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Molecular analysis of the interaction between the *Bacillus subtilis* trehalose repressor TreR and the *tre* operator

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Abstract The trehalose operon of *Bacillus subtilis* is subject to regulation by induction, mediated by the repressor TreR, and by carbon catabolite repression (CCR). For in vitro investigations, TreR from *B. subtilis* was overproduced and purified. Its molecular mass, as estimated by SDS-PAGE, is 27 kDa. Size fractionation under native conditions yielded a size estimate of 56 kDa, indicating that TreR exists as a dimer in its native state. Analysis of its interaction with various DNA fragments shows that TreR is able to recognize two *tre* operators with different efficiencies, and indicates cooperative binding. Previous results have suggested that CCR of the *tre* operon occurs by a mechanism in which the specific regulator, TreR, may be involved independently of the central component, CcpA. The data presented here indicate that the TreR-*tre* operator interaction is influenced by several effectors. Thus, the presence of trehalose-6-phosphate, as well as glucose-1-phosphate and sodium chloride, inhibits *tre* operator binding. Glucose-6-phosphate can act as an anti-inducer, which might reflect its additional role in CCR exerted by glucose.

Key words Sugar metabolism · Phosphotransferase system (PTS) · Transcription · Carbon catabolite repression · Gene regulation

Introduction

The utilization system for the disaccharide trehalose in *Bacillus subtilis* 168 belongs to the class of

phosphoenolpyruvate-dependent phosphotransferase systems (PTS) (Helfert et al. 1995). It is composed of a putative specific enzyme II^{Tre} containing only B and C domains, which is encoded by *treP*, and the cytoplasmic phospho- α -1,1-glucosidase (TreA), which is encoded by *treA*. TreA hydrolyzes trehalose-6-phosphate to glucose and glucose-6-phosphate (Gotsche and Dahl 1995; Helfert et al. 1995; Schöck and Dahl 1996a). Before entering glycolysis, the glucose is further phosphorylated by an ATP-dependent glucose kinase, which we have recently identified (Skarlatos and Dahl 1998). Both coding sequences for the trehalose system, *treP* and *treA*, are organized in an operon that is regulated negatively at the transcriptional level by the trehalose repressor TreR, which contains a N-terminal α -helix-turn- α -helix sequence motif typical of DNA-binding proteins (Dodd and Egan 1990; Schöck and Dahl 1996a). Trehalose is phosphorylated by the specific trehalose permease TreP during translocation across the membrane, and trehalose-6-phosphate acts as the molecular inducer (Schöck and Dahl 1996a). The gene coding for the repressor (*treR*) is located downstream of the *tre* operon (Helfert et al. 1995; Schöck and Dahl 1996a). Inactivation of *treR* by insertional mutagenesis leads to constitutive expression of the *tre* operon (Schöck and Dahl 1996b). On the basis of amino acid sequence homologies, TreR has been classified as a member of the FadR-GntR regulatory protein family; so far, this family has not been well characterized (Haydon and Guest 1991; Schöck and Dahl 1996a). Sequence analysis of the promoter region of the *tre* operon has revealed two palindromes with sequence similarities to potential operator structures recognized by the FadR-GntR family (Fig. 1) (Schöck and Dahl 1996b).

The *tre* operon is also subject to carbon catabolite repression (CCR) by glucose, fructose and mannitol (Helfert et al. 1995; Dahl 1997). Two potential *cis*-acting catabolite responsive elements (*cre*), each composed of a 14-bp palindrome (Hueck and Hillen 1995), are located in the promoter region and the coding sequence of *treP* (Schöck and Dahl 1996a). CcpA is the central *trans*-

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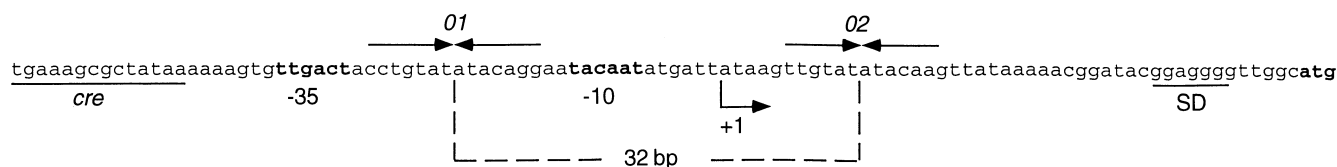


