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Molecular Cloning of *Escherichia coli* K-12 Hexuronate System Genes: *exu* Region

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Lambda transducing bacteriophages carrying the *exu* region (min 66) of *Escherichia coli* K-12 (λ p*exu*) were previously isolated. A restriction map of these phages is presented. Starting from the λ p*exu* phage deoxyribonucleic acid, various endonuclease-generated *exu* fragments were subcloned into multicopy plasmid vectors, using in vitro recombination techniques. The precise location of the *exu* genes, relative to the endonuclease sites, was determined. Plasmids carrying *uxaC* and *uxaA* genes overproduced the corresponding enzymes 30- to 40-fold. When these plasmids were expressed in an in vitro protein-synthesizing system, two polypeptides of 50,500 and 53,000 molecular weights appeared and were identified as the *uxaC* and *uxaA* gene products. A 2.6-kilobase-pair deoxyribonucleic acid fragment was shown to code for a functional *exuR* repressor which controls the expression of the *exu* region. Plasmids containing this fragment overproduced the regulatory protein. It was possible to localize the operator region, *uxaCo*, which overlapped a *Pst*I endonuclease site, and to confirm the transcriptional direction of the *uxaC-uxaA* operon from *uxaC* to *uxaA*.

The aldohexuronates, glucuronate and galacturonate, are degraded according to the Ashwell pathway (2). Galacturonate, tagaturonate, and fructuronate are able to induce the synthesis of enzymes A, II, III′, and IV′ (Fig. 1; 17, 25). The *exu* regulon involves the *exu* region (17, 19) located at min 66 (3) and the *uxaB* operon (enzyme III′) located at min 52 (Fig. 1). The *exu* region includes the *exuR* regulatory gene and the three structural genes *uxaC* (enzyme II), *uxaA* (enzyme IV′), and *exuT* (aldohexuronate transport system, protein A). The *exu* regulon is under the negative control of the *exuR* regulatory gene product (20).

As a first step in studying the genetic regulation of hexuronate metabolism in vitro, various lambda transducing particles carrying either the whole or a part of the *exu* region were previously isolated from a lysogen which had integrated a λ phage in the *exuR* regulatory gene (14).

In this paper, the physical characterization of these phages is presented as well as the restriction map of the *exu* region. We also report the molecular cloning of the *exu* region into multicopy plasmids, using the *exu* transducing phages as a source of DNA. Strains showing about 30-fold increased production of enzymes II and IV′ were obtained, and the operator of the *uxaC-uxaA* operon was localized on an endonuclease-generated fragment.

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 *Escherichia coli* K-12 derivatives.

Culture media. Media for growth were identical to those described by Portalier et al. (20). The minimal medium used was M63, pH 7.2 (26), or M9, pH 7.2 (15). Solid media contained glucose (5 g/liter), glycerol (5 g/liter), glucuronate (2.5 g/liter), or galacturonate (2.5 g/liter). When needed, ampicillin, tetracycline, chloramphenicol, and kanamycin were added at final concentrations of 25 μg/ml.

Chemicals and enzymes. D-Fructuronate was synthesized in our laboratory (J. Robert-Baudouy, thesis, Université Claude Bernard, Lyon, France, 1971).

*Eco*RI, *Bam*HI, and *Hin*dIII restriction enzymes and T4 DNA ligase were obtained from Miles Laboratories, the *Sal*I enzyme was from BRL Laboratories, and *Sma*I, *Pst*I, and *Bgl*II enzymes were from Boehringer Mannheim.

Chloramphenicol was from Sigma Chemical Co., tetracycline, ampicillin, and kanamycin were from Serva Feinbiochemica, and spectinomycin was from The Upjohn Co.

L-[^{35}S]methionine (700 to 1,300 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England.

Enzyme induction and extract preparation. The conditions for induction and extraction of the hexuronate system enzymes were outlined previously (25).

Enzyme assays. D-Altronate:NAD oxidoreductase and D-altronate hydrolyase were assayed according to previously published methods (22). In situ plate assays for D-altronate:NAD oxidoreductase activity were performed on clones grown overnight on glycerol agar medium as described by Portalier and Stoeber (21). Only clones derepressed or induced for D-altronate:

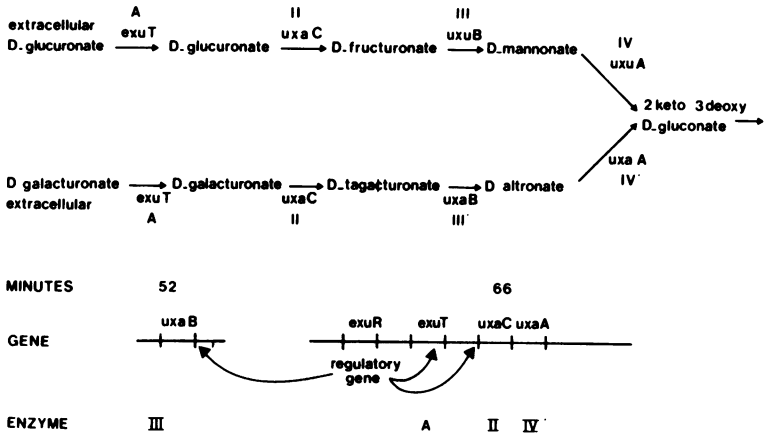

FIG. 1. *Degradative pathway of hexuronates in E. coli K-12. II. Uronate isomerase (EC 5.3.1.12); III, mannonate oxidoreductase (EC 1.1.1.57); IV, mannonate hydrolyase (EC 4.2.1.8); III', altronate oxidoreductase (EC 1.1.1.58); IV', altronate hydrolyase (EC 4.2.1.7); A, aldohexuronate transport system.*

