Use of *uxu-lac* Fusion Strains to Study the Regulation of the *uxuAB* Operon in *Escherichia coli* K12

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(Received 15 April 1983; revised 10 June 1983)

In the hexuronate system of *Escherichia coli*, the *uxuAB* operon is negatively controlled by the UxuR and ExuR repressors. A Mudlac phage was used, by the method of Casadaban, to construct strains where a truncated *lacZ* was fused to *uxuB*. From these fusion strains, deletions of various lengths extending into the *uxu* region were created *in vivo* by selecting temperature-insensitive mutants. Operator-constitutive mutations were also selected for in such strains and their preliminary analysis is presented. Large amounts of either ExuR or UxuR repressor caused a strong decrease of the constitutive expression of the *uxuAB* operon in the operator mutants. The implications of this repression for the presence of one or two operator sites in the *uxuAB* operon are discussed.

INTRODUCTION

D-Glucuronate is degraded in *Escherichia coli* according to the Ashwell pathway (Ashwell, 1962) (Fig. 1). The *uxu* region located at min 97 on the *E. coli* linkage map (Bachmann, 1983) consists of the *uxuAB* operon and the *uxuR* regulatory gene. These genes are transcribed in the same direction (Ritzenthaler *et al.*, 1980; Ritzenthaler & Mata-Gilsinger, 1982). The *uxuA* and *uxuB* genes code for D-mannonate hydrolyase (EC 4.2.1.8) and D-mannonate:NAD⁺ oxidoreductase (EC 1.1.1.57), respectively; mannonic amide (a gratuitous inducer), fructuronate and glucuronate are able to induce the synthesis of the *uxu* enzymes (Robert-Baudouy *et al.*, 1974). The expression of the *uxuAB* operon is primarily controlled by the *uxuR* gene product and secondarily by the ExuR repressor (Robert-Baudouy *et al.*, 1981). This latter repressor exerts a total control on the *exuT*, *uxaCA* (min 68) and *uxaB* genes (min 52) of the galacturonate pathway (Portalier *et al.*, 1980). The UxuR repressor also controls negatively, although only partially, the expression of the *uidA* gene at min 36 (Novel & Novel, 1976*b*). The number of operator sites in the *uxuAB* regulatory region was not known.

Only a few mutations in the *uxu* region were available. One purpose of this work was to isolate a set of *uxu* deletions, especially of the kind extending into the *uxuR* gene but leaving intact the *uxuAB* operon. Mutations in the operator–promoter region are also critical for the dissection of the genetic region involved in the regulation of the *uxuAB* operon. The use of the Mud(Ap^r *lac*) phage (Casadaban & Cohen, 1979) is especially convenient for isolating these kinds of mutations. An *uxu::Mud(Ap^r *lac*)* fusion strain allows the easy selection of *uxu* deletions as temperature-insensitive mutants. Regulatory mutations may be selected by growth on lactose minimal medium. Phage MudII301 (M. Casadaban, unpublished data cited in Beny *et al.*, 1982) is a derivative of MudI (Casadaban & Cohen, 1979) with the loss of eight codons of *lacZ*, all of *trp* and some of the Mu right end. It contains a truncated *lacZ* gene that may become functional downstream from the translational start of an actively transcribed gene; a fused protein is obtained, formed from the NH₂-terminus of this gene-product grafted to the truncated β -galactosidase.

Fig. 1. Degradative pathway of hexuronates and hexuronides in *E. coli* K12. The different steps are catalysed by the following enzymes: A, β -glucuronidase (EC 3.2.1.31); 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 4.1.2.14). The gene symbols next to the arrows denote the structural genes of the corresponding enzymes. At the bottom of the figure is given the location on the *E. coli* chromosome of these structural genes and of the corresponding regulatory genes.

