Transcriptional regulation of the *Escherichia coli rhaT* gene

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Transcriptional regulation of the *rhaT* gene, one of the operons forming the rhamnose regulon in *Escherichia coli*, was studied by fusing its complete or deleted promoter to the reporter gene *lacZ*. Analysis of β -galactosidase activities induced in these constructions grown under different conditions predicted the presence of two putative control elements: one for the RhaS regulatory protein and activating the gene not only by L-rhamnose but also by L-lyxose or L-mannose, the other for cAMP-catabolite repression protein and activating this gene in the absence of glucose. Anaerobiosis increased the promoter function two- to threefold with respect to the aerobic condition. Experiments involving complementation of strains containing the *rhaT*-promoter fusion and carrying a deletion in the *rhaS* and/or *rhaR* genes with plasmids bearing the rhamnose regulatory genes showed that *rhaT* is controlled by a regulatory cascade, in which RhaR induces *rhaSR* and the accumulated RhaS directly activates *rhaT*.

Keywords: rhamnose transport, *rhaT*, regulation, transcription, *Escherichia coli*

INTRODUCTION

L-Rhamnose is metabolized in *Escherichia coli* by a set of catabolic enzymes encoded by the genes of the rhamnose regulon, which is composed of two operons. One, *rhaBAD*, encodes rhamnulose kinase (*rhaB*), rhamnose isomerase (*rhaA*) and rhamnulose-1-phosphate aldolase (*rhaD*) (Badía *et al.*, 1989; Power, 1967). The other, *rhaT*, encodes the rhamnose transport system (Baldomà *et al.*, 1990). The rhamnose regulon is under the control of the regulatory locus *rhaC* (Power, 1967), containing two genes, *rhaR* and *rhaS* (Chen *et al.*, 1987; Tobin & Schleif, 1987). The complete rhamnose system has been cloned and sequenced, and several of its putative regulatory sequences in the promoter and terminator fragments have been identified (García-Martín *et al.*, 1992; Moralejo *et al.*, 1993; Tate *et al.*, 1992; Tobin & Schleif, 1987). Rhamnose metabolism is under catabolite repression by glucose, which is overcome by the binding of cAMP-catabolite repression protein (CRP) to a consensus recognition sequence found in the promoter of *rhaBAD* (Egan & Schleif, 1993). The rhamnose regulon is also induced by the pentose L-lyxose (Badía *et al.*, 1991), which is structurally similar to L-rhamnose and is metabolized by the

enzymes of the rhamnose pathway in mutants able to utilize this pentose. Tobin & Schleif (1990a) found that RhaR protein directly activates *rhaSR* transcription in response to L-rhamnose. Later, Egan & Schleif (1993) showed that induction of *rhaBAD* involves a regulatory cascade in which the induction of *rhaSR* results in the accumulation of RhaS, which in turn induces *rhaBAD* expression. In this report we show that *rhaT* is also under the RhaR–RhaS cascade for induction by L-rhamnose.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells were grown aerobically or anaerobically as described previously (Boronat & Aguilar, 1979) on Luria broth or minimal medium. For growth on minimal medium L-rhamnose, L-mannose, L-lyxose or D-glucose were added to a concentration of 10 mM for aerobic conditions or 20 mM for anaerobic conditions. Casein acid hydrolysate, used only in aerobic conditions, was prepared at 1% (w/v). Where indicated, ampicillin was used at 100 $\mu\text{g ml}^{-1}$, chloramphenicol at 20 $\mu\text{g ml}^{-1}$, kanamycin at 30 $\mu\text{g ml}^{-1}$, streptomycin at 25 $\mu\text{g ml}^{-1}$ and thiamin at 20 $\mu\text{g ml}^{-1}$.

DNA manipulation. Plasmid DNA was routinely prepared by the boiling method (Holmes & Quigley, 1981). For large-scale preparation, a crude DNA sample was purified on a column (Qiagen). DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). The DNA sequence was

Abbreviations: CRP, catabolite repression protein; tsp, transcriptional start point.