TABLE 1. *Bacterial strains, bacteriophages, and plasmids*

Designation	Relevant genotype	Reference
Bacterial strains		
1357	*exuT6 argG thyA recA rpsL*	This laboratory
1864	*uxaC2 metC thyA argG recA rpsL*	This laboratory
1865	*uxaA1 metC thyA recA rpsL*	This laboratory
1866	*Δ(exuR'-exuT-uxaC-uxaA) argG recA rpsL*	Derived from 1576 (14)
1475	*exuR argG his*	This laboratory
HB101	*leu pro hsdM hsdR recA rpsL*	H. W. Boyer
159 (λ *ind*)	*gal* UV^s *rpsL* (λ *ind*)	(27)
Bacteriophages		
λ wild type	*cI857*	M. Gottesman
λ p*exu2*	*cI857 (exuR') exuT uxaC uxaA*	This laboratory (14)
λ p*exu3*	*cI857 exuR exuT uxaC uxaA*	This laboratory (14)
λ p*exu4*	*cI857 (exuR') exuT*	This laboratory (14)
λ p*exu5*	*cI857 (exuR') exuT uxaC*	This laboratory (14)
λ p*exu6*	*cI857 exuR exuT uxaC uxaA*	This laboratory
Bacterial plasmids		
pBR322	bla^+ tet^+	6
pACYC177	bla^+ aac^+	8
pACYC184	tet^+ cat^+	8

NAD oxidoreductase activity become blue under these conditions. Hexuronic isomerase was measured by a coupling method described in a previous paper (19). β-Lactamase activity was measured as described by Sykes and Nordström (29).

Preparation of phage DNA. Purification of phage and phenol extraction of DNA were carried out as described by Allet et al. (1).

Isolation and manipulation of plasmid DNA. Plasmid DNA was amplified in growing cultures by addition of 150 μg of chloramphenicol per ml (9). Spectinomycin (250 μg/ml) was used for the amplification of plasmids carrying the *cat* gene. Extraction and purification of plasmid DNA was achieved by the alkaline extraction procedure described by Birnboim and Doly (5) for rapid analysis of the restriction endonuclease digestion pattern. The cleared lysate technique of Guerry et al. (11) was also used. Further purification of the DNA was done by dye-buoyant density centrifugation in CsCl gradients containing ethidium bromide (23).

Restriction endonucleases were used as recommended by the manufacturers. Digestions with two or more endonucleases requiring different buffers were performed sequentially, using the low-salt buffer first. The reaction was terminated by heating the initial digestion product for 10 min at 60°C before digestion with the second endonuclease.

Recombinant plasmids were constructed in vitro by ligation of endonuclease-generated fragments with T4 DNA ligase at a concentration of 1 U/ml at 8°C in 66 mM Tris-hydrochloride (pH 7.6)–6.6 mM $MgCl_2$–10 mM dithiothreitol–0.5 mM ATP for 10 to 20 h. DNA concentrations were varied, depending on the desired outcome, in accordance with the considerations of Dugaiczyk et al. (10). DNA ligation was monitored by the electrophoresis of small samples of the reaction mixture taken at intervals.

E. coli cells were prepared for transformation with plasmid DNA by the method described by Mandel and Higa (13) as modified by Wensink et al. (30).

Agarose and polyacrylamide gel electrophoreses. Electrophoresis of DNA was carried out in 1% agarose gels in 20 mM sodium acetate–40 mM Tris-acetate (pH 8.0), using a vertical slab gel apparatus; electrophoresis was performed usually at 25 mA for 14 h at room temperature, and gels were photographed as described in reference 6. *Hin*dIII- and *Eco*RI-generated fragments of bacteriophage λ were used as molecular-weight standards in gels.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro products, samples were made 10% glycerol–2% sodium dodecyl sulfate–5% β-mercaptoethanol, boiled for 8 min, and electrophoresed on 10, 12.5, or 15% sodium dodecyl sulfate-polyacrylamide gels according to Laemmli (12) and Studier (28).

Conditions for cell-free protein synthesis. The conditions used for cell-free protein synthesis and preparation of S-30 were those of Zubay et al. (31). Synthesis was performed in a 0.1-ml reaction mixture for 2 h at 37°C. The incubation mixture contained 40 mM Tris-acetate (pH 8.2), 1.4 mM dithiothreitol, 55 mM potassium acetate, 27 mM ammonium acetate, 15 mM magnesium acetate, 7 mM calcium chloride, 0.25 mM each 20 amino acids, 2.2 mM ATP, 0.6 mM each GTP, UTP, and CTP, 21 mM trisodium phosphoenolpyruvic acid, 0.5 mM cyclic AMP, 100 μg of *E. coli* tRNA per ml, 27 μg each of folinic acid, FAD, triphosphopyridine nucleotide, and pyridoxine-HCl per ml, 11 μg of *p*-aminobenzoic acid per ml, and 16 mg of polyethylene glycol 6000 per ml. The total divalent cation concentration was optimized for each S-30 fraction: the optimum amount was determined for each preparation as measured by incorporation of L-[^{35}S]-methionine into acid-precipitable material in the DNA-directed cell-free system. Plasmid DNA was used at a concentration of 100 μg/ml; S-30 was used at 6.5 mg of protein per ml.