Table 1. *Bacterial strains, phage and plasmids*

Designation	Relevant genotype	Source or reference
<i>E. coli</i> strains		
MC4100	<i>araD139 ΔlacU169 rpsL thi</i>	Casadaban (1976)
MAL315	(<i>Mucts dII301(Ap^rlac)</i>) <i>mal::Mucts araD139 Δ(araAIOC leu)7697 Δ(proAB lacIZYA)X111 rpsL</i>	M. J. Casadaban via J.-C. Patte
KL723	<i>thr-1 leuB6 proA2 his-4 recA argE3 thi rpsL</i> with F104*	Low (1972)
290	as MC4100, but <i>uxuB100::Mud(Ap^rlac)</i>	This study
291	as MC4100, but <i>uxuB101::Mud(Ap^rlac)</i>	This study
290-S†	as MC4100, but $\Phi(uxuB-lacZ)100$	This study
291-S†	as MC4100, but $\Phi(uxuB-lacZ)101$	This study
290-1	as 290-S, but <i>uxuAo100</i>	This study
290-2	as 290-S, but <i>uxuAo101</i>	This study
290-3	as 290-S, but <i>uxuAo102</i>	This study
291-1	as 291-S, but <i>uxuAo103</i>	This study
291-2	as 291-S, but <i>uxuAo104</i>	This study
1583	<i>argH trp rpsL uxuAo10</i>	L. Cova
Phage		
Mud(Ap ^r lac)	<i>cts dII301(Ap^rlac)</i>	M. J. Casadaban
Plasmids		
pACYC184	<i>cat⁺ tet⁺</i>	Chang & Cohen (1978)
pRE101	<i>cat⁺ exuR⁺</i>	Ritzenthaler <i>et al.</i> (1981)
pRU101	<i>cat⁺ uxuR⁺</i>	Ritzenthaler & Mata-Gilsinger (1982)

* F104 is an F-prime covering the *uxuR-uxuAB* region.

† 290-S and 291-S are temperature-resistant mutants of 290 and 291, respectively, that have undergone a deletion of the *Mu c* and *kil* genes from Mud(Ap^rlac).

METHODS

Bacterial strains, phage and plasmids. The bacterial strains were *E. coli* K12 derivatives. Bacterial strains, phage and plasmids used in this investigation are listed in Table 1.

Culture media. Media for growth were those described by Miller (1972). Synthetic medium was M63 (pH 7.2) or M9 (pH 7.2) and contained either glycerol (5 g l⁻¹), lactose (5 g l⁻¹), glucuronate or galacturonate (2.5 g l⁻¹). Glucuronate/MacConkey media (Difco) contained this compound at 15 g l⁻¹. Ampicillin was used at a final concentration of 10 µg ml⁻¹ and chloramphenicol at 25 µg ml⁻¹.

Chemicals. Mannonic amide, fructuronate and mannonate were synthesized in our laboratory (Robert-Baudouy *et al.*, 1974). D-Glucuronate and D-galacturonate were purchased from Sigma, and chloramphenicol and ampicillin from Serva Feinbiochemica.

Enzyme induction and extraction. The conditions for induction and extraction of the enzymes of the hexuronate system were as outlined previously (Robert-Baudouy *et al.*, 1974; Novel *et al.*, 1974).

Enzyme assays. D-Mannonate:NAD⁺ oxidoreductase, D-mannonate hydrolyase and β-glucuronidase were assayed by previously published methods (Portalier & Stoeber, 1972*b*; Robert-Baudouy & Stoeber, 1973; Novel & Novel, 1976*a*). *In situ* plate assays for D-mannonate:NAD⁺ oxidoreductase and β-glucuronidase activities were performed on clones grown overnight on glycerol agar medium as described by Portalier & Stoeber (1972*a*) and Novel & Novel (1976*a*). β-Galactosidase was assayed by the method of Miller (1972) in exponentially growing cells.