Table 1. *E. coli* strains

Strain	Relevant genotype	Source or reference
ECL1	HfrC <i>phoA8 relA1 tonA22 T2'</i> (lambda)	Lin (1976)
TE2680	F ⁻ λ^- IN(<i>rrnD-rrnE</i>)/ Δ (<i>lac</i>)X74 <i>rplS galK2</i> <i>recD::Tn10d-Tet trpDC700::putPA1303::</i> [Kan ^s Cam ^r <i>lac</i>]	Elliot (1992)
GD246	MC4100 <i>glpF::Tn10</i> Φ (<i>glpK::lacZ</i>) <i>pcnB</i> Δ <i>rbs7</i>	Sweet <i>et al.</i> (1990)
DF903	Δ (<i>rha-pfkA</i>)15 <i>pfkB1</i>	Daldal & Fraenkel (1981)
Δ RHA2	F ⁻ Δ (<i>rhaT-rhaR</i>) Δ <i>lacU169 endA hsdR thi</i>	E. C. C. Lin (unpublished)
CGSC4833	<i>thi-1 rhaC4 lacZ82 gal-33</i>	B. Bachmann (Coli Genetic Stock Center)
JA140	TE2680 (pRS550-PV1:: <i>lacZ</i>)	This study
JA141	TE2680 (pRS550-PV2:: <i>lacZ</i>)	This study
JA142	TE2680 (pRS550-PV3:: <i>lacZ</i>)	This study
JA143	TE2680 (pRS550-PV4:: <i>lacZ</i>)	This study
JA144	TE2680 (pRS550- <i>PrbaB::lacZ</i>)	This study
JA145	GD246 Δ (<i>rha-pfkA</i>)15, but <i>glp</i> ⁺	This study
JA146	GD246 Δ (<i>rhaT-rhaR</i>), but <i>glp</i> ⁺	This study
JA147	JA145 (pRS550-PV1:: <i>lacZ</i>)	This study
JA148	JA145 (pRS550- <i>PrbaB::lacZ</i>)	This study
JA149	JA146 (pRS550-PV1:: <i>lacZ</i>)	This study
JA160	JA146 (pRS550- <i>PrbaB::lacZ</i>)	This study

determined by the dideoxynucleotide chain-termination procedure of Sanger *et al.* (1977). Double-stranded plasmid DNA was used as the template.

RNA preparations and Northern-blot experiments. For preparation of total RNA, cells of a 25 ml culture grown to OD₆₅₀ 0.5 were collected by centrifugation at 5000 *g* for 10 min and processed as described by Belasco *et al.* (1985). Northern-blot hybridization was performed with each RNA sample (10 μ g), following the procedure described previously (Moralejo *et al.*, 1993).

Primer extension analysis. This was performed by the procedure of Hu & Davidson (1986), which involved hybridization of mRNA to a single-stranded DNA template and annealing of a radiolabelled DNA primer to the template at a site upstream from the 5' end of the mRNA. Extension of the primer by T4 DNA polymerase should stop at the 5' end of the hybridized mRNA; therefore, the 3' end of the growing DNA chain marks its position. T4 DNA polymerase was purchased from Promega.

Construction of *rhaT* promoter deletions and *rhaT-lacZ* fusions. Deletions were generated from plasmid pPV1 (Fig. 1) either by the Promega exonuclease III/S1 nuclease system or by restriction enzyme digestion. Restriction products that had 5' or 3' overhanging ends were converted to blunt ends with Klenow fragment of *E. coli* DNA polymerase I, and cloned into Bluescript vector previously digested with *Sma*I. The resulting plasmids were sequenced and those with the desired fragments in the correct orientation were digested with *Eco*RI and *Bam*HI to be subcloned into the ampicillin and kanamycin resistance vector pRS550 (Simons *et al.*, 1987).