Newly synthesized polypeptides were labeled by the addition of 100 μCi of L-[^{35}S]methionine per ml, instead of 0.25 mM methionine, and visualized by fluorography (7) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Phage infection of UV-irradiated cells. The method of Murialdo and Siminovitch (16), as modified by Springer et al. (27), was adapted for phage infection of UV-irradiated cells. Cells were grown at 37°C in minimal medium containing 1.6 g of galacturonate and 4 g of maltose per liter. Labeling was performed with 15 μCi of L-[^{35}S]methionine per assay. The labeled extracts were analyzed on sodium dodecyl sulfate-polyacrylamide gels as described for cell-free protein synthesis.

RESULTS

Physical characterization of λ p*exu* transducing phages and restriction map of the *exu* region. The restriction nuclease map of the λ p*exu* genomes is shown in Fig. 2 and confirms the λ p*exu* phage structures determined previously by genetic analysis (14). λ p*exu*4, λ p*exu*5, and λ p*exu*2, carrying *exuT*, *exuT-uxaC*, and *exuT-uxaC-uxaA* genes respectively, are λ p*gal*-type transducing phages, and in their genomes, the restriction sites located in the wild-type b2 region were replaced by those of the chromosomal substituted DNA. Comparison of the restriction maps of λ p*exu*5 and λ p*exu*2 revealed that *exuT* and *uxaC* were localized between ΔP′ and the *Bam*HI-3 site and that the *Bam*HI-3 site was probably within the *uxaA* gene.

The recombinant phages λ p*exu*3 and λ p*exu*6 transduced the whole *exu* region and were derived from a cross between λ p*exu*2 carrying the left part of the *exu* region and two independent λ *spi* phages carrying the right part (14). Single or multiple endonuclease digestions showed that a substitution-deletion occurred on the right side of *att* for the two recombinant phages: λ p*exu*3 and λ p*exu*6 had 0.7 and 5.2 kilobase pairs less than one of the parental phages, λ p*exu*2. The bacterial DNA inserted on the right of *att* did not possess restriction sites for the enzymes used in this work.

The *exu* region restriction map has been established by analyzing the cleavage pattern of the different transducing and wild-type phage genomes, except for the *Pst*I sites. Since λ DNA contains 18 *Pst*I sites, it was easier to map these sites in the three *exu Bam*HI fragments subcloned in pBR322. From the λ p*exu*6 phage, it was determined that the four genes belonging to the *exu* region (*exuR*, *exuT*, *uxaC*, and *uxaA*) were carried on a 9-kilobase-pair DNA fragment, and this fragment contained three *Bam*HI, three *Pst*I, two *Sal*I, one *Sma*I, one *Eco*RI and one *Bgl*II restriction sites (see Fig. 3).

Genetic and physiological evidence for the presence of the *exu* genes on the pRE plasmids. The restriction fragments of the *exu* region transduced by the λ p*exu*6 phage were subcloned into plasmid vector pBR322 (6) or pACYC177 (8). The resulting pRE plasmids are shown in Fig. 3.

A secondary attachment site, *att* ΔOΔ′, of bacteriophage λ lies within the *exuR* gene (14), and this site is located near the chromosomal *Eco*RI site of the transducing phages (see Fig. 2). Plasmids pRE1, pRE2, pRE3, pRE8, and pRE19, which contain this region, probably carry part of or the whole regulatory gene. In strain 1475, the *exu* regulon enzyme synthesis is constitutive. The constitutive expression of *uxaB* disappeared in transformants 1475(pRE2) and 1475(pRE3) but persisted in transformants 1475(pRE1) and 1475(pRE19). Thus, *Eco*RI-1 and *Pst*I-1 sites lie within the *exuR* gene. Strains carrying plasmid pRE2 or pRE3 grew very slowly on galacturonate or glucuronate as the sole carbon source. To determine whether *exuR*