Fig. 1 Structure of the promoter region of the trehalose operon. The DNA sequence presented encompasses the promoter with -35 and -10 regions, indicated in **bold**, the transcription start point (+1) previously determined (Schöck and Dahl 1996b), the ribosome binding site (SD) at an appropriate distance from the start codon (**bold**) of *treP*, the first gene in the *tre* operon. Two inverted repeats denoted as *O1* and *O2* represent the putative operators to which the trehalose repressor TreR binds. The centers of *O1* and *O2* are 32 bp apart corresponding two three turns of the DNA helix. The potential catabolite responsive element (*cre1*) of the *tre* operon is also indicated, and differs in one position from the proposed consensus sequence (Hueck et al. 1994). A second presumed *cre* element is located 372 bp downstream of the transcriptional start point of the *treP* gene as described previously (Schöck and Dahl 1996a)

acting component of the CCR system (Henkin et al. 1991; Hueck and Hillen 1995), which interacts directly with *cre* (Fujita et al. 1995; Gösseringer et al. 1997). This interaction requires the HPr from the phosphoenolpyruvate-dependent phosphotransferase system, which must be phosphorylated by an ATP-dependent kinase at its regulatory phosphorylation site Ser46 (Deutscher et al. 1995; Fujita et al. 1995; Ye and Saier 1996; Galinier et al. 1998; Reizer et al. 1998). In vitro, in the presence of glucose-6-phosphate and at low pH, an HPr-independent interaction between CcpA and *cre* is observed (Gösseringer et al. 1997). Inactivation of CcpA leads to an almost complete loss of CCR for many catabolite repressed genes (Hueck and Hillen 1995). However, as we have demonstrated previously, inactivation of CcpA, or an S46 A mutation in the HPr (Deutscher et al. 1994; Reizer et al. 1996), leads to the loss of fructose repression without obviously affecting glucose repression of the trehalose system (Helfert et al. 1995; Dahl 1997; Skarlatos and Dahl 1998). Therefore, we postulated that additional regulatory mechanisms must be involved in repression of the *tre* operon by glucose (Gotsche and Dahl 1995; Helfert et al. 1995; Dahl 1997). One possible mechanism could be mediated via the trehalose repressor. As mentioned above, the trehalose-specific permease contains only the B and C domains of PTS enzyme II. For trehalose uptake, the A domain of the glucose enzyme II is necessary, which in turn results in an inducer-exclusion effect when glucose and trehalose are both present in the growth medium (Dahl 1997).

In order to elucidate further the mode of function of TreR and to analyze the signals that mediate CCR of the *tre* system, we overproduced and purified the trehalose repressor of *B. subtilis*. The purified protein was used for in vitro studies that revealed its ability specifically to bind DNA containing two operators. Our results suggest that this ability depends on the cooperative binding of TreR dimers. This interaction is influenced by various potential effectors, which can function as inducer, co-inducer or anti-inducer.

Materials and methods

Bacterial strains

Escherichia coli strain DH5 α (BRL) was used for plasmid construction and amplification. Cells were grown in Luria broth (Sambrook et al. 1989) supplemented with ampicillin (100 μ g/ml) for selection. Standard procedures were used for *E. coli* transformation (Sambrook et al. 1989). *B. subtilis* strain MD182 [*trpC2*, *treR*::*aphA3*, *amyE*::(*treP*-*spoVG*-*lacZ*, *cat*)] was described previously (Schöck and Dahl 1996b). Transformation of *B. subtilis* with plasmid DNA was carried out using a one-step procedure (Kunst et al. 1994). *B. subtilis* was grown in Luria broth medium (Sambrook et al. 1989), or MC competence medium (Kunst et al. 1994). *B. megaterium* strain WH320 (Rygus and Hillen 1991) was used for overproduction of TreR. Transformation of plasmids into *B. megaterium* was carried out with protoplasts as described (Puyet et al. 1987). *Bacillus* strains carrying plasmid pFS7 (see below) were selected on medium containing 10 μ g/ml tetracycline. For growth, plates or cultures were incubated overnight at 37°C.

Molecular biological techniques

Restriction enzymes, DNA polymerase and T4 ligase were used as recommended by the manufacturers. DNA manipulations, SDS-PAGE and standard procedures to extract plasmids from *E. coli* were carried out as described (Laemmli 1970; Sambrook et al. 1989). Plasmids were prepared using a Nucleobond kit (Macherey and Nagel, Düsseldorf, Germany). DNA fragments or PCR products were purified using a Nucleospin kit (Macherey and Nagel).

