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Construction of Hybrid Plasmids Containing the *Escherichia coli uxaB* Gene: Analysis of Its Regulation and Direction of Transcription

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The *uxaB* gene of *Escherichia coli*, encoding for altronate oxidoreductase involved in the hexuronate degradative pathway, was isolated on a ColE1-*uxaB* hybrid plasmid from the Clarke and Carbon bank. The restriction map of this plasmid was established. The *uxaB* gene was mapped on a 1.5-megadalton *Hin*dIII-*Kpn*I DNA fragment. Use of an in vitro gene fusion between *uxaB* and *lacZ* genes led to the determination that *uxaB* is transcribed from the *Kpn*I towards the *Hin*dIII restriction sites. Gene amplification in cells containing various *uxaB* hybrid plasmids allowed us to show a gradation in the level of repression of *exu* operator sites by the *exuR* regulatory gene product.

Escherichia coli K-12 is able to grow on galacturonate, which is degraded according to the Ashwell pathway (2). Galacturonate, tagaturonate, and fructuronate induce the synthesis of enzymes A, II, III′, and IV′ (Fig. 1) (36, 40). The *exu* regulon involves the *exu* region located at min 66 on the *E. coli* calibrated map and the *uxaB* gene (enzyme III′) located at min 52 (Fig. 1) (26, 27, 28). The *exu* regulon is subjected to the negative control of the *exuR* gene product. Various lambda transducing particles carrying the *exu* region were previously isolated from a lysogen that had integrated a lambda phage in the *exuR* regulatory gene (24). Endonuclease-generated *exu* fragments of lambda *exu* DNA were subcloned into multicopy plasmid vectors and characterized (35).

In this work we report the isolation of gene *uxaB* on a ColE1 plasmid vector from the collection of Clarke and Carbon (13) and the molecular cloning of this gene into multicopy plasmids. The *uxaB* gene product was amplified in cells containing *uxaB* hybrid plasmids. The regulatory region of the *uxaB* gene was localized on a 0.8-megadalton (Md) DNA fragment. It was shown that the *exuR* repressor has a differential affinity for the operators of the *uxaCA* and *uxaB* operons. *uxaB-lacZ* fusions were formed on a Casadaban et al. plasmid vector (10), using in vitro recombination techniques. This procedure led to the production of hybrid proteins with β-galactosidase activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The bacterial strains were *E. coli* K-12 derivatives.

Culture media. Media for growth were identical to those described by Portalier et al. (27). The minimal medium was M63 (pH 7.2) (38) or M9 (pH 7.2) (25). Solid media contained glucose (5 g/liter), glycerol, or galacturonate (5 g/liter). When needed, ampicillin, tetracycline, or chloramphenicol was added at a final concentration of 25 mg/liter. Colicin E1 was prepared from strain JF390 according to the method described by Spudich et al. (39).

Chemicals and enzymes. D-Galacturonate was purchased from Sigma Chemical Co. *Eco*RI, *Bam*HI, and *Hin*dIII restriction enzymes and T4 DNA ligase were obtained from Miles Laboratories; *Bgl*II, *Sma*I, *Sal*I, *Kpn*I, and *Pst*I were from Boehringer Mannheim. Chloramphenicol was from Sigma Chemical Co., and tetracycline and ampicillin were obtained from Serva Feinbiochemica Heidelberg.

Preparation of crude cell extracts. Cells were grown to the early stationary phase in antibiotic-containing medium. After centrifugation they were washed with 20 mM phosphate buffer, pH 7, concentrated 20 times in the same buffer, and disrupted at 18,000 lb/in^2 in a chilled French pressure cell (Aminco).

Enzyme induction and assays. The conditions for induction of altronate oxidoreductase, uronate isomerase, and altronate hydrolyase were described by Robert-Baudouy et al. (36). Altronate-oxidoreductase and altronate hydrolyase were assayed as described previously (31). In situ plate assays for altronate oxidoreductase activity were performed on clones grown overnight on glycerol-agar medium as described by Portalier and Stoeber (30). Only clones derepressed or induced for altronate oxidoreductase became blue. Uronate isomerase was measured by a coupling method described in a previous paper (28). β-Lactamase activity was measured as described by Sykes and Nordström (41), and β-galactosidase was measured as described by Miller (25).