Genetical methods. Transduction with phage P1*kc* and conjugation were carried out according to Miller (1972). Cells were prepared for transformation with plasmid DNA by the method described by Mandel & Higa (1970). Lysates of the Mud(Ap^rlac) phage were prepared by thermoinduction of the lysogenic strain MAL315 and used to prepare ampicillin-resistant lysogens of strain MC4100 as previously described (Casadaban & Cohen, 1979). The lysogens were selected by plating on glucuronate/MacConkey plates with ampicillin. White colonies (Glucuronate⁻) appeared at a frequency of about 10⁻³.

Isolation of temperature-insensitive mutants. Derivatives of the Mud(Ap^rlac) lysogens that grew at 42 °C were selected on solid medium. After selection, independent mutants were purified once at 30 °C and then tested for temperature-insensitive growth and for other relevant phenotypes.

Table 2. *Enzyme activities in the stabilized *uxuB-lacZ* fusion strains and in their operator-constitutive derivatives*

Strain	Inducer (mannonic amide, 5 mM)	Specific activity [nmol min ⁻¹ (mg dry wt) ⁻¹]		
		Mannionate oxidoreductase (<i>uxuB</i>)	Mannionate hydrolyase (<i>uxuA</i>)	β -Galactosidase (<i>uxuB-lacZ</i>)
MC4100	—	30	ND	
	+	1500	48	
290-S	—	10	ND	5
	+	10	66	1225
291-S	—	15	ND	7
	+	10	51	1095
290-1	—		22	424
	+		49	1144
290-2	—		27	485
	+		45	1120
290-3	—		29	518
	+		47	1185
291-1	—		19	349
	+		44	1110
291-2	—		18	324
	+		45	1160

ND, Not detectable (≤ 2).

RESULTS

*Isolation of strains containing *uxuB* : *Mud*(Ap^r *lac*) fusions*

In this experiment, only Mu insertions in the *uxuB* gene were sought, for the following reason: after a Mu insertion in *uxuB*, the *uxuA* gene would still be expressed since the *uxuAB* operon is transcribed from *uxuA* to *uxuB*. Therefore, when mutations affecting the regulation of this operon are selected, the resulting phenotype can be examined by following not only the expression of the *lacZ* gene but also that of the *uxuA* gene. This allows verification that the two genes are still coordinately expressed: thus undesirable events that could happen during the selection procedure, such as Mu-catalysed rearrangements, Mu transpositions or mutations in the *lac* genes, can be detected and discounted.

Strain MC4100 was infected with Mu dII301, and *uxu* : *Mu* d(Ap^r *lac*) fusion candidates were screened as white clones on glucuronate/MacConkey plates containing ampicillin. After single-colony purification, 73 mutants were analysed; 41 were unable to catabolize galacturonate and were discarded, since they carried a mutated gene coding for an enzyme of the common hexuronate degradative pathway (*exuT*, *uxaC*, *kdgK* or *kdgA*). The 32 remaining mutants were probably due to an insertion of bacteriophage Mu in one of the genes of the *uxu* region. All had a Lac⁻ phenotype and only two were able to grow on lactose when glucuronate was added as inducer. These two mutants, designated 290 and 291, were retained for further study.

In the fusion strains 290 and 291, β -galactosidase and mannionate hydrolyase synthesis were inducible by mannonic amide (a gratuitous inducer) whereas mannionate oxidoreductase was no longer synthesized (Table 2), proving that the *uxu* mutation in these two strains is located in the *uxuB* gene. To establish that this mutation resulted from the Mu insertion, strains 290 and 291 were transduced to Glucuronate⁺ with a P1 lysate grown on a wild-type strain. Each Glucuronate⁺ transductant lost the ability to express β -galactosidase and became ampicillin- and Mu-sensitive, indicating that the *uxu* mutations were due to the Mu insertion and that the two fusion strains contained single insertions in *uxuB*.

