A DNA Sequence Containing the Control Sites for the uxaB Gene of Escherichia coli

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(Received 7 August 1985; revised 14 October 1985)

The nucleotide sequence of a 286 bp fragment containing the uxaB control region of Escherichia coli has been determined. The transcriptional start of the uxaB gene has been located and the promoter signals identified. Various fragments of the uxaB promoter-proximal region were fused in vitro with the lacZ gene. Results obtained with these fusions indicate that the operator-promoter sites are located on a 110 bp restriction fragment. The determination of the amino acid sequence of the NH2-terminus of the uxaB gene product revealed that the uxaB gene is not initiated with the AUG codon but with the unusual GTG codon. CRP, the cyclic AMP receptor protein, does not bind to the uxaB control region DNA even though expression of the uxaB gene is sensitive to catabolite repression.

INTRODUCTION

Escherichia coli K12 can grow on the aldohexuronates D-glucuronate and D-galacturonate, which are metabolized via the Ashwell pathway (Ashwell, 1962). The first steps in the catabolism of galacturonate involve, in order, products of the structural genes exuT, uxaC, uxaB and uxaA. These genes form the exu regulon. Their expression is subject to negative control by the ExuR repressor, but UxuR, another repressor of the hexuronate system, has no effect on their expression (Portalier et al., 1980; Ritzenthaler et al., 1983). Their expression is also sensitive to catabolite repression by glucose (Hugouvieux-Cotte-Pattat, 1981). The uxaB gene is located at min 52 on the E. coli genome (Bachmann & Low, 1980) and codes for altronate oxidoreductase, the synthesis of which is induced by galacturonate, tagaturonate and fructuronate (Robert-Baudouy et al., 1974).

The exuR and uxuR regulatory genes, and the uxaB gene, have been individually cloned into the plasmid vector pBR322 or its derivatives (Ritzenthaler et al., 1981; Ritzenthaler & Mata-Gilsinger, 1982; Blanco et al., 1983). The regulatory region of uxaB was isolated on a 2.5 kb EcoRI1–EcoRI2 fragment (Fig. 1), and it has been shown that uxaB is transcribed from the EcoRI1 towards the EcoRI2 site (Blanco et al., 1983). The purpose of this work was to determine the nucleotide sequence of the uxaB control region and to locate on this sequence the promoter and the operator. The latter were more precisely located by the construction and expression of uxaB–lacZ fusions formed in vitro on plasmid vectors.

METHODS

Bacterial strains, plasmids and culture media. All the strains were E. coli K12 derivatives: HB101, Pro–Leu–StrR HsdM–HsdR–EndoI–RecA–LacY– (Boyer & Roulland-Dussoix, 1969); MC1061, araD139 A(ara-leu)7697 AlacX74 GalU–HsdR–StrR (Minton, 1984); 71-18, A(lac-pro) F’laclq lacZAM15 Pro+ SupE– (Dente et al., 1983); 1475, ExuR–ArgG–HisA– (Portalier et al., 1980).

The lac fusion plasmids pNM480, pMN481 and pMN482 (Minton, 1984) were used for the detection of uxaB

Abbreviation: SS-DNA, single-stranded DNA.
translational initiation signals. Plasmids pEB3 (uxaB\(^+\)), pEB7 (uxaBo\(^+\)) (Blanco et al., 1983), pRU101 (uxuR\(^+\)) and pRE101 (exuR\(^+\)) (Ritzenthaler et al., 1983), are pACYC184 (tet\(^+\), cat\(^+\)) derivatives (Chang & Cohen, 1978); plasmids pEMBL8 and pEMBL9 (Dente et al., 1983) were used for cloning and sequencing uxaB fragments.

Media for growth were identical to those described by Miller (1972). The synthetic medium was M63 (pH 7.2) and contained glycerol (5 g l\(^{-1}\)). When needed, ampicillin and chloramphenicol were used at final concentrations of 25 \(\mu\)g ml\(^{-1}\).

Chemicals. D-Galacturonate and \(p\)-aminobenzyl \(\beta\)-D-thiogalactosamine agarose were purchased from Sigma, and chloramphenicol and ampicillin from Serva. Purified CRP protein was a gift of B. Blazy. RNA polymerase was obtained from BRL. Fructuronate was synthesized in our laboratory.

