, from which both the *xre* gene and a second gene (ORF10) could be transcribed divergently (see Fig. 4). It was proposed that Xre regulates the transcription of both genes, by binding to these repeats (operators [13]). In this communication we report the overproduction and purification of the Xre protein and demonstrate that it binds to the four operators identified by Wood et al. (13).

The *xre* gene was cloned from *B. subtilis* SO113 DNA by using PCR (2) with two primers designed to introduce *Nde*I sites at both ends of the sequence (primer 1, CATATGATA GGCGGCGGATTGA; primer 2, CATATGTTTTAGAGAA CAGA). Cycling parameters were 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s for 40 cycles. Amplified fragments were filled in with Klenow enzyme and blunt-end ligated into the *Sma*I site of pUC19 to give pXre1. Sequence analysis of the insert in pXre1 confirmed that the 366-bp *xre* sequence was intact. pXre1 was digested with *Nde*I to release the *xre* gene, which was ligated into the *Nde*I site of pET3-a. pET3-a (8) (Table 1) is an expression vector based on pBR322. It carries the T7 gene

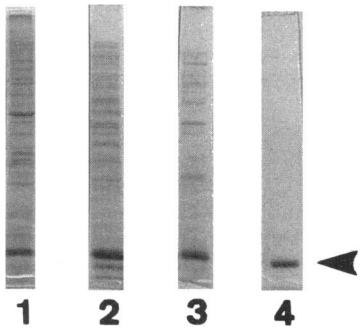

FIG. 1. Overproduction and purification of Xre from crude extracts. Proteins are shown separated by SDS-PAGE (15% polyacrylamide). Lanes: 1, *E. coli* BL21(DE3)/pLysS/pXre2-induced crude extract; 2, unbound protein fraction after adsorption to the anion exchanger DE52; 3, protein fraction eluted from the cation exchanger CM52 at 350 mM KCl; 4, protein fraction eluted from the Xre affinity column (see Materials and Methods) at 300 mM KCl. The arrowhead indicates the position of Xre.

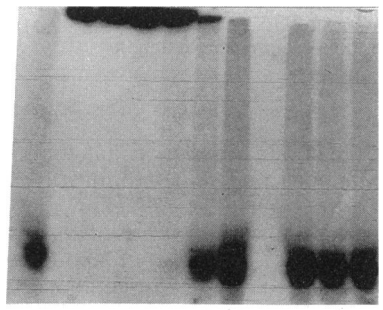

FIG. 2. Gel retardation of O-P DNA with purified Xre. A 0.5-ng portion of labeled DNA was added to 1 μg of cold nonspecific DNA [poly(dI-dC)] and mixed with purified preparations of Xre in a final volume of 10 μl. Xre concentrations used are indicated below. After incubation at 20°C for 30 min, samples were analyzed on 4% polyacrylamide gels in 1× running buffer (7 mM Tris-Cl [pH 7.5], 4 mM sodium acetate, 1 mM EDTA, 0.5 mM β-mercaptoethanol) at room temperature. Lanes: 1 and 10, 200 ng of BSA control per μl; 2, 100 ng of Xre per μl; 3, 75 ng/μl; 4, 50 ng/μl; 5, 32 ng/μl; 6, 20 ng/μl; 7, 16 ng/μl; 8, 8 ng/μl; 9, 2 ng/μl.