Isolation and manipulation of plasmid DNA. Plasmid

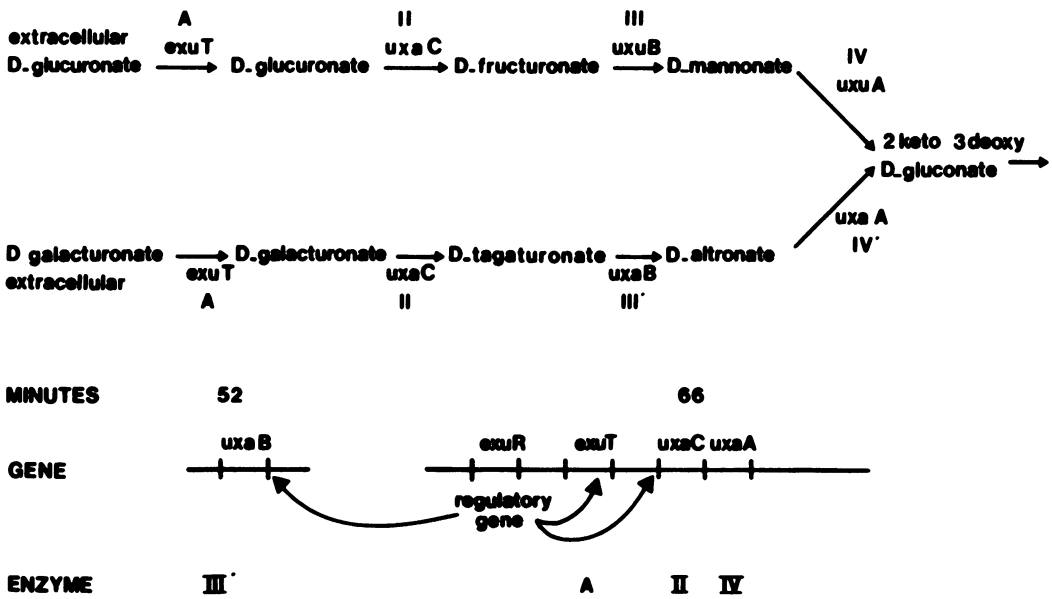

FIG. 1. Degradative pathway of hexuronates in *E. coli* K-12. II, Uronate isomerase (EC 5.3.1.12); III, mannonate oxidoreductase (EC 1.1.1.57); IV, mannonate hydrolyase (EC 4.2.1.8); III', altronate oxidoreductase (EC 1.1.1.58); IV', altronate hydrolyase (EC 4.2.1.7); A, aldohexuronate transport system.

DNA was amplified in growing cultures by adding 150 μg of chloramphenicol per ml (14). Spectinomycin (250 μg/ml) was used for the amplification of plasmids carrying the *cat* gene. Extraction and purification of plasmid DNA were achieved by the alkaline extraction procedure described by Birnboim and Doly (3). The cleared-lysate technique of Guerry et al. (19) was also used. Further purification of the DNA was done by dye-buoyant centrifugation in CsCl gradients containing ethidium bromide (33). Restriction endonuclease digestion and DNA ligation were carried out as described in reference 34. *E. coli* cells were prepared for transformation with plasmid DNA by the method of Mandel and Higa (22) as modified by Wensink et al. (42).

Agarose and polyacrylamide gel electrophoresis. Electrophoresis of DNA and proteins was carried out as described previously (35).

Construction of pEB plasmids. Plasmids pEB1, pEB2, pEB3, and pEB4 were constructed by the following procedure. Plasmid vector pBR322 and plasmid A1 were digested with one or two restriction enzymes, and the resulting fragments were ligated in vitro with T4 DNA ligase. This mixture was used to transform strain HB101. Hybrid plasmid DNA was extracted and digested with the same enzymes used previously for cloning. The cloned A1 fragment was identified after electrophoresis on agarose gel. The *Bgl*II-1–*Bgl*II-2, *Bgl*II-2–*Bam*HI, and *Bgl*II-1–*Bam*HI fragments of A1 were inserted into the *Bam*HI site of pBR322, yielding pEB1, pEB3, and pEB2, respectively. Plasmids with the 8- and 4-Md *Hin*dIII fragments of A1 cloned into the *aac* gene of pACYC177 were designated pEB4 and pEB5, respectively. pEB6 was constructed by cloning the *Eco*RI-3–*Bam*HI fragment of pEB3 into pBR322; pEB7 is plasmid pACYC184 in which the *Eco*RI-2–*Eco*RI-3 fragment of pEB3 is inserted in the *cat* gene. The introduction of the *Eco*RI-2–*Bgl*II-2 fragment of plasmid A1 into pBR322 yielded pEB8. The 8.5-Md *Bgl*II-1–*Bgl*II-2 fragment of A1 was inserted into the *Bam*HI site of pBR322; the resulting plasmid, cleaved with *Kpn*I and recircularized, yielded pEB9. pEB10 was formed from pEB9 by deletion of a DNA fragment between the pBR322 and chromosomal *Pvu*II sites.

Construction of pEBG plasmids. The *Eco*RI-2–*Eco*RI-3 fragment of A1 was inserted into the *Eco*RI site of plasmid pMC1403. pEBG1 was one of the resulting hybrid plasmids and was able to restore a Lac^+ phenotype in strain HB101 by complementation of the *lacY* mutation. The introduction of pEBG1 into strain 2510 did not modify the Lac^- phenotype of this strain. However, Lac^+ revertants occurred spontaneously on lactose minimal medium after 48 h at 37°C at a rate of 1 per 10^7 cells plated. After the homogenization step described by Ritzenthaler and Mata-Gilsinger (34), the structure of the Lac^+ plasmids was identical to that of pEBG1, except for the disappearance of the *Eco*RI site at the *uxaB-lacZ* junction in 40% of the cases.

Two plasmids were chosen for further study: pEBG2, in which the *Eco*RI and *Bam*HI sites of pMC1403 were maintained at the *uxaB-lacZ* junction; and pEBG3, which had lost these two sites.