Enzyme induction, preparation and assay. The conditions for induction, extraction and assay of altronate oxidoreductase were as outlined previously (Robert-Baudouy et al., 1974; Portalier & Stoeber, 1972). \(\beta\)-Galactosidase was assayed by the method of Miller (1972) in exponentially growing cells. All the specific activities reported are the means of at least three separate assays.

Isolation and analysis of plasmid DNA. This was done as described by Blanco et al. (1983) and Maniatis et al. (1982).

3'-labelling of DNA fragments. EcoRI–HindIII fragments purified from pEMBL-E plasmids were 3'-end-labelled with DNA polymerase I Klenow fragment.

Construction of pEMBL-E vectors. The EcoRI–EcoRI\(_1\) fragment of pEB7 bearing the regulatory region of the uxaB gene was purified and introduced into the EcoRI site of pEMBL8 in each of the two possible orientations, yielding pEMBL-E1 and pEMBL-E2 (Fig. 1). In pEMBL-E1, the EcoRI\(_1\) site is located near the laeZ sequence of pEMBL8, whereas in pEMBL-E2 it is EcoRI, that is adjacent to laeZ. The EcoRI–EcoRI\(_1\) fragment possesses a unique NruI site and no SmaI site (Blanco et al., 1983); pEMBL-E1 and -E2 were digested with NruI and SmaI and ligated in vitro with T4 DNA ligase. The resulting plasmids bearing NruI-SmaI deletions were named pEMBL-E3 and pEMBL-E4 (Fig. 1).

The purified EcoRI–EcoRI\(_1\) fragment was digested with Sau3A, and the fragments generated were inserted into the BamHI site of pEMBL8. The pEMBL8 hybrids containing uxaB Sau3A fragments were introduced into strain 71-18 by transformation. Transformant colonies were screened for constitutive altronate oxidoreductase synthesis, since multiple copies of the uxaB operator cause derepression of the uxaB chromosomal gene due to titration out of the ExuR repressor. In clones showing derepression of uxaB, the recombinant plasmids contained a unique 200 bp Sau3A fragment. These plasmids were named pEMBL-E5. The 200 bp Sau3A fragment was transferred from pEMBL8 to pEMBL9 using the EcoRI and HindIII sites flanking the pEMBL linker. The resulting plasmid was named pEMBL-E6.

The NruI–HincII (SalI site of pEMBL polylinker) fragment of pEMBL-E5 was deleted, yielding pEMBL-E7; similarly pEMBL-E8 and pEMBL-E9 respectively resulted from the deletion of the HincII fragment of pEMBL-E5 and pEMBL-E4.

Construction of uxaB–lac fusions. EcoRI–HindIII fragments of pEMBL-E were transferred to the vectors pNN480, -481 and -482, since pNN plasmids possess the same polylinker cloning sites as pEMBL. The resulting pNN vectors were named pNN-Ex according to the fragment Ex inserted. Fusions were made in the three reading frames using pNN480, -481 and -482. \(\beta\)-Galactosidase synthesis was obtained only with one of the three pNN vectors.

SI nuclease mapping. RNA was purified by hot phenol extraction (Salser et al., 1967) from strain 1475 (ExuR\(^-\)) containing the pEB3 (uxaB\(^+\)) plasmid. Cells were grown on minimal medium M63 containing glycerol (0.4%, w/v).

Single-stranded (SS) DNA probes, complementary to the uxaB sequences cloned into pEMBL-E1 and pEMBL-E7, were prepared according to Burke (1984). SS-DNA template was annealed with the universal sequencing primer and the primer was extended with the SS-DNA probe (100 ng; BRL) for 30 min at 37°C. The 32P-label SS-DNA products were sized on denaturing sequencing gels.