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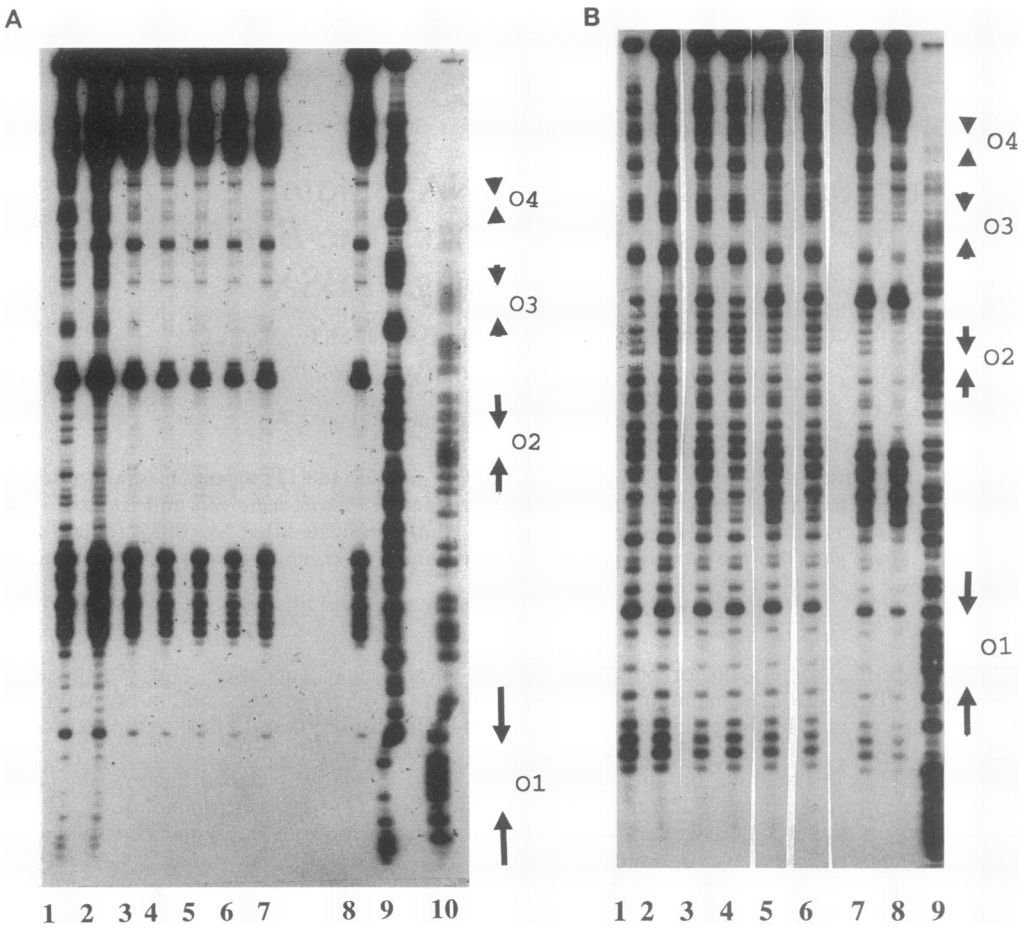

FIG. 3. DNase I footprinting of O-P fragment with Xre protein. The O-P fragment was amplified from pUC19/O-P by using universal primers, digested with *Ava*I and *Pst*I, and Klenow labeled with [α-^{32}P]dCTP. DNA-binding reactions were set up as described for gel retardation reactions, except that 50-μl volumes were used. DNase titrations indicated that 2 U of DNase I (Pharmacia) per reaction routinely gave the most uniform DNA ladder. Digestions were incubated at 20°C for 60 s in the presence of 0.1 mM $MgCl_2$ and 0.05 mM $CaCl_2$. Reactions were stopped with 140 μl of 192 mM sodium acetate–32 mM EDTA–0.14% SDS–64 μg of yeast RNA per ml, and the products were phenol-chloroform extracted and precipitated by the addition of 400 μl of ethanol. Reaction products were resuspended in 3 μl of loading dye (deionized formamide with 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol) and loaded onto a preheated 8% acrylamide gel (running in 1× Tris-borate-EDTA [TBE]). The DNA sequence of protected regions was determined with the aid of a sequence marker generated by Maxam and Gilbert G+A reactions (9). The positions of operator sites O1 to O4 are indicated. (A) Lanes: 1, 3.2 ng of Xre per μl; 2, 6.4 ng/μl; 3, 9.6 ng/μl; 4, 12.8 ng/μl; 5, 16 ng/μl; 6, 19.2 ng/μl; 7, 22.4 ng/μl; 8, 25.6 ng/μl; 9, control (100 ng of BSA per μl); 10, Maxam-Gilbert G+A sequencing reaction. (B) Lanes: 1, control (100 ng of BSA per μl); 2, 0.6 ng of Xre per μl; 3, 0.9 ng/μl; 4, 1.2 ng/μl; 5, 1.3 ng/μl; 6, 1.5 ng/μl; 7, 1.9 ng/μl; 8, 3.0 ng/μl; 9, Maxam-Gilbert G+A sequencing reaction.