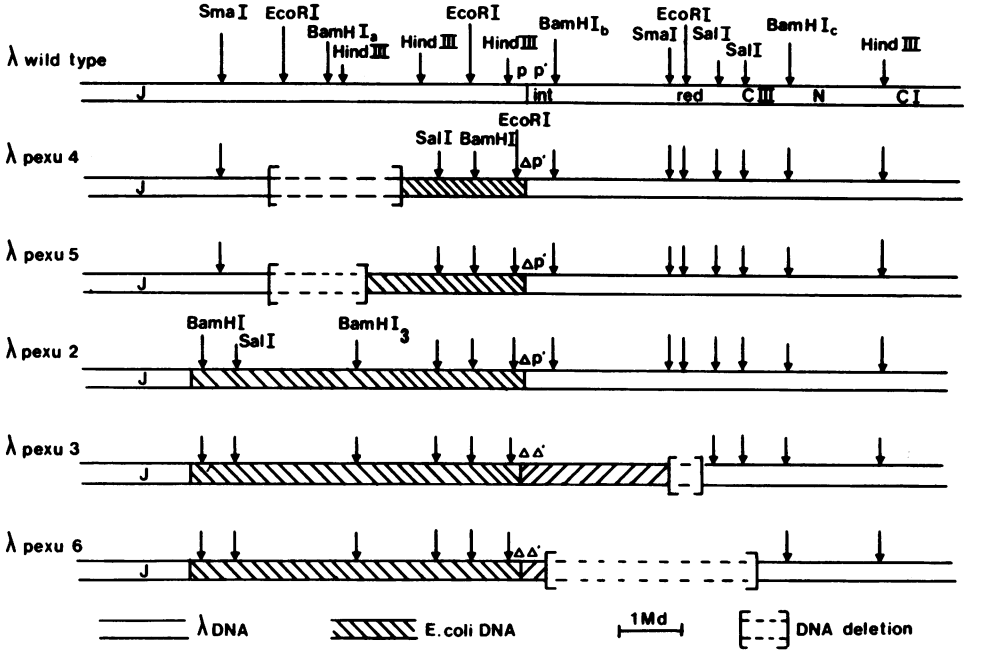

FIG. 2. *Restriction map of λ wild-type and λ pexu transducing phages (region located between the two λ genes, J and cI).*

cloned on a multicopy plasmid was able to further reduce *exu* regulon expression, we measured *exu* regulon enzyme levels in various plasmid transformants. Even in the presence of a high concentration of inducer, *exu* regulon expression in strains bearing the *exuR* plasmids, i.e., pRE2 or pRE3, was twofold and about tenfold lower, respectively, than in the isogenic *exuR* haploid strain (see Table 3). These observations are consistent with the expectation that *exuR* repressor is overproduced in strains bearing *exuR* on multicopy plasmids. Neither pRE2 nor pRE3 carried other *exu* genes since they did not complement mutations in *exuT*, *uxaC*, and *uxaA* genes (Table 2).

As shown by the restriction analysis of λ *pexu* transducing phages, the *exuT* and *uxaC* genes are located between the *att* ΔOΔ' and *Bam*HI-3 sites. The pRE6 and pRE18 transformants of strain 1357 (*exuT* mutation) were able to catabolize galacturonate. In contrast, plasmids pRE5 and pRE13, when introduced into strain 1357, did not allow growth on galacturonate (Table 2). These results indicate that the *exuT* gene is included in the *Sal*I-1–*Pst*I-1 fragment and that the *Bam*HI-2 site is within the *exuT* gene. Plasmids pRE6 and pRE18, in an *exuR*, *uxaA*, or *uxaC* strain, did not complement these mutations (Table 2) and therefore did not contain any one of these three genes in a functional state. In addition, these plasmids did not cause a constitutive synthesis of enzyme III' when introduced in a wild-type strain (Table 2). This point will be discussed below.

All of the following plasmids complemented the *uxaC* mutation but not the *exuT* or *uxaA* mutation: pRE4, pRE14, pRE11, pRE12, and pRE17. These results suggested that the *uxaC* gene is on the *Sal*I1–*Bgl*II-1 fragment. In strain 1864 or HB101 carrying the plasmid pRE4 (Table 3), enzyme II was synthesized at a high level: the uninduced rates were about 20- to 40-fold the normal induced rate of a haploid strain.

Confirmation of the location of *exuR*, *exuT*, and *uxa*C genes on the above mentioned fragments was given by plasmids pRE7 (*Eco*RI-1–*Bam*HI-3) and pRE8 (*Bam*HI-1–*Bam*HI-3) since these plasmids contained the restriction fragments coding for *exuT* and *uxaC* products. Indeed, pRE7 or pRE8 transformants of strain 1866 were able to dissimilate glucuronate, proving the presence of *exuT* and *uxaC* genes. The constitutive synthesis of enzyme III' was abolished in strain 1866(pRE8) but persisted in strain 1866 (pRE7) (Table 3); therefore, pRE8 contained, in addition, a functional *exuR* gene. The induced rate of synthesis of enzyme II in strain 1866(pRE8) is about threefold higher than the uninduced rate, but much lower than in pRE4 transformants also carrying *uxaC* but lacking *exuR*. The *exuR* regulatory gene present on pRE8 prevented the amplification of the synthesis of enzyme II. The *exuT* mutation in strain 1357 was complemented by pRE7 and pRE8 but