Stabilization of the uxu-lac fusions

Because the bacteriophage Mud(Ap^r *lac*) has the *cts62* allele (Bukhari, 1975), which encodes a thermolabile repressor, it appears that even at 30 °C there is residual expression of Mu functions and that Mu can still transpose. Therefore, when used to isolate spontaneous regulatory mutants, the fusion strains containing the prophage give rise to a large proportion of Mud/*lac* transpositions or Mu-catalysed rearrangements that place the *lac* genes under the control of some new promoters (Bukhari, 1976; Faellen & Toussaint, 1980). To eliminate these undesirable events, temperature-resistant derivatives of the two *uxu::Mud*(Ap^r *lac*) fusion strains were selected. Among the temperature-resistant mutants only those ampicillin-resistant, Mu-sensitive clones which preserved the regulatory characteristics of the original fusion strains were retained. By this procedure, deletions of part of Mu were obtained removing the Mu repressor gene and the *kil* gene (Van de Putte *et al.*, 1977). The *bla* gene was still expressed, indicating that the deletions did not extend into the *lac* genes (Fig. 2). The same strategy was employed by Mulligan *et al.* (1982) to stabilize *met-lac* fusions. The stabilized derivatives of strains 290 and 291 were termed 290-S and 291-S, respectively (Table 2).

Isolation and characterization of mutations affecting the regulation of the uxuAB operon

Spontaneous mutants with derepressed expression of the *lac* genes were selected by plating the fusion strains on lactose minimal medium in the absence of glucuronate. Lac⁺ mutants of strains 290-S and 291-S appeared at a frequency of about 5×10^{-6} . Of these mutants, 65% were affected in the *uxuR* gene, as judged by the constitutive expression of the *uidA* gene. Among the remaining clones we expected to find operator-constitutive mutants. All of them contained a wild-type *exuR* allele since none of them showed a constitutive expression of the *uxaB* gene.

Five independent Lac⁺ mutants with no modification of *uidA* expression (putative operator-constitutive mutations) and five others constitutive for the β -glucuronidase synthesis (putative *uxuR* mutations) were examined in greater detail. The F-prime factor, F104, covering the *uxu* region (Low, 1972), was introduced into the ten Lac⁺ strains. As expected, in the presumptive *uxuR* mutants, the constitutive synthesis of the β -glucuronidase and β -galactosidase disappeared upon introduction of the *uxuR*⁺ allele whereas the presence of F104 did not modify the *uxuB-lacZ* gene expression in the presumptive O^c mutants (data not shown). To verify that the mutations which rendered the fusion strains able to grow on lactose were located in the *uxu* region, the mutants were transduced to Glucuronate⁺ with phage P1 grown on a wild-type strain. All Glucuronate⁺ transductants became Lac⁻.

In the five presumptive O^c mutants, β -galactosidase synthesis was accompanied by mannonate hydrolyase synthesis (Table 2); these syntheses were still inducible by mannonic amide; the induction ratio was on average about 3.

Effect of repressor overproduction in regulatory mutants

We attempted to discover if large amounts of ExuR or UxuR repressor had an identical or a different effect on the constitutive expression of the *uxu* operon in the mutant strains. Plasmids pRE101 and pRU101, bearing the *exuR* and *uxuR* regulatory genes, respectively, overproduced the corresponding repressors (Ritzenthaler *et al.*, 1980, 1981). Therefore the expression of the *uxu* operon in the five presumptive O^c mutants was examined in the presence of these plasmids (Table 3). Overproduction of ExuR or UxuR repressors caused a strong but not complete repression of the *uxuB-lacZ* gene expression. The same result was observed in strain 1583, an O^c mutant selected by a totally different strategy (L. Cova, unpublished results): 25% of the constitutive level remained in strain 1583 whereas in the five O^c fusion strains only 10% was retained. In all cases, for the same mutant, the repression exerted by ExuR and that caused by UxuR were approximately the same. These results will be discussed later.