10 promoter (ϕ10) and translation initiation site (s10), followed by a unique *Nde*I site and the T7 transcriptional terminator (T). Insertion in the correct orientation at the *Nde*I site places a gene under the control of the s10 translational initiation site. Insertions of *xre* were found in both the correct (pXre2) and incorrect (pXre3) orientations for expression. pXre2 and pXre3 were introduced into *Escherichia coli* BL21 (DE3) containing the compatible plasmid pLysS (Table 1). pLysS contains the T7 lysozyme gene expressed at low levels; this is a natural inhibitor of the T7 RNA polymerase and modulates expression from pET3-a (10). Transformants were grown to an A_{600} of 5 units and induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 1 h. Cells were isolated from 1 ml of culture and boiled in 200 μl of sodium dodecyl sulfate (SDS)-GLB, and samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide). The pXre2-containing clones overexpressed a 14-kDa protein when compared with known size standards (Fig. 1). This agreed well with the size expected for Xre (13.3 kDa). No plasmid-encoded protein was expressed in any detectable amount on induction of pXre3-containing clones.

Xre was subsequently purified by ion-exchange and affinity chromatography. The cells from 1 liter of *E. coli* BL21(DE3)/pLysS/pXre2 cell culture, as described above, were harvested and resuspended in 40 ml of sonication buffer. Lysozyme was added to a final concentration of 100 μg/ml, and the mixture was incubated at 37°C for 10 min. After sonication to lyse the cells, cell debris was removed by centrifugation. A 10-μl volume of DNase I (10 mg/ml) was added, and the mixture was incubated at 20°C for 30 min. Then 5 ml of cellulose anion exchanger (Whatman DE52) equilibrated with ion-exchange buffer (50 mM KCl, 10 mM Tris-Cl [pH 7.9], 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) was added to 40 ml of

TABLE 1. Bacterial plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pUC19	Ap^r	14
pUC19/x1	1.2-kb *Eco*RI, containing *xre* repressor, cloned into *Eco*RI site of pUC19	13
pLysS	Cm^r T7 lysozyme cloned into *Bam*HI of pACYC184	10
pET3-a	Ap^r ϕ10 promoter and transcription start site	8
pXre1	*xre* gene cloned into *Sma*I site of pUC19	This study
pXre2	*xre* gene cloned into *Nde*I site of pET3-a, positive orientation for expression	This study
pXre3	*xre* gene cloned into *Sma*I site of pUC19, negative orientation for expression	This study
E. coli		
TGI	K-12 Δ(*lac-pro*) *supE thi hsdR* F′, *traD36 proAB lacI lacZ* ΔM15	Amersham
BL21(DE3)	*hsdS gal* (*cIts857 ind1 Sam7 nin5 lacUV5*-T7 gene 1)	10
B. subtilis 168		
SO113	*trpC2 amy-3*	6

sonicated cell extract, mixed gently for 2 h, and gradient eluted by increasing the concentration of KCl in ion-exchange buffer. Xre was found to be present in the unbound fraction (Fig. 1), which was dialyzed overnight at 4°C in the ion-exchange buffer at 50 mM KCl. The unbound fraction was then similarly adsorbed to the cation exchanger CM52 (Whatman); Xre was found to elute at 350 mM KCl in a stepwise gradient (Fig. 1). Xre was estimated to be greater than 50% of the total protein in this fraction. Xre fractions were dialyzed overnight in ion-exchange buffer at 50 mM KCl at 4°C.

Preliminary gel retardation analysis on the operator-promoter (termed O-P fragment) upstream of *xre*, by using crude and partially purified Xre extracts, demonstrated that Xre was binding to this region (results not shown). On the basis of these results, the O-P fragment was used for affinity chromatography. The 151-bp O-P fragment was cloned in pUC19, allowing amplification with M13/pUC universal primers. Maximum amplification was found at 3 mM $MgCl_2$ in the presence of 30 ng of template DNA and was carried out, as described above, for 30 cycles. By using a 24-mer forward primer which was biotinylated at one end (a gift from S. Krogh), amplified products could be fixed to streptavidin-agarose (Sigma). A 200-μl volume of the support was prepared with 1 ml of binding buffer (12% glycerol, 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 4 mM Tris-Cl [pH 7.9], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) containing 500 μg of bovine serum albumin (BSA) per ml and 200 μg of poly(dI-dC). After being mixed for 5 min at 20°C, the support was washed in elution buffer (12% glycerol, 12 mM Tris-Cl [pH 6.8], 1 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol), equilibrated in binding buffer, and mixed with 200 ng of biotinylated target fragment. Dialyzed cation-exchange cell extracts were added to the resin, including an excess of poly(dI-dC), and mixed at 20°C for 1 h. After two washes in an equal volume of binding buffer, proteins were eluted from the support with binding buffer at KCl concentrations up to 1 M. Xre bound to the resin; the majority could be eluted at 300 mM KCl. Purified Xre was dialyzed overnight in binding buffer at 50 mM KCl and analyzed by SDS-PAGE (15% polyacrylamide) (Fig. 1). The Xre protein preparation contained no other detectable proteins.