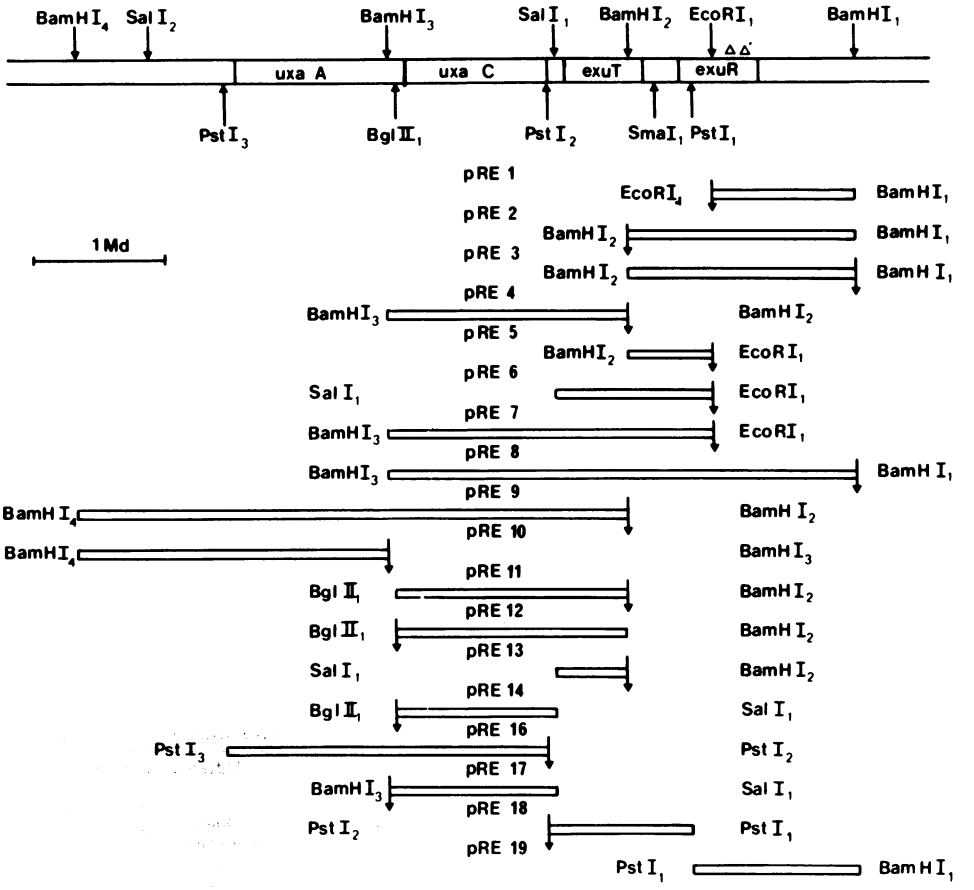

FIG. 3. *Physical maps of recombinant plasmids carrying portions of the exu region. At the top is a map of the exu region indicating appropriate restriction sites (BamHI-1 is phage BamHI-C from Fig. 2). All of the endonuclease-generated fragments were cloned in pBR322, except the pRE19 exu fragment which was integrated in pACYC177. The extremity of the exu-inserted segment near the EcoRI site of pBR322 is labeled with an arrow to show the orientation of the cloned fragment in the vector.*

not by pRE4 (*Bam*HI-3–*Bam*HI-2) and pRE2 (*Bam*HI-2–*Bam*HI-1). Comparison of the structure of these plasmids and their phenotype confirms that the *Bam*HI-2 restriction site lies within the *exuT* gene. The location of *Bam*HI-3 within the *uxaA* gene, as deduced from the restriction analysis of λ p*exu* phages, was borne out by the pattern of plasmids pRE9, pRE10 and pRE4, since when these plasmids were transferred into strain 1865 (*uxaA* mutation), only strain 1865(pRE9) transformants were able to grow on galacturonate (Table 2). Plasmid pRE16 as well as plasmid pRE9 complemented *uxaA* and *uxaC* mutations when they were introduced in strains 1865 and 1864 (Table 2). The fragment *Pst*I-3–*Pst*I-2 appears to carry the two structural genes *uxaA* and *uxaC*.

Location of the operator site of the *uxaC-uxaA* operon. Plasmids pRE4, pRE9, pRE12, pRE14, pRE16, and pRE17 carried the *uxaC* gene. The resulting plasmid transformants of strain 1864 or HB101 exhibited a high rate of enzyme II synthesis, 30- to 40-fold the induced rate of the corresponding haploid strain (Tables 3 and 4).

In addition, all of these plasmids except pRE16 caused a weak constitutive synthesis of enzyme III′ (10-fold the basal level). Constitutive synthesis of enzyme IV′ was also detectable in strains transformed by plasmids carrying *uxaC* but not *uxaA* genes, such as pRE12 or pRE14 (Table 3). This point will be discussed below since it suggests that pRE16 plasmids do not possess a functional operator site.

To check this hypothesis, the *Bam*HI-1–*Bam*HI-2 restriction fragment coding for the *exuR* repressor was inserted in the *tet* gene of plasmid pACYC184 (8), yielding plasmid p184*exuR*. Plasmids pACYC184 and p184 *exuR*, compatible with ColE1-derived plasmids, were

TABLE 2. *Pattern of complementation of the pRE plasmids in various exu mutant strains*[a]

Plasmid	Growth on galacturonate				Altronate oxidoreductase activity[b]	
	1357 (*exuT*)	1864 (*uxaC*)	1865 (*uxaA*)	1866 (Δ*exu*)	HB101	1475 (*exuR*)
pRE1	−	−	−	−	−	++
pRE2	−	−	−	−	−	−
pRE3	−	−	−	−	−	−
pRE4	−	+	−	−	+	++
pRE5	−	−	−	−	−	++
pRE6	+	−	−	−	−	++
pRE7	+	+	−	−	+	++
pRE8	+	+	−	−	−	−
pRE9	−	+	+	−	+	++
pRE10	−	−	−	−	−	++
pRE11	−	+	−	−	+	++
pRE12	−	+	−	−	+	++
pRE13	−	−	−	−	−	++
pRE14	−	+	−	−	+	++
pRE16	−	+	+	−	−	++
pRE17	−	+	−	−	+	++
pRE18	+	−	−	−	−	++
pRE19	−	−	−	−	−	++

[a] Symbols: −, absence of growth or enzyme activity; +, growth or low enzyme activity; ++, presence of large quantity of enzyme.