Purification of uxaB–lacZ-encoded protein. Hybrid \(\beta\)-galactosidase encoded by a uxaB–lacZ fusion was purified by affinity chromatography, from a culture of strain 1475 containing plasmid pEBG1 (Blanco et al., 1983). Cells were disrupted in a French pressure cell and the mixture was centrifuged at 12000 r.p.m. for 15 min. The supernatant was made 36% saturated with ammonium sulphate, and centrifuged at 12000 r.p.m. for 15 min; the resulting pellet was dissolved in buffer (10 mM-Tris pH 7.0, 10 mM-magnesium acetate, 0.1 mM-NaCl, 10 mM-mercaptoethanol), and applied to a 10 ml column of p-aminobenzyl \(\beta\)-D-thiogalactosamine agarose. The eluted
DNA sequence of the \textit{uxaB} regulatory region

The 2.5 kb \textit{EcoRI} fragment containing the \textit{uxaB} regulatory region was purified from pEB7 and cloned into pEMBL-8, in the two orientations, yielding pEMBL-E1 and -E2 (Fig. 1). Various restriction fragments of the \textit{EcoRI}_1–\textit{EcoRI}_2 segment bearing the entire or part of the \textit{uxaB} regulatory region were cloned into pEMBL-8 and pEMBL-9, yielding pEMBL-E3 to -E9 (Fig. 1). The SS-DNA of these plasmids was used as template for the determination of the nucleotide sequence of the \textit{uxaB} control region by the dideoxy chain terminator method of Sanger et al. (1977). Except for the sequence extending from the \textit{Sau3A}_1 to the \textit{EcoRI}_1 sites, the nucleotide sequence shown in Fig. 2 was determined several times in both strands according to the various overlaps obtained.
Identification of the transcriptional start of the uxaB gene

The 5' end of uxaB messenger RNA was located by S1 nuclease mapping using uniformly labelled SS-DNA probes containing the relevant strand of the DNA fragments. The SS-DNA molecules derived from products of S1 mapping were detected and sized on sequencing gels, in comparison to sequencing ladders derived from pEMBL-E vectors. The EcoRI1–NruI, NruI–EcoRI2 and Sau3A1–Sau3A2 SS-DNA fragments were used as probes for S1 mapping. The NruI–EcoRI2 fragment hybridized with mRNA was not cleaved by S1 treatment, showing that the transcription start point is upstream of the NruI site. The sizes of the RNA :DNA hybrid molecules obtained with EcoRI1–NruI and Sau3A1–Sau3A2 were respectively 61 ± 1 bp and 144 ± 1 bp, indicating that the start point of uxaB transcription is at position 110 ± 1 on the nucleotide sequence.

Putative −10 and −35 regions of the uxaB promoter can be identified. The presumptive −10 region, TGTTCGT (nucleotides 100 to 105), is 18 nucleotides distant from the −35 region, TCGGGCG.

Expression and regulation of uxaB–lacZ fusions

All fragments cloned on pEMBL vectors and sequenced were transferred into the fusion probe vectors pNM480, -481 and -482. These vectors carry the lac operon in which the
Table 1. Regulation of uxaB-lacZ gene expression

\[\text{β-Galactosidase activity is expressed in milliunits [nmol product min}^{-1} \text{ (mg dry weight)}^{-1}\text{], and is}
\]
\[\text{normalized to a constant level of β-lactamase activity. All the assays were at 30 °C. The values reported}
\]
\[\text{are means of at least three separate assays, results from which varied by <5% about the mean. No}
\]
\[\text{β-galactosidase activity was detectable in the presence of the hybrid plasmids pNM-E2, -E4, -E6 or -E9,}
\]
\[\text{or that of the parental plasmids pNM-480, -481 and -482.}
\]

<table>
<thead>
<tr>
<th>Growth medium*</th>
<th>Plasmid in trans</th>
<th>\text{β-Galactosidase specific activity* in the presence of:}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pNM-E1</td>
<td>pNM-E3</td>
</tr>
<tr>
<td>Glycerol</td>
<td>none</td>
<td>1410</td>
</tr>
<tr>
<td>Glucose</td>
<td>none</td>
<td>160</td>
</tr>
<tr>
<td>Glucose + cAMP (10 mM)</td>
<td>none</td>
<td>1580</td>
</tr>
<tr>
<td>Glycerol</td>
<td>pACYC184 (control)</td>
<td>1420</td>
</tr>
<tr>
<td>Glycerol</td>
<td>pRE101 (exuR⁺)</td>
<td>20</td>
</tr>
<tr>
<td>Glycerol + tagaturonate (5 mM)</td>
<td>pRE101 (exuR⁺)</td>
<td>290</td>
</tr>
<tr>
<td>Glycerol</td>
<td>pRU101 (uxuR⁺)</td>
<td>1450</td>
</tr>
</tbody>
</table>