Single-copy fusions on the *E. coli* chromosome were obtained by the method of Elliot (1992). Plasmids containing the different *rhaT-lacZ* fusions were linearized with *Xho*I, electrophoresed and purified by Gene-Clean. Strain TE2680 (Elliot, 1992) was transformed with 1.0–2.0 μ g of the linearized DNA. Due to the

presence in strain TE2680 of the *recD::Tn10* mutation and sequences inserted into the *trp* operon that are homologous to sequences in the pRS plasmids, this strain recombines linear pRS550-based plasmids into its chromosome. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1vir lysates were made from the fusion strains and used to transduce the fusions into *pcnB* strains JA145 and JA146, which contained the *rha* deletions of strains DF903 and Δ RHA2, respectively. The *pcnB* Δ *rha* mutants were obtained by P1 transduction of the rhamnose deletion into strain GD246 (Sweet *et al.*, 1990), together with functional *glpF* and *glpK* genes. Transductants were selected for growth on glycerol and screened for the inability to utilize L-rhamnose.

Preparation of cell extracts and enzyme assays. For enzyme assay, the cells were harvested at the end of the exponential phase and the cell extract was prepared as described previously (Boronat & Aguilar, 1979) with 10 mM Tris/HCl buffer, pH 7.3.

The β -galactosidase activity in strains grown under specified conditions was assayed as described by Miller (1972); the values are reported in nmol min⁻¹ (mg protein)⁻¹. L-Rhamnose permease activity was determined by the rate of uptake of L-[¹⁴C]rhamnose [210 mCi mmol⁻¹ (7.7 GBq mmol⁻¹); CEA, Gif sur Yvette, France] by whole cells. Cells were harvested and the assay was performed as described for fucose transport by Hacking & Lin (1976), using a rhamnose concentration of 0.2 mM [0.8 mCi mmol⁻¹ (29.6 MBq mmol⁻¹)]. Isomerase activity was determined as described by Takagi & Sawada (1964), measuring ketose formation by the cysteine-carbazole method (Dische & Borenfreund, 1951) with tagatose as a standard. One unit of isomerase activity was defined as the amount of enzyme that catalysed the formation of 1 μ mol ketose min⁻¹ at 37 °C.

The protein concentration in cell extracts was determined by the Lowry method, with bovine serum albumin as standard.

Fig. 1. Restriction map of plasmid inserts containing the *rhaT* promoter region and sequential deletions thereof, derived from recombinant Bluescript plasmid pCG2. Numbers indicate the terminal end positions of plasmid inserts. The hatched bar represents the Bluescript vector, which was used in all constructions. Tsp and the ATG initiating codon are indicated. Inserts, labelled on the left margin, were cloned into the pRS550 fusion vector, crossed onto the *E. coli* chromosome and assayed for β -galactosidase activity from cells grown on casein acid hydrolysate in the presence or in the absence of L-rhamnose.

RESULTS

Transcription and transcriptional start site of the *rhaT* gene

Total RNA from cells of strain ECL1 grown under non-inducing (casein acid hydrolysate) or inducing (casein acid hydrolysate plus rhamnose, or minimal medium with rhamnose) conditions was prepared as indicated above. Northern-blot hybridization performed with a probe of 420 bp (*Sau3A* fragment from the *rhaT* open reading frame) showed a transcript of 1.1 kb in the RNA preparation obtained from induced cells, while no hybridization band was detected when RNA preparations of non-induced cells were used (Fig. 2).

Fig. 2. Northern blots of total RNA from strain ECL1 grown aerobically on casein acid hydrolysate (lane 1), or casein acid hydrolysate plus rhamnose (lane 2). Hybridization was performed with a 420 bp *Sau3A* probe from the *rhaT* open reading frame.

