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Physical Mapping of the *exuT* and *uxaC* Operators by Use of *exu* Plasmids and Generation of Deletion Mutants In Vitro

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Operons *uxaCA* and *exuT* of the hexuronate system are very closely linked on the *Escherichia coli* genetic map. Using plasmid vectors constructed by Casadaban et al. (J. Bacteriol. **143**:971–980, 1980), we formed *exuT-lacZ* and *uxaA-lacZ* fusions in vitro. The phenotypic properties of the new plasmids allowed us to confirm that the *exuT* and *uxaCA* operons are divergently transcribed. An analysis of these fusion plasmids and derivatives in the presence of multiple copies of the *exuR* regulatory gene demonstrated that the two operons possess separate control regions. The precise location of the operator site relative to endonuclease restriction sites was determined. In addition, deletions of different lengths were generated on *exu* plasmids by restriction enzymes and were recombined into the chromosome. The expression of the *exu* regulon genes in the resulting deletion mutants is in agreement with the postulated location of the *exuT* and *uxaCA* operators in the fusion plasmids.

The aldohexuronates, D-glucuronate and D-galacturonate, can serve as carbon sources for growth of *Escherichia coli* K-12 and are degraded according to the Ashwell pathway (1) (Fig. 1). These compounds enter cells by the same specific transport system (protein I) and are dissimilated in two parallel pathways, which have the same first step (enzyme II). The *exu* region located at 66 min on the *E. coli* linkage map (2) includes the *uxaCA* operon (uronate isomerase and altronate hydrolyase), the *exuT* gene (aldohexuronate transport protein), and the *exuR* regulatory gene (27, 30). Gene *uxaB*, located at 52 min, codes for altronate oxidoreductase. The *exu* regulon comprises four structural genes, *uxaC*, *uxaA*, *exuT*, and *uxaB*, and is subject to negative control by the *exuR* regulatory gene product (31). The *uxu* region located at 97 min consists of two structural genes, *uxuB* and *uxuA*, and the *uxuR* regulatory gene. Expression of the *uxuAB* operon is controlled primarily by the *uxuR* gene product and secondarily by the ExuR repressor (42). Galacturonate, tagaturonate, and fructuronate are able to induce synthesis of the *exu* regulon enzymes, whereas glucuronate works as an inducer only after conversion into fructuronate (27, 41). Tagaturonate and fructuronate enter cells by separate transport systems that are distinct from the system encoded by *exuT*. All of the genes belonging to the *exu* regulon have been cloned into plasmid vector pBR322 (5, 39).

The adjacent operons *exuT* and *uxaCA* have been shown to be transcribed in opposite directions (21). Therefore, each could have its own operator, or the two operons could be transcribed from a single control region, like other operons which are divergently transcribed, such as *bioA.BFCD* (13, 18), *argE.CBH* (34), *araC.BAD* (20, 28), and *malBK.EFG* (19).

In this paper, we show that *uxaCA* and *exuT* are two distinct operons. We fused in vitro the presumed promoter-operator region of each of these operons to the *lac* genes, and we describe the isolation and characterization of these *uxaA-lac* and *exuT-lac* fusions. Various deletions in the *exu* region were also constructed. The physiology of the fusion plasmids and the deletions allowed us to locate the operator sites of the *exuT* and *uxaCA* operons precisely.

MATERIALS AND METHODS

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

Culture media. The media used for growth were identical to those described by Miller (26). The synthetic medium used was M63 medium (pH 7.2) (44) or M9 medium (pH 7.2) (26) and contained either glucose (5 g/liter), glycerol (5 g/liter), glucuronate (2.5 g/liter), galacturonate (2.5 g/liter), tagaturonate (2.5 g/liter), or fructuronate (2.5 g/liter); aldohexuronate-containing MacConkey media (Difco Laboratories) contained 15 mg of glucuronate or galacturonate per ml. When needed, ampicillin, chloramphenicol, and kanamycin were used at final concentrations of 25 μg/ml, and