* M63 medium with the additions shown. Glycerol and glucose were used at 5 g l⁻¹.

regulatory region and the first eight codons of lacZ have been removed and replaced by the same polylinker cloning sites as in pEMBL. They allow the expression of hybrid β-galactosidase when a DNA fragment containing transcriptional and translational signals is introduced in the correct orientation in front of the lac genes. The gene fusion can be made in all three translational readings frames using the three vectors pNM480, -481 and -482. When the inserted fragment contains a promoter but no translational signals, only the lacY gene is expressed.

Transcriptional and translational signals were examined in the three reading frames by introducing pNM derivatives into strain MC1061 (Δlac). β-Galactosidase activity was detected in strain MC1061 containing pNM-E1, pNM-E3, pNM-E5, pNM-E7 and pNM-E8, showing the presence of transcriptional and translational signals in the corresponding DNA fragments. As expected in each case, these signals were only detected with one of the three pNM vectors. All the experiments with the various inserts gave the same result in relation to the translational phase of the uxaB gene. To seek promoter activity in fragments -E2, -E4, -E6 and -E9, pNM vectors bearing appropriate inserts were introduced into HB101 (Lac⁺). In no case did these plasmids restore a Lac⁺ phenotype to strain HB101, demonstrating the absence of a promoter activity in the insert fragments.

These results indicated that transcriptional and translational signals are both included in the 110 bp Sau3A₁-NruI fragment. To prove that these signals belonged to uxaB gene, the regulation of β-galactosidase synthesis was analysed in strain MC1061 containing plasmids pACYC184, pRE101 (exuR⁺) or pRU101 (uxuR⁺) in trans to the fusion plasmid. β-Galactosidase synthesis was specifically repressed upon introduction of pRE101 (exuR⁺); transformation with the control plasmid pACYC184 or with pRU101 (uxuR⁺) had no effect on the expression of the uxaB-lacZ gene. The repression exerted by the ExuR regulatory molecule could be reversed upon addition of tagaturonate as inducer (Table 1).

Hugouvieux-Cotte-Pattat (1981) showed that uxaB expression is sensitive to catabolite repression by glucose, which is reversed by cAMP. The uxaB-lacZ fusions carried by the pNM vectors were also sensitive to this catabolite repression. β-Galactosidase synthesis was strongly decreased in cells grown on glucose; when cAMP was added, the β-galactosidase activity was identical to that observed in glycerol-grown cells (Table 1). The 110 bp Sau3A₁-NruI fragment in pNM-E7 was sufficient to confer sensitivity to glucose repression (Table 1). All the regulatory properties of the expression of the uxaB-lacZ fusions were identical to those observed for the wild-type uxaB gene.

The repression of β-galactosidase synthesis exerted by the exuR product in strains containing
Table 2. Expression of uxaB in strain 71-18 containing the plasmids pEMBL-E1 to -E9

Specific activities are given in milliunits [nmol product min^{-1} (mg dry weight)^{-1}]. Cells were grown at 37 °C to an appropriate cell density in glycerol M63 medium with or without inducer and assayed for enzyme activity after treatment in a French press. The induced activities measured in the presence of the plasmids pEMBL-E1 to -E9 were identical to those obtained with the parental plasmid pEMBL8 or 9. The values reported are means of at least three separate assays, results from which varied by <5% about the mean.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Inducer (galacturonate, 5 mm)</th>
<th>Altronate oxidoreductase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEMBL8 or 9</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>pEMBL-E1</td>
<td>-</td>
<td>1010</td>
</tr>
<tr>
<td>pEMBL-E2</td>
<td>+</td>
<td>490</td>
</tr>
<tr>
<td>pEMBL-E3</td>
<td>-</td>
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</tr>
<tr>
<td>pEMBL-E4</td>
<td>-</td>
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</tr>
<tr>
<td>pEMBL-E5</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>pEMBL-E6</td>
<td>-</td>
<td>410</td>
</tr>
<tr>
<td>pEMBL-E7</td>
<td>-</td>
<td>380</td>
</tr>
<tr>
<td>pEMBL-E8</td>
<td>-</td>
<td>370</td>
</tr>
<tr>
<td>pEMBL-E9</td>
<td>-</td>
<td>450</td>
</tr>
</tbody>
</table>