Fig. 3. Identification of the 5' end of the *rhaT* transcript. The primer-extension products (lane 1) were electrophoresed with a sequencing ladder (lanes A, C, G and T) generated by using the same template and primer. A control reaction without RNA was run in lane 2. A portion of the nucleotide sequence deduced from the sequencing lanes is shown on the right. The most intense extended product is indicated by an arrow; it corresponds to a transcription initiated at the following G nucleotide, labelled tsp.

The site of transcription initiation of the *rhaT* gene was determined by primer extension analysis. Total mRNA was prepared from strain ECL1 grown aerobically on L-rhamnose. The DNA template used in this experiment was derived from plasmid pCG2 (Fig. 1), a deletion obtained from plasmid pCG1 (García-Martín *et al.*, 1992), with an insert containing the *rhaT* gene and 250 bp upstream from the ATG codon. Single-stranded DNA of

Fig. 4. (a) Nucleotide sequence of the *RsaI*-ATG fragment of plasmid pPV1 encompassing the promoter region of *rhaT*. Dashed arrows indicate the inverted repeat corresponding to the putative CRP binding site; solid arrows indicate the inverted repeat corresponding to the proposed L-rhamnose-specific regulator element. The transcription initiation nucleotide (position 1) and the -10 and -35 promoter sequences are indicated. The ribosome-binding site is boxed and the ATG initiation codon is underlined. (b) Alignment of *rhaT* (PT) and *rhaBAD* (PBAD) promoter sequences encompassing the two control elements, which are indicated by the arrows. Vertical lines mark identical nucleotides present in both fragments. The sequences recognized by CRP are boxed and conserved nucleotides in the CRP consensus (de Crombrughe *et al.*, 1984) are in outline (bolder) type.

this template was hybridized with the total mRNA preparation. After annealing with ^{32}P -labelled sequencing primer that was complementary to the Bluescript poly-linker region, the primer was extended with T4 DNA polymerase. The samples were subjected to PAGE in parallel with sequencing mixtures prepared with the same template and primer. Three products differing in one basepair were observed (Fig. 3, lane 1) which were absent when the primer extension reaction was performed without mRNA (lane 2). The smallest and most intense of the products obtained after extension of the primer towards the 5'-end of the hybridized RNA allowed us to define the more frequently used transcriptional start point (tsp) (Fig. 3), the other two being used less often. The tsp was thus located at 40 bp upstream from the ATG and was taken as position number 1 in the *rhaT* promoter sequence reported by García-Martin *et al.* (1992) and presented in Fig. 4(a).

Table 2. β -Galactosidase activities in strains JA140 and JA141 grown under different conditions

Carbon source	Oxygen	β -Galactosidase activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Strain JA140	Strain JA141
L-Rhamnose	+	5770	2430
L-Rhamnose	-	17500	6810
L-Lyxose	+	2660	1450
L-Mannose	+	5860	2705
D-Glucose	+	< 25	< 25
Glycerol	+	< 25	< 25

Transcriptional activation of the *rhaT-lacZ* fusion

The use of β -galactosidase activity as a reporter of the *rhaT* promoter function allowed us to study the effect of different conditions on the expression of *rhaT*. For this purpose the merodiploid strain JA140, containing the fusion pRS550-PV1, in which fragment 1 contains the whole putative *rhaT* promoter (Fig. 1), was grown in the different conditions and β -galactosidase activity was measured in the corresponding extracts (Table 2). The gene was found to be induced not only by L-rhamnose or L-lyxose, as expected, but also by the structurally related sugar L-mannose. The β -galactosidase activities were of the same order for the three sugars and were routinely higher in the cells induced by L-mannose. Growth on glucose or glycerol, non-inducers of the rhamnose regulon, gave undetectable levels of activity (Table 2). In addition, growth in the presence of glucose was shown to produce catabolite repression in the function of the *rhaT* promoter. Experiments in which the time course of the