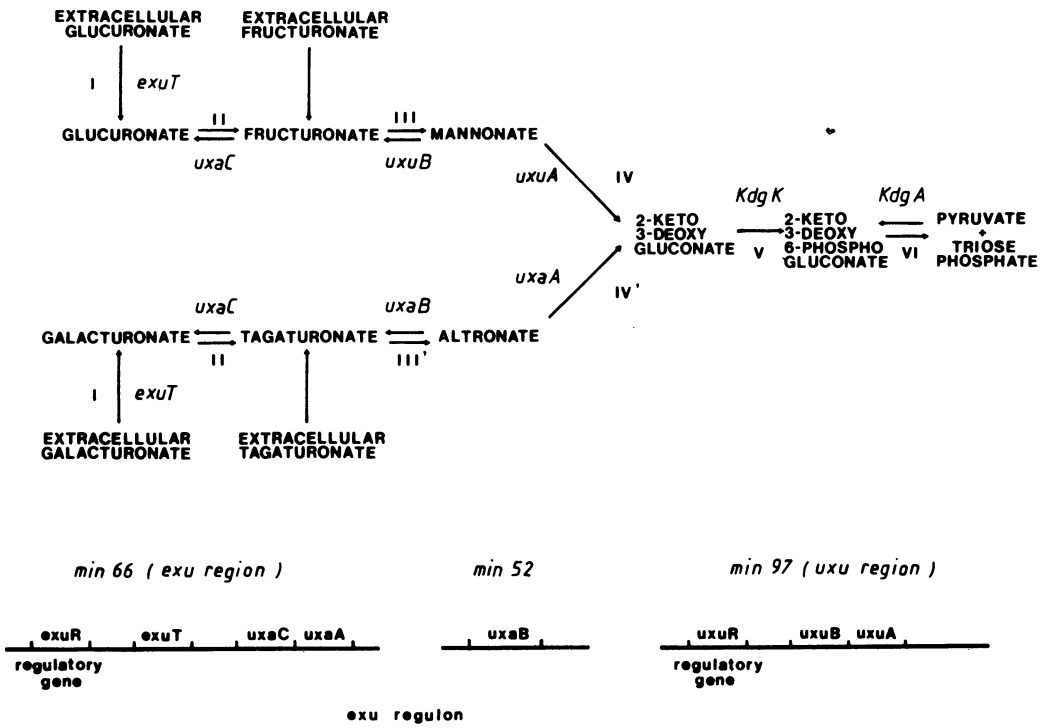

FIG. 1. Degradative pathway of hexuronates in *E. coli* K-12. The different steps are catalyzed by the following enzymes: I, aldohexuronate transport system; II, uronate isomerase (EC 5.3.1.12); III, mannonate oxidoreductase (EC 1.1.1.57); IV, mannonate hydrolyase (EC 4.2.1.8); III', altronate oxidoreductase (EC 1.1.1.58); IV', altronate hydrolyase (EC 4.2.1.7); V, 2-keto-3-deoxygluconate kinase (EC 2.7.1.45); VI, 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.2.1.14). The structural genes of the enzymes are indicated, and the locations on the *E. coli* chromosome of some of these structural genes and of the corresponding regulatory genes are shown at the bottom.

tetracycline was added at a concentration of 15 μg/ml.

Chemicals and enzymes. Intermediate substrates of the hexuronate pathway (tagaturonate and fructuronate) and mannonic amide (gratuitous inducer) were synthesized in our laboratory (41). D-Glucuronate and D-galacturonate were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction enzymes were purchased from Boehringer Mannheim France SA, and chloramphenicol, tetracycline, ampicillin, and kanamycin were obtained from Serva Feinbiochemica. [U-^{14}C]glucuronate (potassium salt) was purchased from the Radiochemical Centre, Amersham, England.

Genetic methods. Transduction with phage P1 or phage λ and conjugation were performed by the methods of Miller (26).

Enzyme induction and preparation. The conditions used for induction and extraction of the enzymes of the hexuronate system have been described previously (41).

Enzyme assays. β-Galactosidase was assayed by the method of Miller (26) in exponentially growing cells. D-Altronate:NAD oxidoreductase, altronate hydrolyase, and uronic isomerase were measured by previously described methods (30, 33, 40). The assay for the glucuronate uptake experiments was carried out as described previously (27). In situ plate assays for D-altronate:NAD oxidoreductase activity were performed with clones grown overnight on glycerol agar medium containing or lacking inducer and treated with toluene, as described by Portalier and Stoeber (32). Only the clones derepressed or induced for D-altronate:NAD oxidoreductase activity became blue under these conditions.

Isolation and analysis of plasmid DNA. The procedures used for isolation of plasmid DNA (4, 17, 36), construction, cloning, restriction endonuclease cleavage, and gel analysis of recombinant plasmids (14, 24, 43, 38), and transformation of *E. coli* with plasmid DNA (23) have been described previously.

Details of plasmid pREG construction. The chromosomal *Bam*HI fragment of plasmid pRE4 has a molecular weight of 1.85×10^6 and includes the *uxaC* gene with the beginning of the *uxaA* gene at one extremity and part of the *exuT* gene at the other extremity (39) (Fig. 2). When this fragment containing a binding site for the *exuR* repressor is inserted into a multicopy plasmid, it titrates out the *exuR* repressor present in the cell and derepresses the *uxaB* operon, resulting in constitutive synthesis of the altronate oxidoreductase enzyme (39).

(i) Plasmids pREG8, pREG9, pREG10, and pREG11. Plasmids pRE4 (Fig. 2) and pMC874 (11) were mixed, digested with endonuclease *Bam*HI, ligated with T4 DNA ligase, and used to transform *lac* deletion strain

TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Designation	Relevant genotype	Source and/or reference
Bacterial strains		
HB101	*leu pro hsdM hsdR recA rpsL lacY*	9
MC4100	*araD139 ΔlacU169 rpsL*	10
2510	As MC4100, but *recA*	This laboratory
EW1b	*hisA tolC argG rpsL*	45
A314	*metB1 kdgA2*	35
2161	As MC4100, but *kdgA2*	A314 × MC4100 recombinant
2987	As 2161, but Δ(*exuT-exuR*) (ΔA)[a]	This study
3013	As 2161, but Δ(*uxaA-uxaC*) (ΔD)	This study
3211	As 2161, but Δ(*uxaA-uxaC*) (ΔC)	This study
3093	As 2161, but Δ(*exuT-uxaC*) (ΔB)	This study
2992	*hisA argG* Δ(*exuT-exuR*) (ΔA)	TolC$^+$ recombinant of 2987 × EW1b
3014	*hisA argG* Δ(*uxaA-uxaC*) (ΔD)	TolC$^+$ recombinant of 3013 × EW1b
3214	*hisA argG* Δ(*uxaA-uxaC*) (ΔC)	TolC$^+$ recombinant of 3211 × EW1b
3094	*hisA argG* Δ(*exuT-uxaC*) (ΔB)	TolC$^+$ recombinant of 3093 × EW1b
1357	*exuT6 argG thyA recA rpsL*	This laboratory
1864	*uxaC2 metC thyA argG recA rpsL*	This laboratory
Bacteriophages		
λ*pexu2*	CI857 *exuT*$^+$ *uxaC*$^+$ *uxaA*$^+$	25
λ*pexu4*	CI857 *exuT*$^+$	25
λ*pexu5*	CI857 *exuT*$^+$*uxaC*$^+$	25
Plasmids[b]		
pBR322	*bla*$^+$ *tet*$^+$	8
pACYC184	*tet*$^+$ *cat*$^+$	12
pMC874	*aac*$^+$ *lacZ lacY*$^+$	11
pRE2	*bla*$^+$ *exuR*$^+$	Derived from pBR322 (39)
pRE3	*bla*$^+$ *exuR*$^+$	Derived from pBR322 (39)
pRE7	*bla*$^+$ *uxaC*$^+$ *exuT*$^+$	Derived from pBR322 (39)
pRE8	*bla*$^+$ *uxaC*$^+$ *exuT*$^+$ *exuR*$^+$	Derived from pBR322 (39)
pRE9	*bla*$^+$ *uxaC*$^+$ *uxaA*$^+$	Derived from pBR322 (39)
pRU12	*bla*$^+$ *uxuR*$^+$	Derived from pBR322 (38)
pRE7ΔB	As pRE7, but Δ(*exuT-uxaC*)	This study
pRE8ΔA	As pRE8, but Δ(*PstI*-1–*PstI*-2)	This study
pRE9ΔC	As pRE9, but Δ(*SalI*-1–*SalI*-2)	This study
pRE9ΔD	As pRE9, but Δ(*PstI*-2–*PstI*-3)	This study

[a] ΔA, ΔB, ΔC, and ΔD are the short designations of the deletions.
[b] Plasmids pRE4, pRE6, pREG8, pREG9, pREG10, pREG11, pREG12, pREG13, and pREG14 are shown in Fig. 2 and 3.

2510 (Table 1). Kanamycin-resistant transformants were selected, and clones that constitutively synthesized D-altronate:NAD$^+$ oxidoreductase were detected by an in situ plate assay; these clones contained plasmid pMC874 with the 1.85-megadalton (Md) fragment of pRE4 inserted into the *Bam*HI site. As expected, both insertion orientations were found. The structures of pREG8 and pREG9 are shown in Fig. 2. These two plasmids were not able to restore a Lac$^+$ phenotype in strain 2510. However, spontaneous Lac$^+$ revertants were obtained on lactose-containing minimal medium after 48 h at 37°C. Each Lac$^+$ colony contained a mixture of Lac$^+$ and Lac$^-$ plasmids since the Lac$^+$ mutation which shifted the translational reading frame between *uxaC* (or *exuT*) and *lacZ* occurred only on one copy of the plasmid. Therefore, a homogenotization step was necessary to get clones with only Lac$^+$ plasmids. This procedure has been used previously for the isolation of a *uxuR-lacZ* fusion (37). The Lac$^+$ derivatives of pREG8 and pREG9, designated pREG10 and pREG11, had structures identical to those of the parental plasmids, as shown by their restriction enzyme patterns after single *Bam*HI, *Sal*I, or *Pst*I digestion or double *Eco*RI-*Bgl*II digestion.

(ii) Plasmid pREG12. Plasmid pREG11 was digested with endonuclease *Pst*I, ligated with T4 DNA ligase, and used to transform strain 2510. Lac$^+$ transformants were detected on lactose-containing MacConkey agar plates containing kanamycin, and their plasmid DNAs were extracted and analyzed by single *Pst*I digestion and double *Sal*I-*Hin*dIII digestion. Plasmid pREG12 had the *Pst*I restriction fragment containing the fused *exuT-lacZ* gene in reverse orientation to that of pREG11.

(iii) Plasmid pREG13. The *Sal*I DNA segment bearing the *exuT-lacZ* hybrid gene was subcloned from plasmid pREG11 into the tetracycline resistance gene of pACYC184 (12); after cleavage of pREG11 and pACYC184 by *Sal*I, ligation, and transformation of strain 2510, plasmid pREG13 (Fig. 2) was isolated from a chloramphenicol-resistant, tetracycline-sensitive, kanamycin-sensitive transformant; this transformant had a Lac$^+$ phenotype. The orientation of the *Sal*I DNA segment was determined by analyzing the