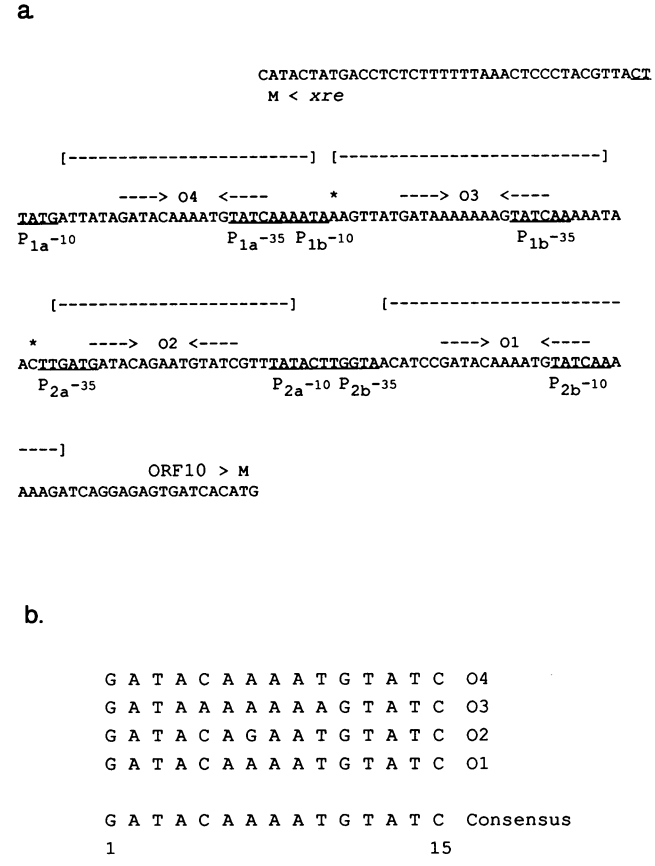

FIG. 4. (a) Summary of DNase I footprinting experiments. The sequence of the O-P fragment between *xre* and ORF10 is shown. Xre-binding sites (O4/O1) are indicated as inverted arrows above the sequence, and the positions of putative divergent promoters ($P_{1a/b}$ and $P_{2a/b}$), as proposed by Wood et al. (13), are underlined. The regions protected from DNase I digestion by Xre are indicated by brackets. Hypersensitive sites between O2/O3 and O3/O4 are indicated by asterisks. (b) Alignment of operator sites O4 to O1. The deduced consensus sequence is shown below. Each base pair has been numbered from 1 to 15 for the purpose of discussion.

Gel retardation experiments (1) were performed by using a radioactive O-P fragment prepared by PCR amplification and end labeled with Klenow enzyme by using [α-^{32}P]dCTP (>3,000 Ci/mmol). When 8 ng of purified Xre per μl and 0.5 ng of DNA were used, no retardation was observed (Fig. 2). However, as Xre concentrations were increased from 16 to 24 ng/μl, a proportion of the DNA was retarded, and by 32 ng/μl, total retardation was seen. These results confirmed the efficacy of the purification protocol. Only free and fully retarded species were observed when purified extracts were used. At saturation, retardation could be successfully inhibited by unlabeled O-P fragment at 5 to 10 ng/μl (results not shown). Competition experiments with nonspecific DNA suggested that retardation was specific to Xre/O-P interactions.

For DNase I footprinting, the O-P fragment (PCR amplified from pUC19/x1) was cut with *Ava*I and *Pst*I and Klenow labeled with [α-^{32}P]dCTP. DNA-binding reactions were carried out as described for gel retardation reactions, except that they were in 50-μl volumes; 2 to 5 μl of the reaction mixture was subjected to gel retardation analysis to check binding.

At protein concentrations that retarded the O-P fragment

during electrophoresis, four sites within the labeled fragment were protected from DNase digestion (Fig. 3); these sites were found to be centered at previously identified 15-bp palindromic sequences with a consensus of GATACAAAATGTATC (13) (Fig. 4). The four sites may have different affinities for Xre. O1 and O2 appeared to be bound to some extent at Xre concentrations below those at which O3 and O4 were bound (Fig. 3).