Generation of deletions in the uxu region

The *uxuB-lacZ* fusion strains were used to create deletions of various lengths in the *uxu* region. The structure of the *uxuB::Mud*(Ap^r *lac*) is shown in Fig. 2: the *c* end of Mu is adjacent to the *uxuR* gene. Temperature-insensitive mutants derived from either strain 290 or strain 291

Table 3. Effect of the *exuR* and *uxuR* regulatory genes on the expression of the Φ *uxuB-lacZ* gene in the operator-constitutive mutants

Strain	Specific activity of β -galactosidase [nmol min ⁻¹ (mg dry wt) ⁻¹] in the presence of:		
	pACYC184 (control)	pRE101 (<i>exuR</i> ⁺)	pRU101 (<i>uxuR</i> ⁺)
290-S	5	3	4
290-1	375	45	25
290-2	435	42	29
290-3	476	57	26
291-S	7	4	4
291-1	319	45	33
291-2	305	35	31

Fig. 2. Deletions generated by the bacteriophage Mud(Ap^r *lac*) in the *uxu* region. A detailed map of the *uxu* region shows the position of MudII301 within the *uxuB* structural gene. P denotes the promoters of the two operons. The arrows show the direction of transcription of the *uxuAB* and *uxuR* operons. A prime (e.g. *uxuB'*) indicates that the gene is interrupted on the side the prime is written. The loss of functions due to deletion formation is indicated by the solid lines; deletion could extend through regions indicated by broken lines. The Roman numerals denote the classes of deletion mutants (see text and Table 4).

were selected at 42 °C on rich medium in the presence or absence of ampicillin, yielding five classes of deletion mutants. The expression of the *uxu* genes, as well as the sensitivity to ampicillin, was examined for each surviving clone (Fig. 2; Table 4).

Survivors of class I did not contain large deletions, since they were Ap^r and the synthesis of β -galactosidase (*uxuB-lacZ*) and mannuronate hydrolyase (*uxuA*) were inducible by glucuronate, proving that the *uxuR* gene was functional. In class II, the deletion reached the *uxuR* gene, the survivors were Ap^r and synthesized β -galactosidase and the *uxuA* and *uidA* gene products constitutively. In class III, the deletion also removed the *bla* gene and the fused *uxuB-lacZ* gene, as shown both by the loss of synthesis of β -galactosidase and by the ampicillin-sensitive phenotype. In classes IV and V, the deletions extended through the *uxuB-lacZ* gene and *uxuA* gene: in class IV an intact *uxuR* gene was conserved whereas in class V it was removed since the mutant strains of class V showed a constitutive low level of β -glucuronidase (*uidA*) activity.

Table 4. Phenotypes of temperature-resistant mutants derived from the *uxuB* : : *Mud*(*Ap^rlac*) fusion strain

Key: +, high enzyme activity; (+), low enzyme activity; —, absence of activity; R, resistant; S, sensitive.

Temperature-insensitive mutants	Inducer (glucuronate, 5 mM)	Mannionate oxidoreductase (<i>uxuB</i>)	β -Galactosidase (<i>uxuB-lacZ</i>)	Mannionate hydrolyase (<i>uxuA</i>)	β -Glucuronidase (<i>uidA</i>)	Ampicillin phenotype
Class I	—	—	—	—	—	R
	+	—	+	+	(+)	
Class II	—	—	+	+	(+)	R
Class III	—	—	—	+	(+)	S
	+	—	—	+	(+)	
Class IV	—	—	—	—	—	S
	+	—	—	—	(+)	
Class V	—	—	—	—	(+)	S
	+	—	—	—	(+)	
290 (control)	—	—	—	—	—	R
	+	—	+	+	(+)	