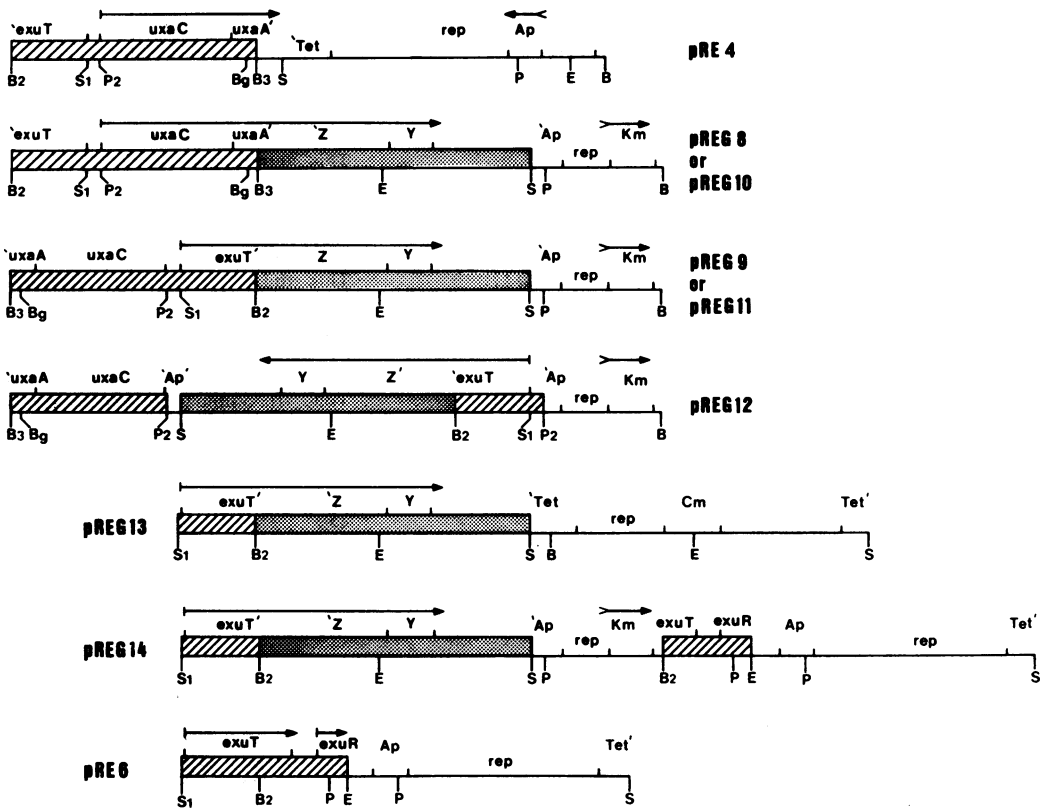

FIG. 2. Structures of the *uxaA-lacZ* and *exuT-lacZ* fusion pREG plasmids. The contruction of these plasmids is described in the text. The maps show the plasmids arbitrarily opened at a *Bam*HI or *Sal*I site. The scale of pMC874 is reduced twofold with respect to pRE4. Plasmid DNA is represented by a thin line, *exu* chromosomal DNA is represented by a double hatched line, and *lac* chromosomal DNA is represented by a double stippled line. The arrows on the plasmids indicate the direction of transcription; primes indicate that the gene is interrupted on the side on which the prime is written. Abbreviations: B, *Bam*HI: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; S, *Sal*I; Bg, *Bgl*II; Ap, *bla* gene; Tet, *tet* gene; Km, *aac* gene; Cm, *cat* gene; Z, *lacZ* gene; Y, *lacY* gene.

restriction enzyme patterns of pREG13 after single digestions with endonucleases *Bam*HI and *Eco*RI.

(iv) Plasmid pREG14. Plasmid pRE6 (Fig. 2) carries a functional *exuT* gene on a *Sal*I-*Eco*RI restriction fragment. The unique *Bam*HI site of pRE6 is located within the *exuT* gene (39). Plasmid pMC874 also possesses a unique *Bam*HI restriction site. After pRE6 and pMC874 *Bam*HI cleavage, ligation, and transformation of strain 2510, ampicillin- and kanamycin-resistant clones were selected; all of these clones had a Lac$^-$ phenotype. The plasmid DNAs of these clones were extracted and analyzed after *Bam*HI, *Sal*I, *Eco*RI, and *Pst*I digestions. The two possible orientations of insertion of pMC874 into pRE6 were found. Only the orientation allowing a fusion between the beginning of *exuT* and *lacZ* was retained. Lac$^+$ revertants of these hybrid plasmids were selected as described above; plasmid pREG14 was one of these revertants, and the structure of this plasmid is shown in Fig. 2.

Construction of deletion plasmids. A series of internal deletions of the *exu* region were introduced into plasmids pRE8 and pRE9 (39) by using restriction enzymes. pRE8 contained the *Bam*HI-1–*Bam*HI-3 fragment of the *exu* region, whereas pRE9 carried the *Bam*HI-2–*Bam*HI-4 fragment of this region. Both *exu* fragments were inserted into the *tet* gene of pBR322 (Fig. 3).

Two deletions were obtained by using endonuclease *Pst*I, which cuts pRE8 and pRE9 at two sites in the cloned *exu* region and at one site in the *bla* gene of the pBR322 portion of the plasmid. The deletion plasmid designated pRE8ΔA had lost the 1.1-Md *Pst*I-1–*Pst*I-2 fragment bearing the *exuT* gene and part of the *exuR* gene (Fig. 3). This plasmid was constructed by partially digesting pRE8 with *Pst*I, ligating with T4 DNA ligase, transforming strain 1357 (*exuT* mutant), and selecting galacturonate$^-$ transformants on galacturonate-containing MacConkey agar plates supplemented with ampicillin. In plasmid pRE9ΔD, the 2.5-Md *Pst*I-2–*Pst*I-3 segment including the entire *uxaCA* operon (Fig. 3) was deleted. This plasmid was derived from pRE9 by the same procedure that was used for pRE8ΔA construction, except that the transformed strain was strain 1864 (*uxaC* mutant). Cleavages of pRE8ΔA and pRE9ΔD with *Bam*HI, *Sal*I, and *Pst*I