pNM-E7 showed that the 110 bp Sau3A1-NruI segment carries the operator site of uxaB. This result was confirmed by the ability of this fragment to titrate out the chromosomal ExuR repressor, leading to constitutive expression of the uxaB gene (Table 2). All the other fragments containing the Sau3A1-NruI segment had the same effect. The level of derepression observed in the presence of pNM-E7 was greater than that obtained in the presence of pEB7, a pACYC184 derivative containing the EcoRI1-EcoRI2 fragment (Blanco et al., 1983). This difference may be explained by the fact that pEMBL and pNM vectors are both pBR322 derivatives affected for copy number control (Dente et al., 1983; Minton, 1984) as found for pAT153 (Twigg & Sherratt, 1980), where the copy number is increased 1.5- to 3-fold.

Peptide sequence of the NH₂-terminus of altronate oxidoreductase

An identical reading frame was found for all the uxaB-lacZ fusions, but no ATG initiator codon could be detected in this reading frame. Fusions performed at the NruI site led to the expression of a hybrid β-galactosidase, suggesting that an initiator codon is located upstream of this site. A putative initiator codon GTG at position 160 of the nucleotide sequence is preceded by a good ribosome-binding site AAGG (Shine & Dalgarno, 1974). To confirm the role of GTG as the initiator codon for the uxaB gene, the amino acid sequence of the NH₂-terminus of altronate oxidoreductase was determined. Hybrid β-galactosidase encoded by the uxaB-lacZ fusion of plasmid pEBG1 was purified to near homogeneity and subjected to Edman degradation. The NH₂-terminal sequence determined was NH₂-Met-Lys-Thr-Leu, which corresponds to the NH₂-terminal sequence of altronate oxidoreductase; it is in complete agreement with the codons from nucleotides 150 to 161. This result shows unambiguously the use of the GTG codon as initiator codon for the uxaB gene.

The uxaB regulatory region has no CRP-binding site

In most known catabolite-activated operons, principally those involved in the catabolism of growth substrates, the activation is mediated by the catabolite activator protein CRP. In the presence of its effector, cAMP, CRP stimulates the initiation of transcription at the promoter of these genes. Since the uxaB and uxaB-lac fusions were sensitive to glucose catabolite repression which was reversed by cAMP, we searched for putative CRP-binding sites in the uxaB sequence. The binding of CRP-cAMP complex to uxaB sequences was tested by the gel electrophoretic method described by Garner & Revzin (1981), Fried & Crothers (1983) and Kolb et al. (1983). The binding of proteins to DNA can be easily detected since it alters the electrophoretic mobility of the DNA fragment. The 3'-end-labelled Sau3A₁-Sau3A₂ fragment
DNA sequence of the uxaB regulatory region

Fig. 3. Test for specific CRP binding to the uxaAB and uxaB promoter regions. Each assay contained 200 μM-cAMP and from 0 to 400 nM-CRP. (a) Migration of the uxaB Sau3A₁–Sau3A₂ fragment and of DNA fragments containing the uxaAB control region; (b) migration of the uxaB Sau3A₁–Sau3A₂ fragment alone. Each restriction fragment was 3'-end-labelled.

was incubated in the presence of various concentrations of CRP, with 200 μM-cAMP, and the protein–DNA mixture was submitted to electrophoresis in 8% polyacrylamide gels. No binding of CRP to the uxaB fragment was found, whereas in the same conditions, an 180 bp DNA fragment containing the uxaAB CRP-binding site was displaced in a well-defined band (Fig. 3).