Plasmid construction

Cloning of *treR* for overproduction of the trehalose repressor was achieved by constructing plasmid pFS7 in two steps. A 1086-bp *BclI*-*SmaI* fragment of plasmid pCH2 (Helfert et al. 1995) was ligated to pWH1520 (Rygus and Hillen 1991), digested with *NruI*/*Bam*HI, yielding plasmid pFS6. An optimized ribosome binding site was introduced by PCR (Mullis and Faloona 1987) using pCH2 (Helfert et al. 1995) as template DNA and the following primer pair: 5'-GTACTAGTTAAGGCAAGGAGGTCCATAATGAA-GGTG-3' and 5'-ACACGGCTCGGCTAC-3'. The PCR fragment was digested with *SpeI* and *EagI*, and the resulting 85-bp fragment was introduced into the appropriate restriction sites in pFS6, leading to pFS7. The DNA fragment obtained by PCR was verified by sequence analysis, using an ABI PRISM 310 sequencer according to the recommendations of the manufacturer (Perkin-Elmer, Foster City, Calif.).

Purification of the trehalose repressor TreR

Three litres of *B. megaterium* strain WH320 carrying plasmid pFS7 was grown in Luria broth at 37°C. At an optical density of $A_{600} = 0.3$ expression of *treR* was induced by addition of 0.5% xylose. Growth was allowed to proceed for another 3 h, then the cells were harvested by centrifugation at 5000 \times g. The resulting cell pellet was washed once in buffer A (50 mM NaCl, 20 mM sodium phosphate pH 6.8, 2 mM dithiothreitol) and resuspended in the same buffer. The cells were sonicated six times, at 0.9-s intervals, for 30 s each at 4°C and 140 W, using a Labsonic U sonicator (B. Braun, Melsungen, Germany). After centrifugation for 30 min

at $40\,000 \times g$ the supernatant was collected. The crude extract was passed over a tentacle-based cation exchanger Fractogel EMD SO_3^- 650 S/ 24–40 μm column (2.6 \times 12 cm; Merck, Darmstadt, Germany), previously equilibrated with buffer A. The column was washed with the same buffer until the absorption at 280 nm and the conductivity showed a stable baseline. Elution was carried out with a 100-ml linear gradient 0.05 M NaCl to 1 M NaCl in buffer A at a flow rate of 1 ml/min. Fractions of 10 ml for column washing and 5 ml for gradient elution were collected and analyzed by SDS-PAGE for the presence of a prominent band of about 27 kDa corresponding to the TreR protein. TreR-enriched fractions were collected and precipitated with ammonium sulfate to 80% saturation under gentle stirring. The precipitate was collected by centrifugation for 30 min at $10\,000\times g$, dissolved in 2 ml of TDDT buffer (50 mM TRIS-HCl pH 7.8, 2 mM dithiothreitol) and loaded on a gel filtration Superdex 75 column (1.6 \times 60 cm, Pharmacia-LKB, Freiburg, Germany), which was equilibrated in the same buffer. Elution was performed isocratically at a flow rate of 0.4 ml/min. Fractions of 1.5 ml were collected and analyzed by spectroscopy and for TreR activity in DNA retardation assays. Protein concentrations of TreR were determined spectroscopically at A_{280} according to Wetlaufer (1962) using a molar extinction coefficient for TreR of $\epsilon = 23100$.

Determination of molecular masses by gel filtration

The molecular mass of isolated TreR was determined on an Äkta purifier FPLC Superdex-200 (HR 10/30) column that had been calibrated with serum albumin (67 kDa, Pharmacia), ovalbumin (43 kDa, Pharmacia), chymotrypsinogen A (25 kDa, Pharmacia) and ribonuclease A (13.7 kDa, Pharmacia). A 10- μg aliquot of purified TreR was loaded on the column and eluted at a flow rate of 1 ml/min with 50 mM TRIS-HCl, pH 8.0.

Isoelectric focussing

Isoelectric focussing (IEF) under native conditions (Robertson et al. 1987; Heukeshoven and Dernick 1988) was performed with the Pharmacia PhastSYSTEM using precast IEF-Phast gels in the pH range from 3.0 to 9.0 with an appropriate protein standard. Electrophoresis was performed at 2000 V and 2.5 mA at 15°C for 410 Vh. After electrophoresis, the gel was fixed in 20% (w/v) trichloroacetic acid, and stained with 0.02% (w/v) Coomassie Blue R250 in 30% (v/v) methanol-10% (v/v) acetic acid. The gel was destained in 30% (v/v) methanol-10% (v/v) acetic acid containing 1% (v/v) glycerol.