[b] The presence of altronate oxidoreductase activity was tested on an in situ plate assay as described in the text (cells grow in the absence of inducer).

TABLE 3. *Activity of the exu regulon enzymes in E. coli strains containing different pRE plasmids*

Plasmid	Functional *exu* gene(s) on plasmid	Strain	Inducer (5 mM)	Differential rate of synthesis[a]		
				Uronate isomerase (II) (*uxaC*)	Altronate hydrolyase (IV') (*uxaA*)	Altronate oxidoreductase (III') (*uxaB*)
pBR322	None	HB101	None	20	ND	30
			Galacturonate[b]	310	40	2,200
			Fructuronate	330	38	2,300
		1475	None	490	160	9,400
		1864	Galacturonate	ND		1,500
		1865	Galacturonate	240	ND	1,600
		1866	None	ND	ND	10,000
			Galacturonate	ND	ND	9,900
pRE2	*exuR*	HB101	Galacturonate[b]	140	25	1,400
		1866	None	ND	ND	20
pRE3	*exuR*	HB101	Galacturonate[b]	50	5	200
			Fructuronate	55	5	150
pRE4	*uxaC*	1864	None	11,800		300
		HB101	None	14,000		500
pRE9	*uxaC uxaA*	1865	None	8,500	380	350
			Galacturonate	9,900	480	2,000
pRE8	*exuR exuT uxaC*	HB101	Galacturonate	330	45	2,700
		1866	None	45	ND	750
			Galacturonate	140	ND	1,200
pRE7	*exuT uxaC*	1866	None			9,500
pRE12	*uxaC*	HB101	None	7,860	25	370
pRE14	*uxaC*	HB101	None	7,670	22	400
pRE13	None	HB101	None	15	ND	25

[a] Differential rates of synthesis are given in milliunits (nanomoles of product per minute) per milligram of dry weight. When the *exu* gene is carried on the plasmid, the corresponding enzyme activity is brought back to a constant level of β-lactamase activity. All assays were done at 37°C. ND, Activity not detectable.

[b] In the presence of 5 or 50 mM inducer, the enzyme levels were identical.

TABLE 4. *Effect of repressor added in trans on synthesis of exu enzymes coded for by pRE16 and pRE17*[a]

Plasmid	Functional *exu* gene on plasmid	Plasmid in trans	Enzyme (U)		
			II	III′	IV′
pRE17	*uxaCo uxaC*	pACYC184	7,500	420	
pRE17	*uxaCo uxaC*	p184 *exuR*	35	15	
pRE16	*uxaC uxaA*	pACYC184	4,460	30	490
pRE16	*uxaC uxaA*	p184 *exuR*	4,190	25	470

[a] Strain 1864, carrying the two compatible plasmid types pRE and pACYC184, were grown on glycerol, chloramphenicol, and ampicillin or tetracycline, without inducer, and assayed for the *exu* regulon enzymes. p184 *exuR* is derived from pACYC184, with *exuR* integrated in the tetracycline resistance gene.

introduced in strains 1864(pRE16) and 1864(pRE17) by transformation. The transformants were selected for resistance to chloramphenicol and ampicillin or tetracycline. Enzyme II expression of pRE17 was placed under the control of the *exuR* repressor when p184 *exuR* was added in trans and was less than 1% of the level measured in the presence of pACYC184 (Table 4). Enzyme III′ expression was also reduced in the presence of the *exuR* repressor.

This result implies that pRE17 contained an intact operator site which is able to bind the *exuR* repressor. In contrast, p184 *exuR* had no effect on the expression of the *uxaA-uxaC* genes included in pRE16. This confirms that pRE16 does not contain a functional operator site.

Protein synthesis directed by λ *pexu* phages and by pRE plasmids. A second line of evidence confirming the presence of the *exu* region on the vectors was given by two experiments: the analysis of radioactively labeled proteins synthesized in UV-irradiated cells (16) upon infection with purified λ p*exu* lysates, and in vitro assays using the system described by Zubay et al. (31). In both cases, proteins were labeled with L-[^{35}S]methionine and separated on sodium dodecyl sulfate-polyacrylamide gels.

In the first experiment, strain 159 (λ *ind*) was used as host. Lanes d and i of Fig. 4 show the residual background protein synthesis of the extract of the noninfected strain 159 (λ *ind*). Likewise, when control phage λ wild type infected strain 159 (λ *ind*), only background protein was visible (Fig. 4, lanes c and h). When λ p*exu*2 (*exuT-uxaC-uxaA*) or λ p*exu*6 (*exuR-exuT-uxaC-uraA*) infected strain 159 (λ *ind*), two bands appeared (Fig. 4, lanes b and g). Infection by λ p*exu*5 (*exuT-uxaC*) resulted in the synthesis of one protein (Fig. 4, lanes a and f). No additional band appeared after infection by λ p*exu*4 (Fig. 4, lane e). The protein of 50,500 molecular weight was identified as the *uxaC* gene product, due to its comigration with purified enzyme II in sodium dodecyl sulfate-polyacrylamide gels. Similarly, the product of the 53,000 molecular weight protein corresponded to the *uxaA* gene product.