There is a high degree of similarity among the four sites of Xre binding. The consensus for these sites is GATACAAAAT GTATC (Fig. 4). The footprinting studies with Xre revealed DNase I-hypersensitive sites between O2 and O3 and between O3 and O4 (Fig. 4); these suggest that the DNA may be distorted by the binding of Xre. The apparent operator sites and flanking base pairs, especially toward the 3′ ends, are A+T rich, which also suggests DNA flexibility. The Xre operator sites are similar in this respect to the operator sites of phage 434, which are A+T rich, especially within the central region (11). In comparison, the λ operator consensus (TATCACC GCCGGTGATA) is G+C rich (7).

Although the physiological roles of the operator sites remain to be demonstrated by the introduction of point mutations in each of the sites, it is interesting to speculate that the binding of Xre at O1 and O2 would block both putative promoters for the rightward operon (as identified by Wood et al. [13]). The evidence presented in the accompanying paper shows that genes distal to open reading frame ORF10 are involved in regulating transcription of the late operon (3). We propose that Xre directly blocks rightward transcription and is therefore critical to maintaining the lysogenic state. Binding of Xre to O3 and O4 would block the putative leftward promoters from which *xre* is expected to be expressed. Thus, Xre is likely to regulate its own synthesis. This kind of arrangement has been well characterized in the immunity regions of lambdoid phages, in which an autoregulated repressor protein is expressed divergently from an operon which is also controlled by the repressor (7).

To search for additional operator sites within the PBSX genome and elsewhere on the chromosome, we synthesized a pool of oligonucleotides (GATANANAANGTATC) based on the sequences of the four apparent operator sites (Fig. 4). The 15-mers (10 pmol) were end labeled and used to probe *B. subtilis* SO113 chromosomal digests. Hybridization was observed only to the 1.2-kb *Eco*RI fragment x1, which includes the O-P fragment (results not shown). Xre presumably acts only at sites O1 to O4.

In conclusion, the evidence shows that *xre* encodes the PBSX primary repressor, Xre. Xre binds to four sites in the region between the divergently transcribed genes *xre* and ORF10. Xre binding to operators O1 and O2 may repress rightward transcription of ORF10 and other downstream middle genes which are important for lytic growth (3). Xre may regulate transcription of its own gene by binding to O3 and O4, thereby preventing leftward transcription and expression of *xre*.

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REFERENCES

1. **Butler, G.** 1989. Ph.D. thesis. University of Dublin, Ireland.
2. **Erlich, H. A.** 1989. P.C.R. technology. Stockton Press, New York.
3. **McDonnell, G. E., H. Wood, K. M. Devine, and D. J. McConnell.** 1994. Genetic control of bacterial suicide: regulation of the induction of PBSX in *Bacillus subtilis*. J. Bacteriol. **176**:5820–5830.
4. **Okamoto, K., J. A. Mudd, J. Mangan, W. M. Huang, T. V. Subbaiah, and J. Marmur.** 1968. Properties of the defective phage of *Bacillus subtilis*. J. Mol. Biol. **34**:413–428.
5. **O'Kane, C., M. A. Stephens, and D. J. McConnell.** 1986. Integrable α-amylase plasmid for generating random transcriptional fusions in *Bacillus subtilis*. J. Bacteriol. **168**:973–981.
6. **Ortlepp, S. A., J. F. Ollington, and D. J. McConnell.** 1986. Integrable α-amylase plasmid for generating random transcriptional fusions in *Bacillus subtilis*. Gene **23**:267–276.
7. **Ptashne, M.** 1992. A genetic switch. Blackwell Scientific Publications, Oxford.
8. **Rosenberg, A. H., B. N. Lade, D. Chui, S. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
9. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
10. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
11. **Wharton, R. P., E. L. Brown, and M. Ptashne.** 1984. Substituting an α-helix switches the sequence-specific DNA interactions of a repressor. Cell **38**:361–369.
12. **Wood, H. E., M. T. Dawson, K. M. Devine, and D. J. McConnell.** 1990. Characterization of PBSX, a defective prophage of *Bacillus subtilis*. J. Bacteriol. **172**:2667–2674.
13. **Wood, H. E., K. M. Devine, and D. J. McConnell.** 1990. Characterisation of a repressor gene (xre) and a temperature-sensitive allele from the *B. subtilis* prophage, PBSX. Gene **96**:83–88.
14. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.