DNA retardation experiments

In order to obtain radioactively labeled DNA fragments for DNA retardation assays, plasmid pCH2 (Helfert et al. 1995) was used as a template for PCR. PCR (Mullis and Faloona 1987) was carried out with Vent DNA polymerase (Stratagene, La Jolla, Calif.) in the presence of [α - ^{32}P]dATP (10 μCi). The following sets of oligonucleotides were used to obtain labeled DNA fragments harboring either operator 1 (*O1*), operator 2 (*O2*) or both. Amplification using the primer pair 5'-CCCAAGCTTGGGCATAGAGGCTCACCTGC-3' and 5'-CCGCTCGAGCGGCACCGCCGACTGCTTCGAC-3' resulted in a DNA fragment of 271 bp, containing the promoter region with the operators *O1* and *O2*; 5'-CCCAAGCTTGGGAATACAATATGATTATAAGTTGTATATACAAGT-3' and 5'-CCGCTCGAGCGGCACCGCCGACTGCTTCGAC-3' yielded a DNA fragment of 136 bp, containing the operator *O2*; while 5'-CCCAAGCTTGGGCATAGAGGCTCACCTGC-3' and 5'-CCGCTCGAGCGGATAATCATATTGTTTCTG-3' generated a DNA fragment of 166 bp, containing operator *O1*. All PCR products were ethanol precipitated before further use in DNA retardation experiments.

Between 0.07 and 14 pmol of TreR dimer was incubated with radioactively labeled operator DNA (see above) and 2 μg of

competitor DNA (sonicated pWH912; Dahl et al. 1994) in a total volume of 25 μl of 20 mM TRIS-HCl pH 8.0, 20 mM EDTA pH 8.0, 0.5 mM dithiothreitol and 3% Ficoll for 15 min at 21°C. Depending on the experiment, the pH of the reaction buffer was varied as indicated or kept stable at pH 8.0, and supplemented with NaCl, fructose, fructose-6-phosphate, fructose 1,6-bisphosphate, glucose, glucose-1-phosphate, glucose-6-phosphate, trehalose-6-phosphate or trehalose in the concentrations and combinations indicated. Electrophoresis of the samples was carried out for 4 to 6 h at 180 V on 1 mm thick 5% PAA gels in 90 mM TRIS, 90 mM boric acid, 2 mM EDTA, 10% glycerol pH 8.3. The gels were exposed and analyzed in a phosphorimager (Fujifilm, BAS-1500). Relative intensities of radioactively labeled DNA are expressed in units of photo-stimulated luminescence (PSL) or in relation (percentage) to the total amount of radioactively labeled DNA in each reaction mixture. The ratio of protein to DNA is expressed with respect to TreR dimers.

Results

Overproduction and purification of TreR

As observed in previous studies, *treR* is weakly expressed in *B. subtilis* and does not form a predominant protein band on SDS gels (Helfert et al. 1995; Schöck and Dahl 1996b). Therefore, TreR was overproduced using a ribosome binding site that was optimized according to Vellanoweth (1993) in combination with the strong xylose-inducible promoter *xyIA* (Rygu et al. 1991; Rygu and Hillen 1992). The resulting plasmid, pFS7, can replicate in *E. coli* – thus allowing for amplification – as well as in *B. megaterium* and *B. subtilis*. In the latter organism the plasmid complements a *treR* mutation (Schöck and Dahl 1996b), reconstituting the trehalose-inducible phospho- α -1,1-glucosidase activity when cells were grown in 10 mM xylose and 10 mM trehalose (data not shown). These data demonstrate that the plasmid-encoded TreR repressor protein is expressed and functional in vivo in *B. subtilis*. However, even in *B. subtilis* cells containing pFS7, no predominant band of the expected protein size could be observed on SDS gels (data not shown). Using *B. megaterium* strain WH320 as a host for plasmid pFS7, a prominent band at 27 kDa in the soluble fraction of crude cell extracts is visible after xylose induction (Fig. 2A), in agreement with the calculated molecular mass of 27 693 Da (Schöck and Dahl 1996a; Kunst et al. 1997). TreR was purified to homogeneity by a combination of cation exchange chromatography, ammonium sulfate precipitation and gel filtration using an FPLC system (Fig. 2A). The total amount of purified TreR obtained from a 3-l culture volume was 5 mg. The purity of TreR was estimated to be 95% from SDS-PAGE by densitometry. Isoelectric focussing of purified TreR gave an isoelectric point of 7.1, which is in the range of the calculated value of 6.82 (Schöck and Dahl 1996a; Kunst et al. 1997). Size fractionation carried out by gel filtration under native conditions, together with a set of molecular weight standards, yielded a molecular weight estimate of 56 kDa (Fig. 2B). These data strongly suggest that TreR is a dimer in its native state.