In vitro polypeptide synthesis directed by various pRE plasmids confirmed these results. In addition to the 29,000-molecular-weight β-lactamase protein, a protein with a molecular weight of 50,500 coded by *uxaC* (enzyme II) was seen in the presence of plasmids pRE4, pRE7, pRE8, and pRE9 (Fig. 5, lanes b, c, d, and e), which complemented the *uxaC* gene in vivo.

Plasmids that complemented the *uxaA* gene in vivo, such as pRE9 (Fig. 5, lane e), directed the synthesis of a protein of 53,000 molecular weight coded for by the *uxaA* gene (enzyme IV′).

In the case of plasmids pRE7 and pRE8 containing *exuT* (Fig. 5, lanes b and c), a protein of 37,000 molecular weight was detectable. This band presumably represented the aldohexuronate transport system, in the non-membrane-bound state.

When plasmids pRE2 and pRE8 carrying *exuR* were used as templates, a band of 21,000 molecular weight was observed (Fig. 5, lanes a and c), which probably presented the *exuR* repressor.

All of these results confirm the previous analysis of the different pRE plasmids. The molecular weights of the proteins synthesized in vitro by this technique were in agreement with the coding capacity of the DNA fragments containing the *uxaA* and *uxaC* genes as deduced from the restriction and cloning experiments.

DISCUSSION

In accordance with the genetic map established previously, the gene order *exuR-exuT-uxaA-uxaC* was confirmed by subcloning different restriction fragments from λ p*exu*6 into multicopy plasmid vectors.

Our cloning data show that the four genes of the *exu* region are linked very closely, since <7.4 kilobase pairs are sufficient to encode the four proteins: *exuR* repressor, aldohexuronate transport protein, and enzymes II and IV′. The size of the *exu* genes was deduced from the location of these genes with respect to the restriction sites and from the plasmid DNA-directed protein synthesis data. *uxaA* and *uxaC* are located on a *Pst*I endonuclease-generated fragment of 3.85 kilobase pairs able to code for a 140,000-molecular-weight polypeptide. The molecular-weight sum of *uxaA* and *uxaC* gene products is 103,000. Therefore, most of the *Pst*I fragment contains only these two genes.

It was demonstrated that the *exu*R gene overlaps the *Pst*I-1 and *att* ΔOΔ′ sites, which are separated by 0.6 kilobase pair. This result suggests that the *exuR* repressor molecular weight

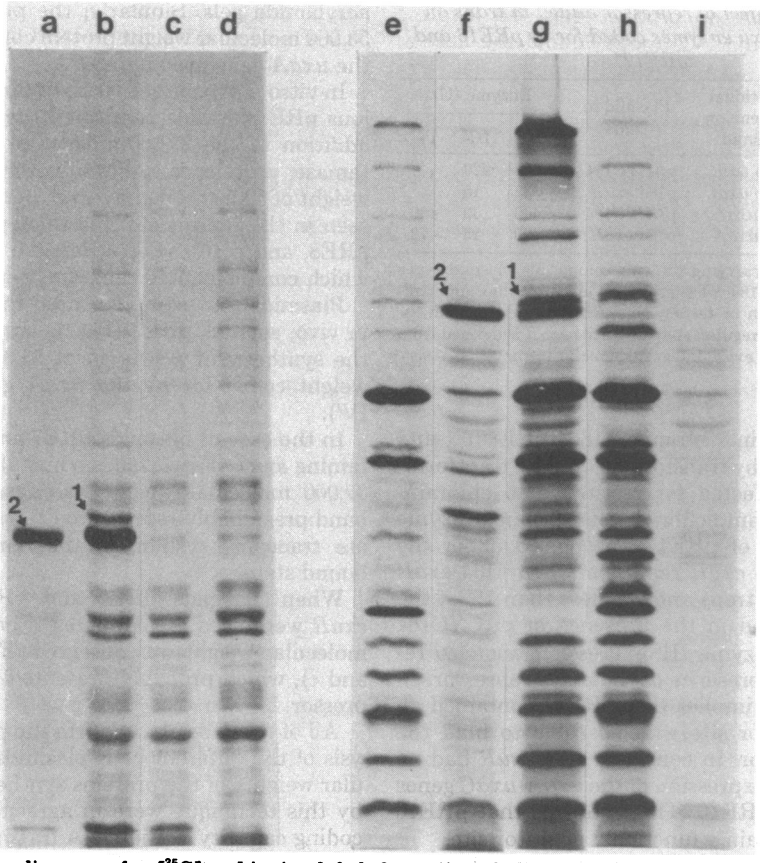

FIG. 4. *Autoradiogram of L-[^{35}S]methionine-labeled proteins obtained after phage infection of UV-irradiated cells and separated on sodium dodecyl sulfate-polyacrylamide gel (10% in lanes a to d and 15% in lanes e to i). The cells were strain 159 (λ ind). The phages were as follows: lanes a and f, λ pexu5; lane b, λpexu2; lane e, λ pexu4; lane g, λ pexu6; lanes d and i, no phage; lanes c and h, λ wild type. Two proteins were identified as the uxaC (arrow 2) and uxaA (arrow 1) gene products.*