RESULTS

Identification of a ColE1 hybrid plasmid carrying the *uxaB* gene. The ColE1 hybrid plasmids of the Clarke and Carbon colony bank were transferred by conjugation into strain 1862 (*uxaB*). Clones of strain 1862 able to grow on galacturon-

TABLE 1. Bacterial strains and plasmids

Designation	Relevant genotype	Reference
Bacterial strain		
1862	*uxaB met arg thy recA*	This laboratory
JF390	*Paracoli* Ca 57 (Colc)	18
JA200	*ΔtrpE5 thr leu lacY recA*	13
HB101	*pro leu rpsL hsdM hsdR endoI lacY recA*	6
MC4100	*ΔlacU169 ara rpsL*	9
2510	As MC4100 but *recA*	This laboratory
2422	As MC4100 but *exuR*	This laboratory
GR501	*relA1 spoT1 lig-251*(Ts)λ^-	16
AT998	*dapA16 relA1 spoT1* λ^-	7
AT978	*dapE9 relA1 spoT1* λ^-	7
JP1449	*gtlX351 thr-1 leuB6 lacZ4 rpsL8 supE44* λ^-	37
HK656	*argHBCE thr leu rpsL ptsI*(Ts) *tgs*	21
HK717	*argHBCE thr leu rpsL ptsI*(Ts) *cysA*	21
HK758	*argHBCE thr leu rpsL ptsI*(Ts) *crr*	21
CB84	*nirF84 thr leu lacY1 tonA22 ana-1 rpsL*	1
1475	*exuR argG his*	This laboratory
1351	*dsdA6*	E. McFall
Plasmid		
pBR322	*bla*$^+$ *tet*$^+$	5
pACYC177	*aac*$^+$ *bla*$^+$	12
pACYC184	*tet*$^+$ *cat*$^+$	12
pMC1403	*bla*$^+$ *lacZ' lacY*$^+$	10
p184*exuR*	*cat*$^+$ *exuR*$^+$	34
p184*uxuR*	*cat*$^+$ *uxuR*$^+$	34

ate minimal medium in the presence of colicin E1 were selected; all of the analyzed clones carried a plasmid which had a molecular weight of 11.5×10^6. After transformation by this plasmid, strain 1862 showed a galacturonate$^+$, colicin E1-immune phenotype. This result confirms the presence of the *uxaB* gene on the selected plasmid.

A ColE1-*uxaB* hybrid plasmid was also isolated by direct selection: as in the case of the *lac* operator (23), the multiple copies of a plasmid containing a binding site for the *exuR* repressor titrated out the *exuR* repressor present in the cell and thereby derepressed the *exu* operons, resulting in a constitutive synthesis of altronate oxidoreductase. This approach was used to isolate ColE1 hybrid plasmids that caused a constitutive altronate oxidoreductase synthesis. The Clarke and Carbon collection was screened for the presence of altronate oxidoreductase activity in the absence of inducer on an in situ plate assay described above. Of 500 clones tested, 1 presented weak constitutive synthesis of altronate oxidoreductase. The plasmid contained in this strain had the same molecular weight as those selected by conjugation with strain 1862.

All of these plasmids presented the same *Eco*RI patterns after electrophoresis on agarose gel. So we assumed that we had isolated the same ColE1-*uxaB* hybrid plasmid by the two methods; this plasmid was called A1.

Detection on plasmid A1 of bacterial genes located at the 52-min region. Gene *uxaB* was previously located at 52 min on the *E. coli* linkage map. The presence of bacterial genes of this chromosomal region on plasmid A1 was

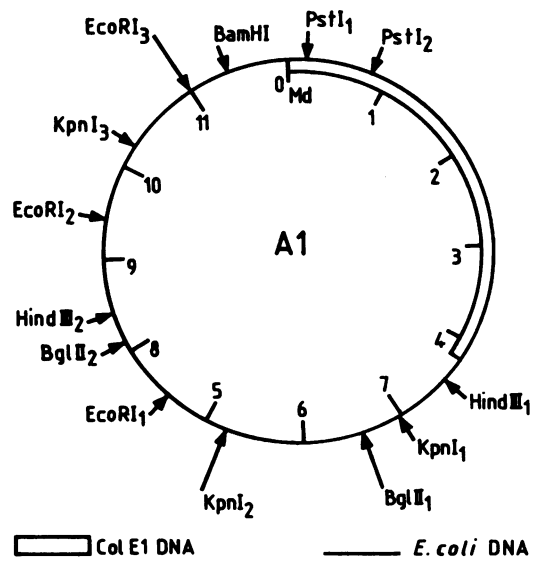

FIG. 2. Restriction map of plasmid A1. The ColE1 vector is indicated by the double line, and *E. coli* chromosomal DNA is represented by the single line.