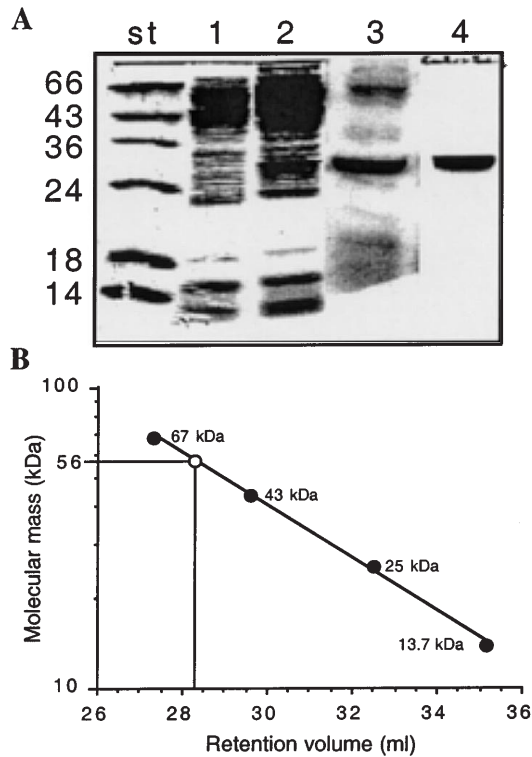


Fig. 2A, B Overproduction and purification of TreR. **A** SDS-PAGE showing the enrichment of TreR during purification. The first lane (marked st) contains molecular weight standards with sizes indicated in kDa; lane 1, soluble fraction of crude extract from *B. megaterium* strain WH320 harboring plasmid pFS7 before xylose induction; lane 2, soluble fraction of crude extract from *B. megaterium* strain WH320 harboring plasmid pFS7 after 3 h of xylose induction; lane 3, fraction containing TreR collected from the cation exchange column; lane 4, purified TreR protein after gel filtration. **B** Molecular mass determination by gel filtration. The standard curve obtained by plotting retention volume against molecular mass for four different marker proteins (filled circles) is shown: ribonuclease (13.7 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa) albumin (67 kDa). The retention volume and corresponding molecular weight of purified TreR is given by the open circle

Activity of the purified trehalose repressor

During the purification process pooled fractions containing TreR were shown to contain binding activity for the *tre* operator in DNA retardation experiments (data not shown). Purified TreR was used in increasing concentrations in the presence of a constant amount of radioactively labeled DNA fragment to study protein complex formation. The DNA retardation patterns shown in Fig. 3 were observed with a 271-bp DNA fragment of the *tre* promoter region containing both palindromes representing the putative operators *O1* and *O2* (Fig. 1) (Schöck and Dahl 1996b). DNA forms one or two complexes, depending on the TreR concentration used (Fig. 3A). Thus, it can be concluded that formation of the trehalose repressor-*tre* operator complex does not require any additional protein from the crude extract. At lower protein concentrations (at a TreR:DNA ratio of 0.2:1), only one complex was observed, while at higher

TreR concentrations two distinguishable complexes were formed. At a TreR:DNA ratio of 40:1 only the less rapidly migrating complex is found (Fig. 3B). This demonstrates that this larger complex contains a larger number of TreR molecules, and that saturation of the