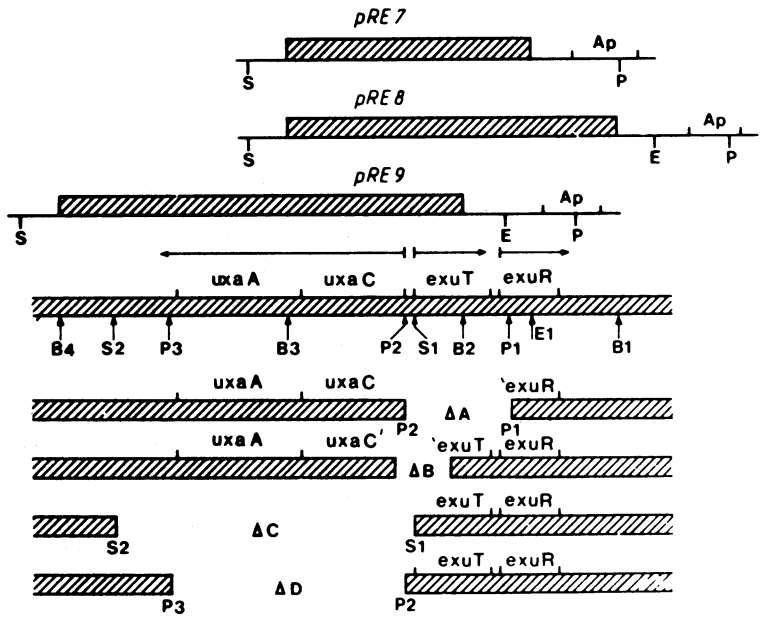

FIG. 3. Structures of in vitro-generated deletions in the *exu* region. The construction of these deletions is described in the text. For other details see the legend to Fig. 2. The physical structures of the three pRE plasmids in which the deletions were generated are shown at the top. In the middle is a map of the *exu* region which indicates the restriction sites. The four in vitro-generated deletions integrated into the chromosome are shown at the bottom.

yielded the expected patterns.

One deletion plasmid was constructed by using restriction enzyme *Sal*I. Three *Sal*I sites are present in plasmid pRE9, two in the cloned *exu* region enclosing the *uxaCA* operon (Fig. 3) and one in the inactivated *tet* gene. After partial digestion of pRE9 with *Sal*I, ligation, and transformation of strain 1864, 20 galacturonate$^-$ ampicillin-resistant transformants were analyzed for their plasmid DNAs; one transformant contained a plasmid in which only the 3.1-Md *Sal*I-1–*Sal*I-2 fragment had been removed. This plasmid was characterized by using endonucleases *Bam*HI, *Sal*I, and *Pst*I and was designated pRE9ΔC. Plasmid pRE7 contains functional *uxaC* and *exuT* genes on the *Eco*RI-1–*Bam*HI-3 fragment (39) (Fig. 3). Plasmid pRE7ΔB was a derivative of pRE7 and was isolated from a galacturonate$^-$ pRE7 transformant of strain 1864, which spontaneously appeared as a white clone on a galacturonate-containing MacConkey agar plate supplemented with ampicillin. A restriction enzyme analysis of pRE7ΔB revealed that this spontaneous deletion was 0.6 Md long and included the *Pst*I-2 and the *Sal*I-1 sites. When introduced into strain 1357 or 1864, this plasmid did not allow growth of the transformants on galacturonate; in contrast, it was able to complement by recombination all of the *uxaC* mutations (M. Mata-Gilsinger, Ph.D. thesis, Université Claude Bernard, Lyon, France, 1978), but not the *exuT* mutations located to the left of the *Bam*HI-2 site (Mata-Gilsinger and Ritzenthaler, submitted for publication). Therefore, the ΔB deletion removed the beginning of the *uxaC* and *exuT* genes and their regulatory regions.

RESULTS

Determination of the *exuT* direction of transcription. To locate the promoter of the *exuT* gene, we used *lac* fusion plasmid pMC874 (11). This plasmid includes *lac* operon in which the operator-promoter sites of the *lacZ* gene and the first amino acids have been removed and replaced by a unique *Bam*HI restriction site. The *lac* genes can be regulated by any promoter cloned in the correct orientation at the *Bam*HI site. The hybrid *lacZ* gene is expressed as a hybrid protein with β-galactosidase activity only when the translational reading of the gene on the *Bam*HI-generated fragment is in frame with the reading of the *lacZ* gene.

The 1.85-Md *Bam*HI fragment of plasmid pRE4 was inserted into pMC874 in the two possible orientations, yielding plasmids pREG8 and pREG9 (Fig. 2). The construction of these plasmids is described above.