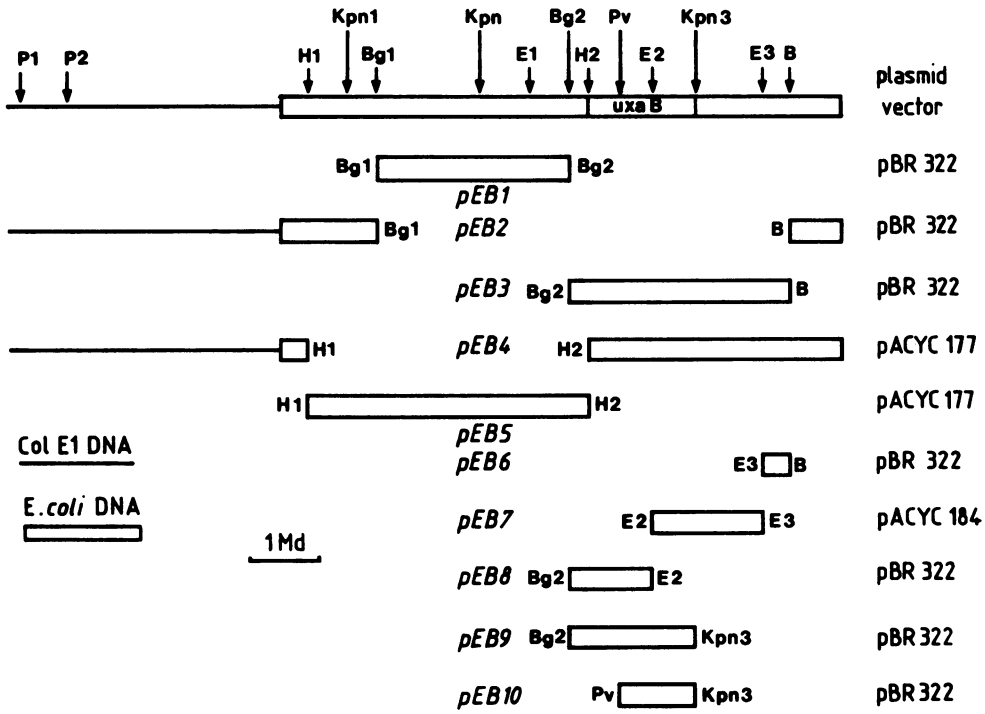

FIG. 3. Physical maps of recombinant plasmids carrying portions of the *uxaB* chromosomal region. At the top is a map of plasmid A1. P, *Pst*I; H, *Hin*dIII; Kpn, *Kpn*I; Bg, *Bgl*II; E, *Eco*RI; B, *Bam*HI; P, *Pvu*II.

tested by introducing plasmid A1 into appropriate mutant strains. Selection was performed for colicin E1 immunity and complementation of the chosen mutant allele.

Complementation of *gtlX*, *lig*, *ptsI*, *crr*, *cysA*, *tgs*, *nirF*, *dapE*, *dapA*, and *dsdA* mutations was tested after transformation of strains JP1449, GR501, HK758, HK717, HK756, CB84, AT978, AT998, and 1351. In no case did these transformant strains manifest a wild-type phenotype; therefore, no other bacterial gene was detected on plasmid A1.

Restriction map of plasmid A1. The cleavage map of plasmid A1 was determined by analyzing, on agarose gels, its cleavage patterns obtained after single or double digestions by various restriction endonucleases. The two *Pst*I sites on ColE1 DNA were used as references to localize all other sites. The *uxaB* region contains two restriction sites for endonucleases *Hin*dIII and *Bgl*II, three for *Eco*RI and *Kpn*I, and one for *Bam*HI, but neither *Sma*I, *Pst*I, nor *Sal*I cleaved the inserted DNA (Fig. 2). In addition, the *Bgl*II-2–*Bam*HI fragment contains only one *Pvu*II restriction site. Plasmid A1 has a molecular weight of 11.5×10^6; since the ColE1 DNA alone has a molecular weight of 4.2×10^6 (17), the size of the inserted chromosomal fragment is estimated to be 7.3 Md. This restriction map presents no analogy with the published cleavage map of the *dsd* region (8); therefore, plasmid A1 does not bear *dsd* genes in accord with the absence of complementation of a *dsdA* mutation by this plasmid.

Location of *uxaB* with respect to restriction sites. We subcloned the various endonuclease-generated fragments of plasmid A1 into pBR322, pACYC177, or pACYC184 plasmid vectors. The resulting plasmids, designated pEB1 to pEB10, are represented in Fig. 3. The genotypes of pEB plasmids were determined by their ability to complement the *uxaB* mutation in transformants of strain 1862 or to give rise to constitutive synthesis of altronate oxidoreductase in HB101 transformants. Results are listed in Table 2. Among the pEB plasmids, only pEB3, pEB4, and pEB9 allowed the dissimilation of galacturonate by strain 1862. Thus, a functional *uxaB* gene is located on the *Hin*dIII-2–*Kpn*I-3 fragment of plasmid A1. Moreover, pEB6, pEB7, pEB8, and pEB10 were not able to complement the *uxaB* mutation. Therefore, the *uxaB* gene overlaps the *Eco*RI-2 and *Pvu*II sites. Altronate oxidoreductase activity was measured in strains 1862 and HB101 carrying plasmids A1, pEB3, pEB4, or pEB9 in the absence of inducer. Under these conditions, a high level of altronate oxidoreductase synthesis was observed, attesting to the presence of an active *uxaB* gene on the plasmids (Table 3). In contrast, plasmids pEB6,

pEB7, and pEB8 did not modify *uxaB* gene expression in strain 1862.

Location of the operator site *uxaBo*. In spite of the absence of a functional *uxaB* gene on plasmid pEB7, strain HB101(pEB7) exhibited a low altronate oxidoreductase activity corresponding to about 15% of the enzyme activity in an induced HB101(pBR322) transformant strain. This result suggests the presence on the *Eco*RI-2–*Eco*RI-3 fragment of a sequence allowing derepression of the chromosomal *uxaB* gene. This sequence should be the operator site of the *uxaB* gene since the gene is under the negative control of the *exuR*-encoded repressor.

Detection of *uxaB* promoter activity and transcription direction of gene *uxaB*. For demonstrating the presence of the *uxaB* promoter on the *Eco*RI-2–*Eco*RI-3 fragment of plasmid A1, the transcription probe plasmid pMC1403 is especially well suited (10). This *lac* fusion plasmid includes a *lac* operon fragment in which the regulatory region of the *lacZ* gene and the codons of the first eight amino acids have been removed and substituted by a short polynucleotide sequence containing unique *Bam*HI, *Eco*RI, and *Sma*I cleavage sites. The segment of *lacZ* that remains determines sufficient primary structure to yield a functional β-galactosidase; insertions into the *Eco*RI, *Bam*HI, or *Sma*I site of a DNA fragment carrying both transcriptional and translational start signals led to the formation of an operon fusion in which *lacZ* and *lacY* genes were transcribed from the promoter sequence of the inserted fragment. A hybrid *lacZ* gene is expressed as a hybrid protein with β-galactosidase activity only if the translational reading signals of the gene on the inserted fragment are in frame with that of the *lacZ* gene.