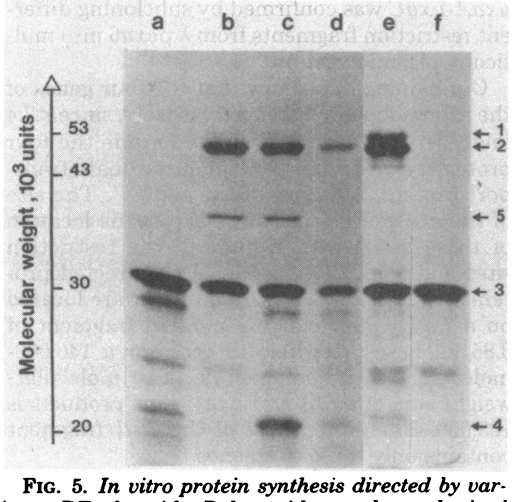

FIG. 5. *In vitro protein synthesis directed by various pRE plasmids. Polypeptides newly synthesized in vitro in the presence of L-[^{35}S]methionine were visualized by autoradiography after separation by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel. Slot a, pRE2; slot b, pRE7; slot c, pRE8; slot d, pRE4; slot e, pRE9; slot f, pBR322. The arrows indicate the positions of the proteins specified by the plasmid DNA: 1, enzyme IV'; 2, enzyme II; 3, β-lactamase; 4, presumptive exuR repressor; 5, presumptive aldohexuronate transport protein.*

(monomer) is greater than 20,000. This conclusion is in agreement with the detection of the presumptive 21,000-molecular weight *exuR* polypeptide from plasmid pRE2 DNA in a cell-free transcription-translation system.

The *exuT* gene was shown to be located on the *Pst*I-1-*Sal*I-1 fragment having a coding capacity for a 60,000-molecular weight polypeptide. In agreement with this, the molecular weight of the presumptive product of *exuT* was found to be 37,000 in the in vitro protein-synthesizing system.

Induced or uninduced strains carrying plasmids which have inserted *uxaA* and *uxaC* genes

such as pRE9 do produce elevated levels of enzymes II and IV', about 30-fold the induced rate of a wild-type isogenic haploid strain. When *exuR* is present on plasmids carrying the *uxaC* gene (pRE8), the synthesis of enzyme II strongly decreases although the copy number of the plasmid remains unchanged. The repressor coded for by the *exuR* gene in pRE8 prevents the expression of the *uxaC* gene. We demonstrated that a strain carrying a plasmid with the *exuR* gene (pRE2 and pRE3) overproduces the *exuR* repressor, since even in the presence of a high concentration of inducer, the chromosome expression of *uxaA* and *uxaC* genes is much lower than that of a wild-type strain. The repression ratio in a strain carrying pRE3 is clearly larger than that observed with pRE2. In these two plasmids, the same cloned *Bam*HI fragment lies in opposite orientations relative to the pBR322 sequences. A read-through from an active external promotor could explain the difference observed in the repression ratio. The use of the pRE3 plasmid should facilitate the purification of the *exuR* repressor since the normal amount of this protein is extremely low.

A comparison of the pRE16 and pRE17 plasmids allows the location of the regulatory region (operator) of the *uxaC-uxaA* operon. Both plasmids overproduce enzyme II, but *uxaC* gene expression is strongly reduced when a compatible plasmid carrying *exuR* is added in trans in strains containing pRE17. In contrast, in strains carrying pRE16, the enzyme II and IV' levels are not affected. Therefore, pRE16 partially or entirely lacks the operator region (*uxaCo*) of the *uxaC-uxaA* operon. Further evidence for the location of *uxaCo* is given by plasmids which have included the *Pst*I-2 site. We can assume, by analogy with the *lac* operator (4, 24), that multiple copies of a plasmid, each containing one binding site for the *exu* repressor, will titrate out the *exuR* repressor present in the cell and thereby derepress the *uxaB* operon, resulting in a constitutive synthesis of enzyme III'. Indeed, when endonuclease-generated fragments overlapping the *Pst*I-2 site are present in the pRE plasmids, a weak but significant constitutive synthesis of enzyme III' is observed. Likewise, when a plasmid carries the *uxaCo* and *uxaC*, but not *uxaA*, genes (pRE12 and pRE14), a low constitutive synthesis of enzyme IV' is detectable, due to the derepression of the *uxaC-uxaA* operon on the chromosome. In contrast, neither pRE16 nor pRE18 induces the constitutive synthesis of enzyme III'. Thus, the *uxaCo* site overlaps the *Pst*I-2 restriction site.

When the operator site of *uxaC* is missing (as in pRE16), both enzyme II and enzyme IV' syntheses become insensitive to the *exuR* repression control. This observation confirms that the *uxaC* and *uxaA* genes possess the same operator, that they belong to the same operon, and that the transcriptional direction of this operon is from *uxaC* to *uxaA*, as suggested previously (14).

The *exuT* gene has been located on the *Pst*I-1–*Sal*I-1 and fragment and can be expressed even if the *uxaA* region is absent (pRE7); the *uxaC-uxaA* region is expressed as well, even if the *exuT* region is missing (pRE16, pRE17). Consequently, the structural gene *exuT* does not belong to the *uxaC-uxaA* operon and forms an independent operon, although it is linked very closely to the first operon, as suggested by Nemoz et al. (17).

Cloning of the operator region of the *uxaC-uxaA* operon will be very helpful for selecting operator-constitutive mutations, tight-binding repressor mutations which overcome operator-constitutive mutations (18), and for determining the nucleotide sequence of this operator site. The pRE plasmids will also be invaluable for studying the structure-function relationships of the *exuR* regulatory protein and the aldohexuronate transport protein. Such studies are currently in progress.

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