When plasmids pREG8 and pREG9 were introduced into strain 2510 (*lacZY* deletion) and into strain HB101 (*lacY* mutation), a Lac$^+$ phenotype on lactose-containing MacConkey agar plates was restored only in the transformants of strain HB101 (6). This indicates that the *Bam*HI fragment cloned into pMC874 possessed a promoter activity in the left and right directions

since the *lacY* gene was expressed on pREG8 as well as on pREG9. The fact that β-galactosidase was not synthesized in the transformants of strain 2510 suggests that the *uxaC* and *lacZ* codons in pREG8 and the *exuT* and *lacZ* codons in pREG9 were translationally out of phase. Selection for growth on lactose-containing minimal medium allowed isolation of mutations (probably microdeletions), shifting the translational reading frame between *uxaC* (or *exuT*) and *lacZ* on plasmid pREG8 (or pREG9). This procedure yielded Lac$^+$ revertant fusion plasmids in which an active hybrid β-galactosidase was synthesized. pREG10 and pREG11 are Lac$^+$ derivatives of pREG8 and pREG9, respectively. Introduction of plasmid pREG10 or pREG11 into strain 2510 resulted in a Lac$^+$ phenotype. When plasmid pRE2 carrying the *exuR* regulatory gene was added in *trans* in these transformants, β-galactosidase synthesis was strongly reduced, suggesting that transcription of the two fused genes was regulated like transcription of the original *exuT* and *uxaA* genes, by the ExuR repressor (Table 2). These results confirm the divergent transcription of the *exuT* and *uxaCA* operons.

TABLE 2. Effect of *exuR* repressor on expression of the *exu* regulon genes in the presence of pREG plasmids

lac fusion plasmid	Plasmid in *trans*	Sp act of:[a]			
		β-Galactosidase		Uronate isomerase (*uxaC*)	Altronate oxidoreductase (*uxaB*)
		exuT-lacZ	*uxaA-lacZ*		
pREG10	None		805		
	pRU12 (*uxuR*$^+$)		785		
	pRE2 (*exuR*$^+$)		40		
pREG11	None	1,490		1,950	210
	pRU12	1,440		1,980	220
	pRE2	90		40	15
pREG12	None	1,530		2,100	240
	pRU12	1,485		2,150	210
	pRE2	80		50	10
	pRE3	60		60	10
pREG13	None	1,550		10	20
	pRU12	1,590			
	pRE2	1,460			
pREG14	None	830			
	pRU12	720			
	pRE2	705			

[a] Specific activities are given in milliunits (nanomoles of product per minute) per milligram of dry weight. Strain 2510 carrying the plasmids indicated was grown at 37°C to an appropriate cell density in glycerol-containing M9 minimal medium and was assayed after treatment in a French press for enzyme activities.

The hybrid proteins encoded by *exuT-lacZ* and *uxaA-lacZ* were detected in crude extracts on sodium dodecyl sulfate-polyacrylamide gels (data not shown). Extracts from strain 2510 carrying pREG10 or pREG11 failed to produce a peptide band in the normal position for β-galactosidase (molecular weight, 116,000). Two new bands appeared, and the estimated molecular weights of the corresponding polypeptides were 123,000 and 120,000 for pREG10 and pREG11. This finding is consistent with the interpretation that strains bearing these plasmids produce hybrid β-galactosidase.

Analysis of the expression of the *exu* region genes on pREG plasmids and their derivatives. To determine whether *uxaCA* and *exuT* are controlled by a single operator or are transcribed from two different control regions, various plasmids were analyzed. Plasmid pREG12 is a derivative of pREG11 in which the *Pst*I restriction fragment containing the *exuT-lacZ* gene has been inverted (Fig. 2).

The activities of the *exu* region enzymes were measured in strain 2510 carrying pREG11 or pREG12; β-galactosidase synthesis reflected the expression of the *exuT* gene. The isomerase and β-galactosidase activities were identical in the presence of pREG11 or pREG12, and *uxaB* gene expression was weakly derepressed (Table 2). When plasmid pRU12 containing the *uxuR* regulatory gene was added in *trans* in strain 2510(pREG11) or 2510(pREG12) transformants, we observed no significant effect on the expression of the *exuT* and *uxaCA* operons, as expected, since the UxuR repressor does not affect the expression of the *exuT*, *uxaCA*, and *uxaB* operons (42). In contrast, the addition of plasmid pRE2 or pRE3 carrying the specific *exuR* regulatory gene resulted in strong repression of isomerase and β-galactosidase synthesis (Table 2). Since the inversion of the *Pst*I fragment in pREG12 did not inactivate the control region of the *exuT* and *uxaCA* operons, we assumed that these operons possess their own operators which are located on opposite sides of the *Pst*I-2 site.

To assure that the regulatory region of the *exuT* operon was located to the right of the *Pst*I-2 site (Fig. 2, plasmid pREG11), plasmids pREG13 and pREG14 were constructed. In these new plasmids containing *exuT-lacZ* gene fusions, the *exu* region located to the left of the *Sal*I-1 site has been removed (Fig. 2). When the resulting plasmids were introduced into strain 2510, β-galactosidase synthesis was not modified by the addition of multiple copies of *exuR* in *trans*, suggesting that no functional *exuT* operator was borne by plasmids pREG13 and pREG14

(Table 2). Thus, the *exuTo* operator may be partially or entirely located to the left of the *Sal*I-1 site.

Mapping of *exuTo* and *uxaCo* operators with restriction enzyme deletions. Starting with plasmids pRE7, pRE8, and pRE9 (Fig. 3), we generated four partial deletions of the cloned *exu* region as described above, yielding plasmids pRE8ΔA, pRE9ΔC, pRE9ΔD, and pRE7ΔB. These deletions were recombined by homologous recombination onto the chromosome, using the following characteristic of the hexuronate system: growing a strain carrying a *kdgA* mutation on glycerol plus galacturonate results in the accumulation of toxic 2-keto-3-deoxy-6-phosphogluconate and then death (35). Secondary mutations or deletions earlier in the galacturonate pathway prevent accumulation of the poisonous compound and allow growth in the presence of this sugar.