TABLE 2. Preliminary characterization of the pEB plasmids[a]

Plasmid	Growth on galacturonate of strain 1862 (*uxaB*)	Altronate oxidoreductase activity in strain HB101[b]
pBR322	−	−
A1	+	
pEB1	−	−
pEB2	−	−
pEB3	+	++
pEB4	+	++
pEB5	−	−
pEB6	−	−
pEB7	−	+
pEB8	−	−
pEB9	+	++
pEB10	−	+

[a] −, Absence of growth or of altronate oxidoreductase activity; +, growth or low enzyme activity; ++, presence of a large quantity of enzyme.

[b] Presence of altronate oxidoreductase activity was tested by an in situ plate assay as described in the text (cells grown in absence of inducer).

TABLE 3. Altronate oxidoreductase specific activity in strains 1862 and HB101 containing pEB plasmids

Plasmid	Functional bacterial gene on the plasmid	Inducer	Altronate oxidoreductase sp act[a] in strain:	
			1862 (*uxaB*)	HB101
pBR322	None	None	15	30
		Galacturonate (5 mM)	25	2,200
A1	*uxaB*	None	1,060	
pEB3	*uxaB*	None	4,060	5,650
pEB4	*uxaB*	None	4,500	4,800
pEB7	*uxaBo*	None	24	392
pEB9	*uxaB*	None	4,927	4,787

[a] Specific activities are given in milliunits (nanomoles of product per minute) per milligram of dry weight.

The *Eco*RI-2–*Eco*RI-3 fragment was inserted into the unique *Eco*RI cleavage site of plasmid pMC1403, yielding plasmid pEBG1 (Fig. 4). When this plasmid was introduced into strain HB101 (*lacY*), it conferred the Lac$^+$ phenotype, suggesting the presence on the *Eco*RI-inserted fragment of a promoter sequence which allows the expression of the *lacY* gene on the plasmid. Introduction in HB101(pEBG1) of plasmid p184*exuR* (carrying the *exuR* gene) inhibited growth on lactose, whereas plasmid pACYC184 or p184*uxuR* (carrying the *uxuR* gene) had no effect. This result demonstrates that the *Eco*RI fragment contains a promoter activity specifically repressed by the *exuR*-encoded repressor. Despite the presence of the *exuR* chromosomal repressor in strain HB101(pEBG1), the transformants could grow on lactose because the high copy number of plasmid pEBG1 caused a partial titration of the repressor molecules. This hypothesis is confirmed by the weak derepression of chromosomal *uxaB* gene expression which was observed in these transformants (data not shown). The presence of the *uxaBo* operator and *uxaBp* promoter on the *Eco*RI-2–*Eco*RI-3 fragment, the orientation of this DNA fragment on plasmid pEBG1, and the location of the entire *uxaB* gene on the *Hin*dIII-2–*Kpn*I-3 segment suggested that the transcription direction of the *uxaB* gene was from *Kpn*I-3 to the *Hin*dIII-2 site.

Characterization of *uxaB-lacZ* gene fusions. Strain 2510(pEBG1) was unable to metabolize lactose but could grow on melibiose at 42°C, proving that the *lacY* gene was expressed on plasmid pEBG1. The absence of β-galactosidase

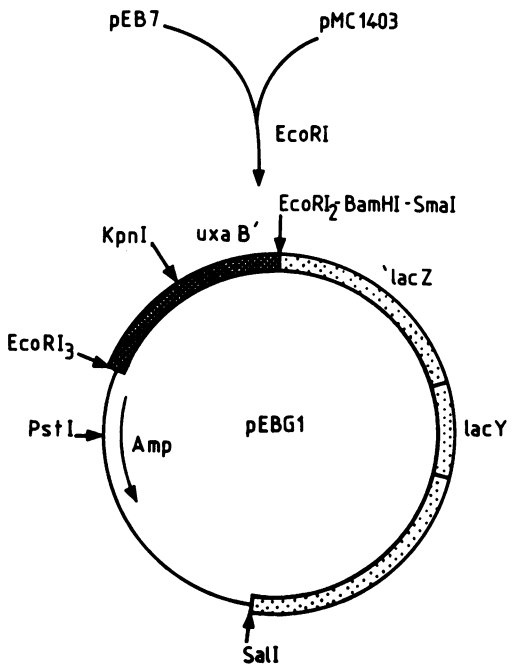

FIG. 4. Construction and structure of plasmid pEBG1. Plasmid DNA is represented by a thin line; chromosomal DNA, by a thick hatched line; *lac* chromosomal DNA, by a thick dotted line. Primes, as in *uxa*B' or *lac*Z', indicate that that part of the gene on the side of the prime is deleted. pEBG1 is an 8.3-Md plasmid.

activity can be explained by the fact that the *uxaB* and *lacZ* codons are translationally out of phase. However, growth on lactose is powerful enough to select mutations (probably microdeletions) shifting the translational reading frame between *uxaB* and *lacZ* on plasmid pEBG1. This procedure yielded Lac$^+$ revertant fusion plasmids in which an active hybrid β-galactosidase was synthesized. pEBG2 and pEBG3 are two of such plasmids and are described above. Introduction of the plasmids into strain 2510 resulted in the complete Lac$^+$ phenotype. When plasmid 184*exuR* was added in trans, the resulting clone exhibited a strong decrease of β-galactosidase synthesis. pACYC184 or p184*uxuR* had no effect on the β-galactosidase level. The two other types of regulation found in the *uxaB* operon, i.e., induction by tagaturonate and catabolite control, also apply to the *uxaB-lacZ* gene (data not shown). These results imply that transcription of the fused gene is regulated, as is that of the original *uxaB* gene, by the *exuR* gene product.