Fig. 5. Time course of β -galactosidase activity of strain JA140 grown aerobically on rhamnose (●), rhamnose plus glucose (▲), or rhamnose plus glucose plus 5 mM cAMP (■). A rhamnose-grown inoculum was used to start 200 ml of each of the three cultures; 10 ml samples were taken at 1 h intervals for determination of the β -galactosidase activities. The results are means of three determinations.

enzyme activity was monitored for three cultures of strain JA140, grown on (i) L-rhamnose, (ii) L-rhamnose plus D-glucose, or (iii) L-rhamnose plus D-glucose plus 5 mM cAMP, showed how glucose repression, indicated by very low β -galactosidase activities, was partially abolished by the presence of cAMP (Fig. 5).

L-Rhamnose is metabolized aerobically as well as anaerobically in *E. coli* (Boronat & Aguilar, 1979). Activities of the enzymes of the rhamnose pathway, such as rhamnose isomerase, were always found to be two to three times higher in anaerobic than in aerobic conditions (not shown). The β -galactosidase reporter activity also showed that the presence or absence of oxygen affects the expression of *rhaT*: β -galactosidase activities were again between two and three times higher for the cells grown under anaerobic conditions (Table 2).

Analysis of deletions into the *rhaT* promoter

To identify the sequences important for *rhaT* promoter function, deletions upstream of the transcriptional start site were generated. A partial deletion of fragment 1 (position -144 to +383) generated fragment 2 (-93 to +383) with a 5' end-point 50 bp shorter (see Fig. 1). Fragments 3 (-67 to +90) and 4 (-28 to +338) were generated from fragment 1 by digestion with *Bsr*I and *Taq*I, respectively, and they had 5' end-points that were 77 and 116 bp shorter. The deletions were cloned into pRS550 and then recombined onto the chromosome as single copies as indicated above. Growth on casein acid hydrolysate in the presence of L-rhamnose of strains JA142 and JA143, containing the fusions of fragments 3 and 4 respectively, gave no β -galactosidase activity, indicating that these deletions essentially abolished transcription (Fig. 1). Neither L-lyxose nor L-mannose was able to activate the β -galactosidase activity in these strains (not shown). Growth of strain JA141, containing the fusion of fragment 2, on L-rhamnose, L-lyxose or L-mannose yielded intermediate levels of activity (Table 2), whereas growth of strain JA140 with the complete *rhaT* promoter in the same conditions displayed, as indicated above, full induction with higher levels of β -galactosidase activity.

Role of RhaR and RhaS in *rhaT* expression

To determine whether *rhaT* was also under the regulatory cascade mediated by *rhaR* and *rhaS* described by Egan & Schleif for *rhaB* (Egan & Schleif, 1993), we complemented strains having deletions in the *rha* genes with plasmids pJTC105, pJTC108 and pJTC114, containing *rhaSR*, *rhaS* or *rhaR* respectively. In these plasmids the cloned genes were expressed from the *tac* promoter present in the vector (Tobin & Schleif, 1990b). Experiments were run in parallel with the corresponding vector pTAC12 as control.

The *pcnB* strain JA147, carrying the deletion $\Delta(rhaSR)$ 15 of strain DF903 (Daldal & Fraenkel, 1981) and the fusion of *lacZ* with the *rhaT* promoter, was used in

the complementation experiments. Hybridization of a Southern blot of strain DF903 DNA with several probes of *rha* genes indicated that the deletion starting at *pfkA* ended between *rhaA* and *rhaD* and consequently deleted both *rhaS* and *rhaR*. Complementation was revealed by the β -galactosidase activity produced by the *rhaT-lacZ* fusion present in strain JA147. The *pcnB* background was used to avoid plasmid toxicity effects.

As a control, complementation experiments were run in parallel with strain JA148, carrying the *rha-pfkA* deletion and the fusion with the promoter of *rhaBAD*. Strain JA148 was constructed by initially introducing the *rhaB* promoter fusion (Moraljo *et al.*, 1993) into TE2680 (forming JA144) and the fusion was subsequently transduced into JA145 by P1 transduction.