The deletion in plasmid pRE8ΔA removed the entire *exuT* gene and part of the *exuR* regulatory gene (Fig. 3). The *exuT* gene is transcribed in the same direction as the *exuR* gene, but these two genes belong to different operons (21). Therefore, integration of deletion ΔA into the chromosome results in constitutive expression of the *exu* regulon structural genes. Plasmid pRE8ΔA was introduced into strain 2161 (*kdgA* mutation). From the resulting transformants mutants able to grow on glycerol plus aldohexuronate (glucuronate and galacturonate) were selected. Only the clones that constitutively synthesized altronate oxidoreductase and were not able to grow on glycerol plus tagaturonate or fructuronate were retained. In the same way, the deletions in plasmids pRE7ΔB, pRE9ΔC, and pRE9ΔD (Fig. 3) were transferred onto the chromosome by plating the corresponding transformants of strain 2161 onto medium containing tagaturonate plus glycerol; only the clones that were able to grow on glucuronate plus glycerol but were poisoned on fructuronate plus glycerol were retained. In these clones grown on tagaturonate plus glycerol, altronate oxidoreductase activity was detectable. These growth phenotypes correspond to double mutations lying in *uxaA* and *uxaC* or *uxaA* and *exuT*. The frequency of the double mutants was incompatible with the frequencies of two single spontaneous mutations; therefore, the isolated clones probably integrated the deletions from the plasmids by recombination. The presumed deletion strains were then cured of the initial plasmid by many generations of growth without antibiotic selection.

The four *exu* mutant strains (strains 2987, 3013, 3211, and 3093 [Table 1]) were used as donors in the transduction of strain EW1b (*tolC* mutation) by phage P1. The frequencies of cotransduction of the glucuronate$^-$ galacturonate$^-$ phenotype with *tolC* were 7 to 20%, corresponding to mutations located in the *exu* region (27). The four deletions were also characterized precisely by transduction to galacturonate$^+$ with various λ p*exu* transducing phages (25). Phage λp*exu*4, carrying *exuT* and a part of *uxaC*, was able to restore growth on galacturonate when it was introduced into strain 2992 (deletion ΔA) or into strain 3094 (deletion ΔB), whereas λ p*exu*5, carrying *exuT* and *uxaC*, and λ p*exu*4 failed to complement the *exu* mutation of strain 3014 (deletion ΔD) or the *exu* mutation of strain 3214 (deletion ΔC). Only λ p*exu*2, transducing operons *exuT* and *uxaCA*, complemented the two last deletions. Our complementation results are in agreement with the expected extents of the four deletions introduced into the chromosome. In addition, reversion tests (26) on galacturonate-containing minimal medium verified that these mutations were deletions (data not shown).

The activities of the *exu* regulon enzymes were determined in the four deletion strains. As expected, in strain 2992 (ΔA), uronic isomerase, altronate hydrolyase, and altronate oxidoreductase were constitutively synthesized, proving that the *exuR* gene was not functional. This constitutive synthesis was completely abolished by adding plasmid pRE2 (data not shown). Therefore, the presumed *Pst*I-1–*Pst*I-2 deletion of strain 2992 leaves intact the *uxaC* operator, suggesting that *uxaCo* is located to the left of the *Pst*I-2 restriction site (Fig. 3).

In strain 2992 (ΔA), as well as in strain 3094 (ΔB), the *exuT* gene was not expressed even in the presence of inducer, proving that this gene or promoter was removed by the deletions. In strain 3214 (ΔC), the uninduced level of aldohexuronate transport activity was identical to the induced level, whereas in strain 3014 (ΔD), the synthesis of this protein was normally inducible (Table 3); hence, deletion ΔC affected the binding of the *exuR* repressor.

The expression of *exuT* in the deletion strains was confirmed by determining the conditions under which altronate oxidoreductase was inducible, when various hexuronate intermediates were added. In strain 3094 (ΔB) glucuronate and galacturonate failed to induce altronate oxidoreductase synthesis due to the absence of aldohexuronate uptake into the cell. In strains 3214 (ΔD) and 3214 (ΔC), *uxaB* gene expression was depressed by the addition of galacturonate but was still uninduced in the presence of glucuronate. In this last case, the two aldohexuronates entered the cells and were not degraded; galacturonate was a weak inducer, but glucuronate alone was not able to induce. This demonstrated the presence in the two strains of a functional *exuT* gene and the absence of the *uxaC* gene.

TABLE 3. *exuT* and *uxaB* gene expression in *exu*-deleted strains

Strain	Aldohexuronate transport activity (*exuT* gene) with:		Altronate oxidoreductase activity (*uxaB* gene) with:			
	No inducer	Mannonic amide (5 mM) inducer	No inducer	Galacturonate (5 mM) inducer	Glucuronate (5 mM) inducer	Tagaturonate (5 mM) inducer
EW1b (wild type)	14[a]	100 (7)[b]	−[c]	+	+	+
2992 (ΔA)	2	10	+	+	+	+
3094 (ΔB)	3	10	−	−	−	+
3214 (ΔC)	89	105 (1.2)	−	+	−	+
3014 (ΔD)	13	110 (8)	−	+	−	+

[a] Percentage of maximum value induced in the wild type.
[b] The numbers in parentheses are induction ratios.
[c] −, No enzyme activity (basal level); +, large quantity of enzyme present.