The size of the hybrid β-galactosidase encoded by pEBG2 and pEBG3 was determined by protein gel electrophoresis. Extracts from strain 2510 carrying pEBG3 or pEBG2 failed to show a peptide band at the normal location occupied by the native β-galactosidase protein but produced a new polypeptide whose size is about 120,000 daltons (Fig. 5, lanes c and d). This 120,000-dalton protein, as well as the 116,500-dalton native β-galactosidase, was absent in extracts from strain 2510 carrying pMC1403 or pEBG1 (Fig. 5, lane b). This result establishes the fact that the 120,000-dalton peptide was made as a consequence of the insertion at the *Eco*RI site of pMC1403 of the *Eco*RI-2–*Eco*RI-3 DNA fragment and is most likely the product of the *uxaB-lacZ* hybrid gene.

Gradation in the repression of the *exu* regulon enzyme synthesis. The *exu* regulon enzymes were measured in strains carrying plasmids pEB3 and pEB4. In strain 1862, altronate oxidoreductase synthesis is strongly inducible by galacturonate. The induced level is about 7- to 10-

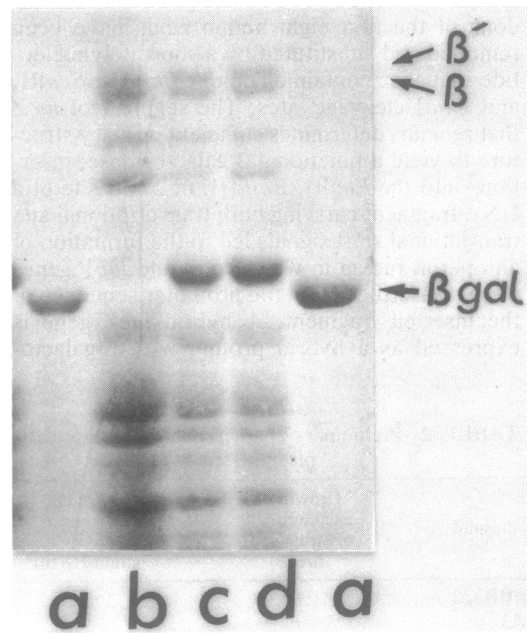

FIG. 5. Identification of the (*uxaB-lacZ*) hybrid proteins in extracts of cells containing plasmid pEBG. Cells were grown at 37°C in M63 glycerol to the early stationary phase; they were then centrifuged, concentrated 10 times in sample buffer, boiled for 5 min, and subjected to electrophoresis on a 5% (wt/vol) polyacrylamide slab gel in the presence of sodium dodecyl sulfate. Slot a, Purified β-galactosidase; slot b, strain 2510(pEBG1) [strain 2510(pMC1403) gave the same result]; slot c, strain 2510(pREG2); slot d, strain 2510(pREG3). Only the upper part of the gel is shown. The positions of the hybrid proteins are shown by small arrows. The positions of authentic *lacZ* polypeptide (116, 500 daltons) and of the β (155,000 daltons) and β' (165,000 daltons) chains of RNA polymerase are shown on the right.

TABLE 4. Activity of *exu* regulon enzymes in *E. coli* strains containing pEB plasmids

Strain	Plasmid	Functional bacterial gene on the plasmid	Inducer (mM)	Sp act[b] of:		
				Altronate oxidoreductase (III') (*uxaB*)	Uronate isomerase (II) (*uxaC*)	Altronate hydrolyase (IV') (*uxaA*)
HB101	pBR322	None	0	30	20	ND
			10	2,200	310	40
	pEB3	*uxaB*	0	6,700	152	28
			10		310	40
1862	pBR322	None	0	15	14	ND
			10	25	320	45
	pEB3	*uxaB*	0	4,300	85	
			10	42,000	340	
	pEB4	*uxaB*	0	5,700	134	12
			10	33,700	305	40
1475	pBR322	None	0	3,400	230	135
			10	3,590	235	135
	pEB3	*uxaB*	0	53,000		
			10	43,600		
	pEB4	*uxaB*	0	73,000		
			10	54,000		

[a] The inducer was galacturonate.
[b] Specific activities are given in milliunits (nanomoles of product per minute) per milligram of dry weight. ND, Not determined.

fold higher than the uninduced rate (Table 4). In strain 1475 (*exuR*), the induced and uninduced levels of the *uxaB* gene product are approximately the same (Table 4). This result suggests that the chromosomal *exuR* repressor is present in sufficient amount to partially repress the expression of the *uxaB* gene on the plasmid in spite of the high copy number of the plasmid.

In strain HB101, as well as in strain 1862, plasmid pEB3 or pEB4 caused a constitutive synthesis of altronate hydrolyase and uronate isomerase: the uninduced level is only about two- to fourfold lower than the induced level. These results are discussed below.