DISCUSSION

The *uxu* region of the hexuronate system in *E. coli* was not well characterized genetically when we began this study: only a few mutations were available. From strains which have the *Mud*(*Ap^rlac*) phage inserted in the *uxuB* gene, deletions of various extents were generated, in particular one extending into the *uxuR* gene without removing the *uxuA* and *uxuB-lacZ* genes. One such mutant has been used to study the regulation of the *uxuAB* operon by the ExuR and UxuR repressors (Ritzenthaler *et al.*, 1983). By selecting Lac⁺ derivatives of the *uxuB-lacZ* strains, regulatory mutations were isolated and characterized. The findings that, in five strains (i) the mutations were *cis*-acting, (ii) the *uxuA* gene and β -galactosidase were both constitutively expressed, and (iii) Glucuronate⁺ transductants became Lac[—], suggested strongly that the five Lac⁺ strains were operator-constitutive mutants. However, another possibility can be considered: the constitutivity might be due to a strong promoter of an insertion sequence lying upstream and close to the *uxuA* gene and its operator site. In this case, the operator sites are functional and this new promoter must be much stronger than the wild-type *uxuAB* promoter since it is able to overcome the repression exerted by the repressor molecules in absence of inducer. For this reason, in the presence of inducer, the level of β -galactosidase and mannionate hydrolyase synthesis should be much higher in the five Lac⁺ derivatives than in the original *uxuB-lacZ* fusion strains. This is not the case (Table 2). Therefore the explanation involving the creation of a new promoter seems unlikely.

Since the *uxuAB* operon is under the dual control of the ExuR and UxuR repressors (Robert-Baudouy *et al.*, 1981), we wanted to know if we could obtain *uxu* operator mutants altered in UxuR repressor binding but leaving unaltered that of the ExuR repressor and vice versa. We have shown in a previous paper (Ritzenthaler *et al.*, 1983) that in mutants with deletions of both *exuR* and *uxuR*, large amounts of either ExuR or UxuR repressor caused a complete repression of the *uxuAB* operon, suggesting that the two repressors, when they are overproduced, are totally interchangeable for the control of the *uxuAB* operon. The putative operator-constitutive mutants were analysed in the same manner, i.e. in the presence of high levels of ExuR or UxuR repressor, by the introduction of plasmids containing the regulatory genes. All the operator mutants derived from the fusion strains showed the same behaviour, although the number of mutants analysed was limited; nevertheless another mutant selected by a different procedure behaved similarly.

The strong but incomplete repression caused by the expression of the *uxuR*⁺ plasmid suggested that the mutated *uxuAB* operator retained a residual affinity for the UxuR repressor. This result was corroborated by the observation that the synthesis of the *uxuA* and *uxuB* gene products was still inducible in these regulatory mutants. An excess of ExuR repressor caused the same decrease in the constitutive expression of the *uxuAB* operon as did the UxuR repressor.

Therefore, the presumptive O^c mutations affected the binding of the ExuR and UxuR repressor equally. The *uxu* operator might consist either of a unique binding site for the two repressor molecules or of two overlapping binding sites. In fact, the presence of two distinct operator sites could be masked if we consider the following hypothetical mechanism of the interchangeability of the two repressors: the *uxuAB* operon may possess two operator sites, one for the specific binding of the ExuR repressor and the other for the binding of the UxuR molecule. The occupancy of these operator sites might lead to different levels of transcription: UxuR binding would result in a higher repression than that exerted by ExuR binding, and complete repression of *uxuAB* should be achieved only when the two sites are occupied. Therefore, the complete repression of the *uxuAB* operon observed in the presence of large amounts of only one repressor could be explained by a weak affinity of the UxuR repressor for the normal ExuR binding site on the *uxuAB* control region and vice versa. The results presented in this paper are compatible with this model; moreover they rule out the hypothesis of two entirely independent operator sites, each specifically responsible for the binding of only one of the two repressor molecules.

This work was supported by grants from the Centre National de la Recherche Scientifique (Laboratoire Propre du CNRS no. 05421 and Action Thématique Programmée Microbiologie 1981 no. 9033), from the Ministère de la Recherche et de l'Industrie (Aide à la Recherche 'Biologie Moléculaire' no. 82 V 1284), and from the Fondation pour la Recherche Médicale Française.

We are indebted to F. Stoeber for his encouragement and material support of this work and to J. Dunn for reading the manuscript. We are grateful to G. Luthaud for technical assistance and to J. Pellissier and S. Ottomani for preparing microbiological media.

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