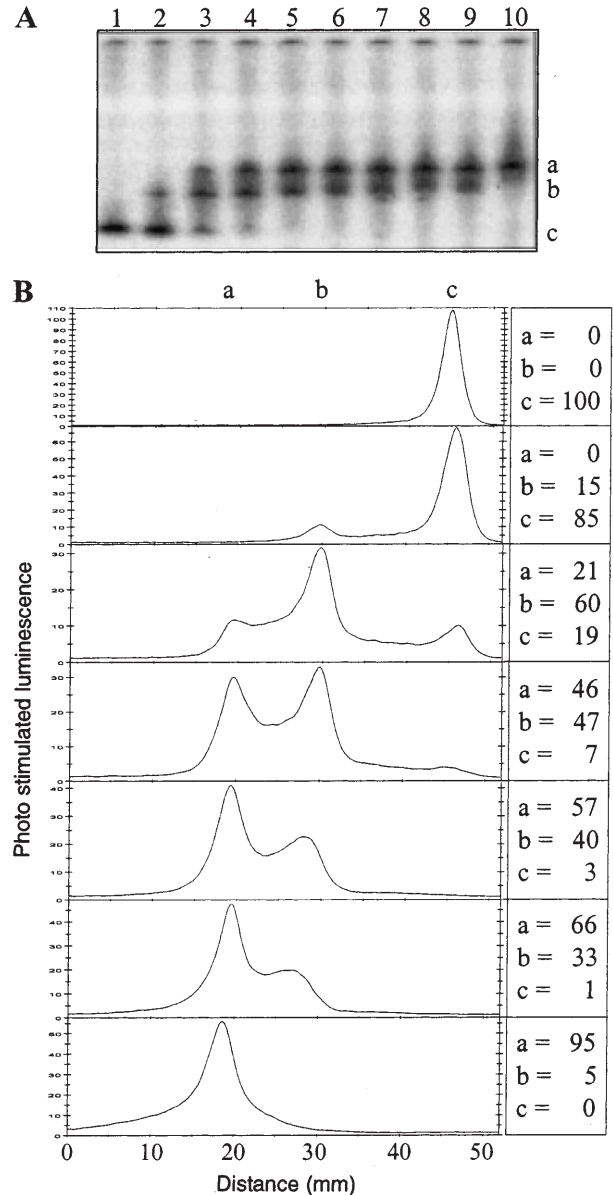


Fig. 3A, B DNA retardation assay demonstrating TreR-*tre* operator interaction. **A** Autoradiograph depicting TreR complex formation with a 271-bp DNA fragment. Free DNA is marked c to the right of the gel, the TreR-*tre* operator complexes are denoted a and b in order of increasing mobility. Lane 1 contains no protein, lanes 2 to 10 contain the same amount of radioactively labeled DNA (0.35 pmol) and increasing concentrations of TreR dimer: 0.07, 0.35, 0.7, 2.1, 3.5, 4.9, 7.0, 10.5 and 14 pmol, respectively. **B** Quantitative analysis of selected lanes of the retardation experiment in **A**. The relative intensities (as photo-stimulated luminescence) of the labeled DNA signals are plotted against the migration distance. Free DNA is denoted as c and the DNA are labeled a and b, as in **A**. On the right the relative intensities of the signals corresponding to free DNA (c) and each of the shifted DNA complexes (a and b) are given as a percentage of the total amount of DNA in each quantified experiment

tre operator with TreR is a two-step process. To verify the specificity of the TreR DNA interaction we analyzed complex formation in the presence of increasing concentrations of unlabeled fragment (Fig. 4). Competition assays were performed with increasing amounts of unlabeled DNA (from 17 to 100 ng/ μ l), and showed that complex formation was nearly completely abolished at 100 ng/ μ l. Based on the quantification data in Fig. 3B, we calculated that at a TreR:DNA ratio of 0.7:1, 50% of the operator DNA is bound by the repressor. At a ratio of 2:1 the quantities of the two complexes are nearly identical. A further increase in TreR concentration results in an increase in the amount of the more severely retarded complex formed. However, between ratios of 6:1 and 30:1 the rate of increase in the concentration of the lower-mobility complex formation is not as drastic as that of the higher-mobility complex at lower protein concentrations. These data suggest that formation of the latter complex is favored over the former. We therefore tested whether the two operators exhibit different affinities for the trehalose repressor.

TreR interacts with two distinct operators

In order to examine the possible function of the two *tre* operators in the regulation of the *tre* operon, we analyzed TreR-DNA complex formation in vitro in DNA retardation experiments. For this purpose, we used DNA fragments containing either operator 1 (*O1*) or operator 2 (*O2*). The quantitative analysis of the data obtained is presented in Fig. 5. DNA fragments of the same region that were deleted for both operators served as a control, and showed no detectable complex formation with TreR (data not shown). TreR can interact with each operator alone, albeit with different efficiencies. About 50% of the DNA was shifted at TreR:DNA

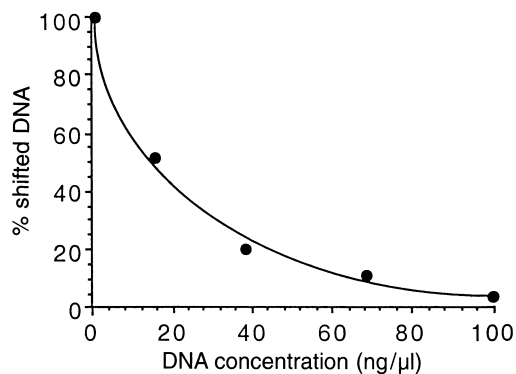


Fig. 4 Competition assay demonstrating the specificity of the interaction of TreR with a DNA fragment containing operators *O1* and *O2*. The radioactively labeled DNA was incubated with TreR under the same conditions as in Fig 3, lane 4, together with the indicated concentrations of an unlabeled DNA fragment containing both operators. The percentage of labeled DNA shifted into complexes was plotted against the concentration of unlabeled competitor DNA added