These results agree with the analysis of the different pREG plasmids; presumed *Pst*I-2–*Pst*I-3 deletion ΔD did not affect the expression of *exuT*, whereas the presumed *Sal*I-1–*Sal*I-2 deletion caused constitutive expression of this gene.

DISCUSSION

To refine our understanding of the genetic organization of the regulatory region of the *uxaCA-exuT* operons, we formed two *exuT-lac* and *uxaA-lac* fusions in vitro by cloning the same *Bam*HI-*exu* fragment into plasmid vector pMC874 in the two possible orientations. In the two resulting plasmids a hybrid β-galactosidase was produced; this finding confirmed that the two *exuT-uxaCA* operons are divergently transcribed. Derivatives of these recombinant plasmids were constructed. An analysis of hybrid β-galactosidase production in the presence of multiple copies of the *exuR* regulatory gene showed that the *exuT* and *uxaCA* genes have different regulatory regions; therefore, they form two distinct operons. The same conclusion was reached by using *exu* deletions which were generated on pRE plasmids and which were supposed to be integrated into the chromosome.

The genetic data presently available allow us to assign limits to the DNA regions that are necessary to regulate and promote transcription at the *uxaCA* and *exuT* operons. On the fusion plasmids, as well as on the chromosomal *exu* deletions, *exuT* is normally regulated even when the DNA region located to the left of the *Pst*I-2 site is removed. In the same manner, deletion of a fragment lying to the right of the *Pst*I-2 site does not affect the expression of the *uxaCA* operon. This suggests that *exuTo* is probably located to the right of the *Pst*I-2 site and that the whole *uxaCA* regulatory region should lie to the left of this restriction site; this result slightly changes the previous location of *uxaCo* (39). Using two *exu* plasmids bearing the *uxaC* gene, one (pRE17), including the *Pst*I-2–*Sal*I-1 segment and the other (pRE16), lacking this restriction fragment, we concluded that *uxaCo* overlaps the *Pst*I-2 site. In strains containing pRE17, *uxaC* gene expression was strongly reduced when a compatible plasmid *exuR* was added in *trans*, whereas in strains harboring pRE16 the protein coded for by *uxaC* was still synthesized in large amounts, although the repressor was overproduced from a plasmid. Under these conditions, the cells contained about one copy of *uxaCo* per copy of *exuR* since these two DNA fragments were carried by two compatible multicopy plasmids. We now feel that this result is best explained by readthrough transcription from the strong pBR322 *bla* promoter in the *Pst*I-*uxaC* fragment of pRE16. The same phenomenon was observed for a *trpR-lacZ* fusion; Bogosian et al. (7) found high expression and no autogenous regulation of the *trpR* gene in the presence of one copy of *trpR-lacZ* gene per copy of a wild-type *trpR* gene. A significant reduction in β-galactosidase production was observed only when strains harboring the *trpR-lacZ* fusion on the chromosome were transformed with plasmids that cause overproduction of the Trp repressor; i.e., when about 20 copies of *trpR* were added per copy of *trpR-lacZ* gene. Kelley and Yanofsky (22) explained these results by readthrough transcription from the pBR322 *tet* promoter located in front of the *trpR-lacZ* fusion. Other evidence for the location of the *Pst*I-2 site on the *uxaCA* operator was obtained from repressor titration tests; only when endonuclease-generated fragments overlapping the *Pst*I-2 site were present on a multicopy plasmid was constitutive derepression of the chromosomal *uxaB* gene observed (39). We now know that the two operators of the *uxaCA* and *exuT* operons are very close and that their repressor affinity is reduced (Mata-Gilsinger and Ritzenthaler, manuscript in preparation). Therefore, *uxaB* gene expression becomes constitutive only when the *uxaC* and *exuT* operators are both

introduced into a multicopy plasmid. From all of these data, it appears that the *exuT* and *uxaCA* operators are located on either side of the *Pst*I-2 site.

Surprisingly, a deletion of the *Pst*I-2–*Sal*I-1 fragment affects the regulation of the *exuT* gene but does not prevent its expression, suggesting that the deletion removes the operator sequences but retains intact the promoter sequences. The hypothesis that an active vector promoter drives expression of the *exuT* gene in plasmid pREG13 or pREG14 is unlikely; the vector DNA sequences near the inserted *exu* region were the distal part of the *tet* gene, and no promoter activity is known in this plasmid region. In addition, when deletion ΔC (*Sal*I-1–*Sal*I-2) was recombined into the bacterial chromosome in the absence of any plasmid DNA, the *exuT* gene was still expressed constitutively. Therefore, *exuTo* should be located on the *Pst*I-2–*Sal*I-1 fragment, and the *exuTp* promoter should lie to the right of *Sal*I-1. The putative location of the *exuT* operator contrasts with the locations of operators in the other operons. In the *lac* (16), *bio* (29), and *trp* (3) systems, the repressor binds close to the transcription initiation site and probably works by excluding RNA polymerase from the promoter. In contrast, the operator of the *gal* operon is located about 60 base pairs before the starting points for transcription of the two *gal* promoters, suggesting a different mechanism for repression (15). The *exuT* operon could be similar to the *gal* operon.

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