Strain JA147 was induced by L-rhamnose, as indicated by the levels of β -galactosidase detected (Table 3), when transformed with pJTC105 (*rhaSR*) or pJTC108 (*rhaS*), which overexpress the RhaS regulatory protein (Tobin & Schleif, 1990a). Complementation with pJTC114 (*rhaR*) showed no induction, as the overexpression of RhaR regulatory protein had no *rhaS* gene to activate. These results were absolutely paralleled by those obtained with strain JA148 containing the *rhaB* fusion (Table 3).

The function of *rhaR* in the cascade model (Egan & Schleif, 1993) for the expression of *rhaT* was ascertained by using a strain deleted in *rhaR* but not in *rhaS* (strain $\Delta RHA2$). As for strain DF903, the extent of the deletion was determined. Hybridization of Southern blots of DNA from the deleted strain with internal probes of the regulatory region showed that the deletion started upstream of *rhaT* and ended in *rhaR*, thus affecting *rhaR* but not *rhaS* (data not shown). This time, fusions of the *rhaT* promoter and the *rhaB* promoter were incorporated into *pcnB* strain JA146, which is isogenic with strain JA145, giving rise to strains JA149 and JA160, respectively (JA145 and JA146 were constructed by P1 transduction of the *glp*⁺ locus from $\Delta RHA2$ into GD246).

Strain JA149 was inducible by rhamnose not only when transformed with pJTC105 or pJTC108 but also when transformed with pJTC114, which complemented *rhaR* in a strain lacking *rhaR* but having *rhaS* encoded in the chromosome (Table 3). The level of expression of the genomic *rhaS* caused a level of β -galactosidase lower than that obtained with the high expression of the plasmid-encoded *rhaS*. Consistently, transformation with the vector gave no induction since the genomic *rhaS* lacked the required *rhaR* product for its expression. These results were again paralleled by those obtained with control strain JA160 (Table 3).

The dependence of the expression of *rhaT* on *rhaSR* function was also confirmed by measuring the rhamnose uptake in the *rhaC*-deficient mutant strain CGSC4833 when transformed with the corresponding plasmids. The transformed cells restored the incorporation of radioactive rhamnose at concentrations that would not permit entry of the sugar in the absence of rhamnose permease, indicating the presence of the transport system under

Table 3. Complementation of *rhaS-rhaR* to express *rhaT* or *rhaBAD* in strains deleted of *rha* genes

Strain	Relevant <i>rha</i> genotype in host	Carbon source*	β -Galactosidase activity in cells bearing plasmid†			
			pJTC105 (<i>rhaSR</i>)	pJT108 (<i>rhaS</i>)	pJTC114 (<i>rhaR</i>)	pTAC12 (vector)
JA147	$\Delta rhaSR/\Phi(rhaT-lacZ)$	L-Rhamnose	2490	2360	< 25	< 25
		CAH	105	160	< 25	< 25
JA148	$\Delta rhaSR/\Phi(rhaB-lacZ)$	L-Rhamnose	3780	5815	< 25	< 25
		CAH	210	105	< 25	< 25
JA149	$\Delta rhaR/\Phi(rhaT-lacZ)$	L-Rhamnose	2550	3930	1575	< 25
		CAH	< 25	< 25	< 25	< 25
JA160	$\Delta rhaR/\Phi(rhaB-lacZ)$	L-Rhamnose	5005	6660	1895	< 25
		CAH	< 25	< 25	< 25	< 25

* CAH, casein acid hydrolysate.