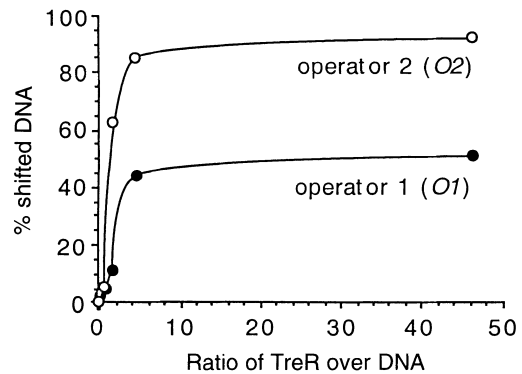


Fig. 5 TreR can bind to each of the two operators independently. Two different labeled DNA fragments, operator 1 (*O1*; filled circles) or operator 2 (*O2*; open circles) were used separately in this set of mobility shift experiments as indicated. The DNA was incubated with increasing concentrations of TreR, resulting in increasing ratios of TreR dimer to DNA as shown. The relative amounts of shifted DNA obtained are plotted as a percentage of the total amount of DNA used in each gel retardation experiment

fragment ratios of 46 for *O1* and of 1.8 for *O2*. These data reflect the different affinities of *O1* and *O2* for TreR and are in agreement with the findings presented in Fig. 3. Furthermore, when the data in Fig. 3 is analyzed with respect to the total amount of shifted DNA (the sum of complexes a and b), 50% complex formation is obtained at a ratio of TreR to wild-type operator (*O1* and *O2*) of 0.7. This can be interpreted as indicative of cooperative binding of TreR as defined by Ackers et al. (1982).

TreR activity is influenced by various effectors

To learn more about TreR-mediated regulation of the *tre* operon, we monitored the TreR-*tre* operator interaction in DNA retardation experiments under various conditions and in the presence of different potential effectors. The addition of substrates, which were suspected of being potential effectors, led to a decrease in the total amount of DNA shifted. Depending on the concentrations used, a decrease in the amount of lower-mobility complex formed is observed before formation of the higher-mobility complex is negatively affected (data not shown). Since this observation correlates directly with an increase in the free DNA signal, we consider only the values for the latter in Fig. 6. All DNA retardation experiments were carried out at a TreR:DNA ratio of 2:1 (cf. Fig. 3). Lowering the pH from 8 to 5.5 nearly completely abolished the binding capacity of TreR (data not shown). Therefore, in the in vitro experiments a constant pH of 8 was used. A negative influence of increasing concentrations of NaCl on the binding capacity of TreR was also observed (Fig. 6A). In principle, this observation reflects the induction mechanism. As has been demonstrated previously using crude cell extracts of *B. subtilis*, increasing concentrations of trehalose-6-phosphate in the DNA retardation experiments led to an

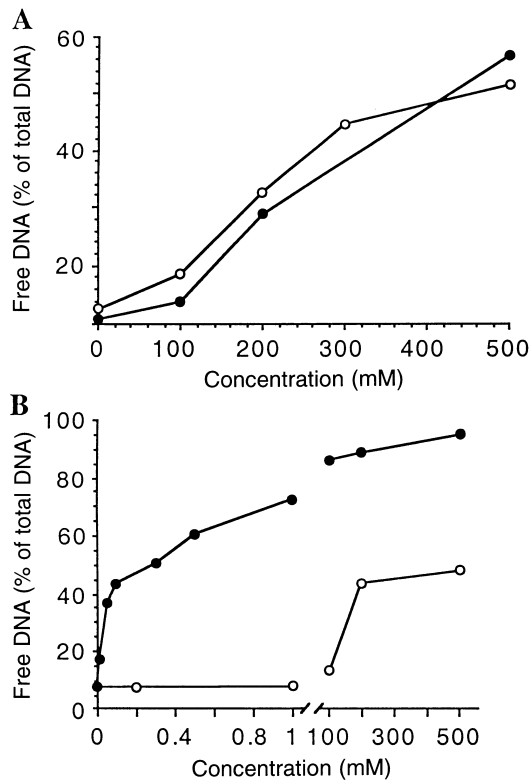


Fig. 6A, B Factors influencing the TreR-*tre* operator interaction. A DNA fragment containing the wild-type operator was incubated with TreR at a ratio of 2:1 (see Fig. 3) and complex formation was examined under different conditions. **A** Addition of the indicated millimolar concentrations of NaCl (filled circles) or glucose-1-phosphate (open circles). **B** Addition of increasing amounts of trehalose-6-phosphate in the absence (filled circles) or presence of 100 mM glucose-6-phosphate (open circles) as indicated. The amount of free DNA is expressed as a percentage of the total amount of DNA used in each gel retardation experiment