DISCUSSION

A ColE1-*uxaB* hybrid plasmid was isolated from the Clarke and Carbon *E. coli* gene bank. No other gene of the 52-min region of the *E. coli* chromosome was present on this plasmid. The subcloning of various restriction fragments from this plasmid into multicopy plasmid vectors allowed us to locate the *uxaB* gene on a 1.5-Md *Hin*dIII-*Kpn*I fragment and to produce elevated levels of altronate oxidoreductase in pEB transformant strains. We demonstrated also that the *uxaB* gene overlaps the *Eco*RI-2 and *Pvu*II sites. The *uxaB* gene product probably had a molecular weight of between 25,000 and 82,000. The presence of the *uxaB* operator-promoter on the 1.7-Md *Eco*RI-2–*Eco*RI-3 fragment was deduced from the properties of the transcription probe plasmid pMC1403, which has inserted this fragment. The *uxaB-lacZ* gene fusions on pEBG2 and pEBG3 coded for the synthesis of hybrid proteins which had β-galactosidase activity but no altronate oxidoreductase activity. These two hybrid β-galactosidases had the same molecular mass, and this was only 3,500 daltons larger than that of the native β-galactosidase. Therefore, the translation initiation site of the *uxaB* gene maps probably very close to the *Eco*RI-2 site.

On plasmid pEBG1, the translational reading frames of *uxaB* and *lacZ* genes were not identical and no β-galactosidase activity was detectable. On Lac^+ revertant plasmids such as pEBG2 or pEBG3, a mutation had probably shifted the *uxaB* and *lacZ* codons translationally in phase. This mutation was certainly a very short deletion located near the *uxaB-lacZ* junction since the restriction patterns of pEBG2 and pEBG3 were not modified compared with those of pEBG1, except that on 40% of the Lac^+ revertant plasmids the *Eco*RI site at the gene fusion point had disappeared. This hypothesis was supported by the results of Betz et al. (4) and Casadaban et al. (11).

The presence of the *uxaB* operator on the *Eco*RI-2–*Eco*RI-3 fragment was also shown by the derepression of chromosomal encoded altronate oxidoreductase synthesis in a wild-type strain transformed with plasmid pEB7 (or pEBG). As a matter of fact, the *uxaB* gene is negatively controlled by the *exuR* gene product

(29), and, as observed with *lacZo* (23) and *uxaCo* (35), the high copy number of *uxaB* operator site in the cell, due to its presence on a multicopy plasmid, partially titrated out the repressor molecules coded for by the *exuR* chromosomal gene, resulting in a constitutive synthesis of the *exu* regulon enzymes.

In cells containing plasmids pEB3 or pEB4, the altronate oxidoreductase activity is strongly inducible, suggesting that in spite of its high copy number in the cell *uxaB* gene expression is still partially repressed by the *exuR* chromosomal gene product. This was not observed when the *exu* regulon genes were present on a plasmid (35): in a pRE9 (*uxaCA*$^+$) transformant strain, the enzyme activities encoded by the *uxaA* and *uxaC* genes were at the same high level in the absence or in the presence of inducer. If the pRE and pEB plasmids have the same copy number in these strains, it is obvious that a difference exists between *uxaB* and *uxaCA* operons with regard to the control of their expression by the *exuR*-encoded repressor. Additional evidence for this phenomenon is given by the behavior in a wild-type strain of plasmids containing various operator sites of the *exu* regulon; the presence of pEB3 or pEB4 (*uxaBo*) in a wild-type strain induced a derepression of the *uxaCA* operon: the enzymatic activities of uronate isomerase and altronate hydrolyase can reach up to 50% of the fully induced haploid level. On the other hand, altronate oxidoreductase activity in strains bearing pRE plasmids (*uxaCo*) represented only 10 to 20% of the fully induced haploid level.

All of these observations suggest that the *exuR*-encoded repressor has a stronger affinity for the *uxaBo* operator than for the *uxaCAo* operator; they are in accordance with the properties of thermosensitive mutant alleles of the *exuR* gene (29). Such a degree of flexibility in controlling the operators of the same regulon by its repressor were previously reported by Pouyssegur and Stoeber (32) for the *kdg* regulon, Cozzarelli et al. (15) for the *glp* regulon, and Jacoby and Gorini (20) for the *arg* regulon.

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LITERATURE CITED

1. **Abou-Jaoudé, A., M. Lepelletier, J. Ratouchniak, M. Chippaux, and M. C. Pascal.** 1978. Nitrite reduction in *E. coli*: genetic analysis of *nir* mutants. Mol. Gen. Genet. **167**:113–118.
2. **Ashwell, G.** 1962. Enzymes of glucuronic and galacturonic acid metabolism in bacteria. Methods Enzymol. **5**:190–208.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1524.
4. **Betz, J. L., and R. Sadler.** 1981. Variants of a cloned synthetic lactose operator. II. Chloramphenicol-resistant revertants retaining a lactose operator in the CAT gene of plasmid pBR325. Gene **15**:187–200.
5. **Bolivar, R., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Bukhari, A. I., and A. L. Taylor.** 1971. Genetic analysis of diaminopimelic acid- and lysine-requiring mutants of *Escherichia coli*. J. Bacteriol. **105**:844–854.
8. **Carothers, A. M., E. MacFall, and S. Palchaudhuri.** 1980. Physical mapping of the *Escherichia coli* D-serine deaminase region: contiguity of the *dsd* structural and regulatory genes. J. Bacteriol. **142**:174–184.
9. **Casadaban, M. J.** 1976. Transposition and fusion of *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
10. **Casadaban, M. J., J. C. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
11. **Casadaban, M. J., J. Chou, and S. Cohen.** 1982. Overproduction of the Tn3 transposition protein and its role in DNA transposition. Cell **28**:345–354.
12. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicules derived from P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
13. **Clarke, L., and J. Carbon.** 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *Escherichia coli* genome. Cell **9**:91–99.
14. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
15. **Cozzarelli, N. R., W. B. Freedberg, and E. C. C. Lin.** 1968. Genetic control of the L-α-glycerophosphate system in *Escherichia coli*. J. Mol. Biol. **31**:371–387.
16. **Dermody, J. J., G. T. Robinson, and R. Sternglanz.** 1979. Conditional-lethal deoxyribonucleic acid ligase mutant of *Escherichia coli*. J. Bacteriol. **139**:701–704.
17. **Dougan, G., M. Saul, G. Warren, and D. Sherratt.** 1978. A functional map of plasmid ColE1. Mol. Gen. Genet. **158**:325–327.
18. **Foulds, J., and C. Barrett.** 1973. Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246: two new classes of tolerant mutants. J. Bacteriol. **116**:885–892.
19. **Guerry, P., D. J. Le Blanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
20. **Jacoby, G. A., and L. Gorini.** 1969. An unitary account of the repression mechanism of *arg* biosynthesis in *Escherichia coli*. I. The genetic evidence. J. Mol. Biol. **39**:73–87.
21. **Kornberg, H., P. D. Watts, and K. Brown.** 1980. Mechanisms of 'inducer exclusion' by glucose. FEBS Lett. **177**(Suppl.):K28–K35.

22. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
23. **Marians, K. J., R. Wu, J. Stawinsky, T. Hozumiand, and S. A. Narang.** 1976. Cloned synthetic *lac* operator DNA is biologically active. Nature (London) **263**:744–748.
24. **Mata-Gilsinger, M., M. Delstanche, and J. Robert-Baudouy.** 1978. Isolation of specialized transducing bacteriophages carrying the structural genes of the hexuronate system in *Escherichia coli* K-12: *exu* region. J. Bacteriol. **133**:549–557.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Nemoz, G., J. Robert-Baudouy, and F. Stoeber.** 1976. Physiological and genetic regulation of the aldohexuronate transport system in *Escherichia coli* K-12. J. Bacteriol. **127**:706–718.
27. **Portalier, R., J. Robert-Baudouy, and G. Nemoz.** 1974. Etudes de mutations affectant les gènes de structure de l'isomérase uronique et de l'oxydoréductase altronique chez *Escherichia coli* K-12. Mol. Gen. Genet. **128**:301–319.
28. **Portalier, R., J. Robert-Baudouy, and F. Stoeber.** 1972. Localisation génétique et caractérisation biochimique de mutations affectant le géne de structure de l'hydrolyase altronique chez *Escherichia coli* K-12. Mol. Gen. Genet. **118**:335–350.
29. **Portalier, R., J. Robert-Baudouy, and F. Stoeber.** 1980. Regulation of *Escherichia coli* K-12 hexuronate system genes: *exu* regulon. J. Bacteriol. **143**:1095–1107.
30. **Portalier, R., and F. Stoeber.** 1972. Dosages colorimétriques des oxydoréductases aldoniques d'*Escherichia coli* K-12: applications. Biochim. Biophys. Acta **289**:19–27.
31. **Portalier, R., and F. Stoeber.** 1972. La D-altronate: NAD-oxydoréductase d'*Escherichia coli* K-12: purification, propriétés et individualité. Eur. J. Biochem. **26**:50–61.
32. **Pouyssegur, J., and F. Stoeber.** 1974. Genetic control of the 2-keto-3-deoxy-D-gluconate metabolism in *Escherichia coli* K-12: *kdg* regulon. J. Bacteriol. **117**:641–651.
33. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1521.
34. **Ritzenthaler, P., and M. Mata-Gilsinger.** 1982. Use of in vitro gene fusions to study the *exuR* regulatory gene in *Escherichia coli* K-12: direction of transcription and regulation of its expression. J. Bacteriol. **150**:1040–1047.
35. **Ritzenthaler, P., M. Mata-Gilsinger, and F. Stoeber.** 1981. Molecular cloning of *Escherichia coli* K-12 hexuronate system genes: *exu* region. J. Bacteriol. **145**:181–190.
36. **Robert-Baudouy, J., R. Portalier, and F. Stoeber.** 1974. Régulation du metabolisme des hexuronates chez *Escherichia coli* K-12: modalités de l'induction des enzymes du systéme hexuronate. Eur. J. Biochem. **43**:1–15.
37. **Russel, R. R. B., and A. J. Pittard.** 1971. Mutants of *Escherichia coli* unable to make protein at 42°C. J. Bacteriol. **108**:790–798.
38. **Sistrom, W. R.** 1958. On the physical state of the intracellularly accumulated substrates of β-galactoside permease in *Escherichia coli*. Biochim. Biophys. Acta **29**:579–587.
39. **Spudich, J. A., V. Horn, and C. Y. Yanofsky.** 1970. On the production of deletions in the chromosome of *Escherichia coli*. J. Mol. Biol. **53**:49–67.
40. **Stoeber, F., A. Lagarde, G. Nemoz, G. Novel, M. Novel, R. Portalier, J. Pouyssegur, and J. Robert-Baudouy.** 1974. Le métabolisme des hexuronides et des hexuronates chez *Escherichia coli* K-12: aspects physiologiques et génétiques de sa régulation. Biochimie **56**:199–213.
41. **Sykes, R. B., and K. Nordström.** 1972. Microiodometric determination of β-lactamase activity. Antimicrob. Agents Chemother. **1**:94–99.
42. **Wensink, P. C., D. J. Finnegan, J. E. Donelson, and D. S. Hogness.** 1974. A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell **3**:315–325.