† Expressed as nmol min⁻¹ (mg protein)⁻¹.**Table 4.** L-Rhamnose isomerase and L-rhamnose permease activities in strain CGSC4883 transformed with different plasmids

Plasmid	L-Rhamnose in growth medium	Specific activity	
		Isomerase*	Permease†
pJTC105 (<i>rhaSR</i>)	+	390	4.5
	—	60	< 0.1
pJTC108 (<i>rhaS</i>)	+	425	4.2
	—	55	< 0.1
pJTC114 (<i>rhaR</i>)	+	450	4.0
	—	32	< 0.1
pTAC12 (vector)	+	30	< 0.1
	—	45	< 0.1

* Expressed as milliunits (mg protein)⁻¹ at 37 °C.† Expressed as nmol min⁻¹ per 10⁹ cells at 30 °C.

inducing conditions (Table 4). As expected, uptake was hardly detectable in the untransformed cells of strain CGSC4833 (data not shown). Likewise, when one of the activities of the *rhaBAD* operon, such as rhamnose isomerase, was determined, it was clearly seen that each of the three plasmids was restoring the activity which was absent in the mutant cell while the vector was unable to restore it even in the presence of rhamnose (Table 4). This result matched those obtained with strain JA149, which allowed us to locate the mutation in strain CGSC4833 in *rhaR*.

DISCUSSION

The determination of the transcriptional start site has allowed us to locate the putative elements of the *rhaT* promoter and subsequently to make the constructions to analyse the regulation of its expression. In this way we

may identify, utilizing the sequence determined by García-Martín *et al.* (1992), putative -10 and -35 boxes, an inverted repeat likely to interact with RhaS and a CRP consensus inverted repeat (see Fig. 4a). The fusions with the deleted *rhaT* promoter indeed indicate the presence of two control elements. One of these, distal to *tsp*, is responsible for the sensitivity to catabolite repression as indicated by the reduced level of expression of strain JA141, which lacks part of the enhancing CRP element. Partial induction by rhamnose in this fusion indicated that the other control element, proximal to *tsp*, is still present in the remaining promoter sequences. This specific element is sensitive to induction by L-rhamnose as indicated by the loss of expression shown by strain JA142, which has a 5' deletion to the -67 position (Figs 1 and 4a).

Some similarities may be pointed out between this promoter and the *rhaBAD* promoter described by Egan

& Schleif (1993). On the one hand, the location of these two putative control elements from *tsp* in the *rhaT* promoter is the same as that described for the control elements of *rhaBAD* (Fig. 4b). On the other hand, there is high similarity between the putative CRP element in *rhaT* and the identified CRP element in *rhaBAD* (9 out of 10 bp match), and also with the core consensus for CRP protein binding (again 9 out of 10 bp match) (de Crombrughe *et al.*, 1984). With respect to the other inverted repeat potentially acting as RhaS binding site in *rhaT*, a significant base-pair matching is also found with that of *rhaBAD*. It has to be pointed out that in *rhaBAD* promoter this inverted repeat has been experimentally proved to be recognized by RhaS (Egan & Schleif, 1994). Both regulatory proteins, RhaR and RhaS, recognize and bind L-rhamnose, becoming in this way activated to induce the corresponding elements of the rhamnose regulon. Induction of the rhamnose system by sugars such as L-lyxose or L-mannose indicates that, due to structural similarity these sugars are also recognized by the two regulatory proteins. However, other structurally related sugars such as L-fucose, D-arabinose or L-galactose, which have a different stereoconfiguration in the hydroxyls at carbons 2 and 4, are not recognized.

The regulation of *rhaBAD* expression was shown to respond to a cascade in which RhaR induced *rhaSR* expression, and RhaS in turn induced *rhaBAD*. Our experiments involving complementation of *rhaS* and *rhaR* with plasmids bearing the regulators show that RhaS also acts as a direct regulator of *rhaT* in the absence of *rhaR*, and that RhaR must be supplied for RhaS synthesis and accumulation in a strain in which *rhaR* has been deleted. Thus the regulatory cascade proposed for the induction of *rhaBAD* is also functional for the stimulation of the *rhaT* promoter. In our experiments, a control fusion with the *rhaBAD* promoter was always run in parallel to validate the comparison.

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