**DISCUSSION**

In vitro fusions of uxaB with the lacZ gene have shown that the entire uxaB control region resides in a 110 bp Sau3A₁–NruI DNA fragment, which contains the uxaB promoter, the uxaB operator and the DNA signals necessary for catabolite repression. The nucleotide sequence of the uxaB control region was determined and we tried to locate the various regulatory signals on this sequence. The transcription start was identified at position 110 ± 1 on the nucleotide sequence. As regards the mRNA start, the examination of the sequence reveals the presence of −10 and −35 sequences which have poor correspondence with the consensus sequences that have been established for *E. coli* promoters (Rosenberg & Court, 1979; Hawley & McClure, 1983). The Bribnow box is imperfect, and the spacing between these two elements (18 bp) is more than the optimal 17 bp (Hawley & McClure, 1983; Aoyama et al., 1983). Such features suggest that this putative promoter would show only weak activity. To correlate the high *in vivo* expression of the uxaB gene or the uxaB–lac fusions with this weak promoter sequence we propose an activation mechanism. Physiological and genetic studies have never shown the action of a specific activator for uxaB expression, so we think that such an activator must have a pleiotropic effect, like the CRP–cAMP complex.

The expression of uxaB and uxaB–lac fusions is sensitive to catabolite repression by glucose, which is reversed by cAMP. The structure of the uxaB promoter is consistent with activation by the CRP–cAMP complex. In fact, examination of the sequences around the transcription start point of CRP-regulated promoters reveal a particularly poor correspondence with the consensus sequences determined for *E. coli* promoters: in most cases the −35 sequence is barely apparent, the Bribnow box is often imperfect, and the spacing between these two elements is more than the optimal 17 bp (De Crombrugghe et al., 1984). Unfortunately no CRP-binding site was detected in the uxaB sequence by alteration of electrophoretic mobility. This result is not surprising since the only putative CRP-binding site, presenting some homology with the consensus sequence AA–TGTGA–N₇–CACA (De Crombrugghe et al., 1984), is located at position 149. The highly conserved sequence TGTGA is present, but the location of this putative site downstream of the promoter makes it unlikely that this sequence has a physiological role. We can exclude the possibility of a CRP-binding site being located upstream of the sequenced fragment because the expression of the uxaB–lac fusion obtained with the Sau3A₁–NruI fragment is sensitive to catabolite repression.
The catabolite repression exerted by glucose on the uxaB–lac fusions is possibly not mediated by the CRP–cAMP complex. The inducer exclusion phenomenon (Kornberg et al., 1980) could affect uxaB gene expression in the wild-type strain, but the uxaB–lac genes carried by multicopy vectors are expressed constitutively, due to repressor titration, thus expression cannot be abolished by an inducer exclusion mechanism. Catabolite repression is not exclusively regulated by the intracellular level of cAMP (Ullman & Danchin, 1980), it affects lac genes in a Δcya strain (Dessein et al., 1978). So it is possible that the catabolite repression of uxaB–lac fusions is mediated by another effector, possibly CMF (Ullman et al., 1976). The catabolite repression mechanism exerted on uxaB expression, and the existence of an activator for uxaB transcription, need further investigation.

The use of GUG as the initiator codon might decrease the rate of initiation of translation of the uxaB mRNA, as is the case for lacI (Steege, 1977). Nevertheless, the features observed in the DNA sequence of uxaB show that the use of the weak GUG initiator codon is compensated by a good Shine–Dalgern sequence, by an optimal spacing separating this sequence from the GUG codon, and by the presence of the AAAA sequence following this codon (Kozak, 1983; Stormo et al., 1982; Gheysen et al., 1982). Thus the use of the weak GUG initiator codon for uxaB is compensated by the organization and the nature of the DNA sequence around the initiator codon. The presence of a weak promoter and GUG codon would be expected to lead to low expression of the uxaB gene. In fact, in induced cells (wild-type or containing uxaB+ plasmids) the uxaB product is synthesized in large amounts, allowing its easy purification (Blanco et al., 1983; Portalier & Stoebler, 1972).

The operator of the uxaB gene was located to within the Sau3A,-NruI fragment. The DNA sequence of this fragment presents some homologies with that of the exuT–uxaCA regulatory region (unpublished observations). We think that the homologous regions are probably the sequence recognized by the ExuR repressor.

This work was supported by grants from the Centre National de la Recherche Scientifique (Laboratoire Propre du CNRS no. UM 380024).

We are indebted to B. Blazy for providing purified CRP protein and to D. Boxer for reading the manuscript. We thank G. Luthaud for typing the manuscript and C. Van Herrewege for help in the preparation of the illustrations.

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DNA sequence of the uxaB regulatory region