increase in free DNA, suggesting that trehalose-6-phosphate is the molecular inducer (Schöck and Dahl 1996b). However, these experiments could not exclude a possible contribution of additional factors present in cell extracts, which might be involved in mediating the induction of *tre* operon expression. Therefore, we studied complex formation between operator DNA and the purified TreR in the presence of increasing concentrations of trehalose-6-phosphate (Fig. 6B). At 250 μ M trehalose-6-phosphate 50% of the input DNA is free. These data clearly confirm that trehalose-6-phosphate is the molecular inducer. However, these data cannot exclude the possibility that additional effectors might be involved in other signal pathways that can also regulate *tre* operon expression via TreR. As depicted in Fig. 6A, increasing concentrations of glucose-1-phosphate also negatively influence formation of the TreR-*tre* operator complex, comparable to the effect of sodium chloride. Other sugars tested in these experiments, such as fructose, fructose-6-phosphate, fructose-1,6-diphosphate, glucose and glucose-6-phosphate, had no significant influence on TreR-*tre* operator interaction. Furthermore, we determined whether the potential effectors could

compete with each other to influence complex formation. For that purpose, we analyzed the effect on complex formation in the presence of increasing concentrations of trehalose-6-phosphate in the presence of fructose, fructose-6-phosphate, fructose-1,6-diphosphate, glucose or glucose-6-phosphate. Only when glucose-6-phosphate was added, was the effect of trehalose-6-phosphate abolished (Fig. 6B). Using glucose-1-phosphate in a similar set of experiments, no interference with trehalose-6-phosphate could be observed. Thus, glucose-6-phosphate specifically interferes in vitro with trehalose-6-phosphate triggered TreR inactivation.

Discussion

The *tre* operon in *B. subtilis* is subject both to control by trehalose and to carbon catabolite repression (CCR) triggered by glucose, fructose and mannitol (Helfert et al. 1995). Deletion of *treR* leads to constitutive expression of the *tre* operon (Schöck and Dahl 1996b). From this, and from the experiments presented in this report using purified TreR, it can be concluded that the induction of the *tre* operon expression is exclusively mediated via the trehalose repressor TreR. Trehalose-6-phosphate was identified as the molecular inducer because it inhibits the interaction between TreR and the *tre* operator in a concentration-dependent manner, and this in turn leads to the transcription of the *tre* operon. On the other hand, the presence of glucose, fructose or mannitol in the growth medium triggers CCR even in the presence of trehalose. In CCR, CcpA is known to be a key component (for an overview see Hueck and Hillen 1995). Inactivation of CcpA leads to the loss of CCR of most catabolite-controlled genes and operons. The *ptsHI* mutation, which leads the replacement of Ser46 by Ala at the regulatory phosphorylation site of HPr (Deutscher et al. 1994; Reizer et al. 1998) has similar effects. However, several examples are known of genes that still show glucose repression in the background of *ccpA* or *ptsHI* mutations: these include glycerol kinase (Deutscher et al. 1994), the maltose-inducible α -glucosidase (sucrase-maltase-isomaltase; Deutscher et al. 1994; Schönert et al. 1998) and the *tre* operon (Helfert et al. 1995; Schöck and Dahl 1996b). Based on these observations, additional mechanisms have been postulated to mediate CCR (Gotsche and Dahl 1995; Helfert et al. 1995, Dahl 1997). One CcpA-independent glucose repression mechanism is based on the effect of inducer exclusion (Dahl 1997). The contribution of glucose kinase to CCR has been also discussed for *Staphylococcus xylosus* (Wagner et al. 1995) and *B. megaterium* (Späth et al. 1997). The product of the glucose kinase-catalyzed reaction, glucose-6-phosphate, has been shown to act as an anti-inducer in this study. We have recently identified the glucose kinase encoded by the *glcK* gene of *B. subtilis* (Skarlatos and Dahl 1998), and there is evidence for a contribution of glucose kinase to CCR (L. Rosana,

P. Skarlatos, S. Gotsche and M. K. Dahl, unpublished results). The xylose systems of *B. subtilis* and *B. megaterium* represent other known cases, in which the repressor is presumably involved in CCR (Dahl et al. 1995; Kraus et al. 1994). While xylose functions as an inducer, glucose-6-phosphate has identified as an anti-inducer in vitro. This is also the case for the TreR-*tre* operator interaction. We tested the effects of several additional sugars capable of influencing the TreR-*tre* operator interaction. Only glucose-1-phosphate was identified as an additional potential effector, which might act as an inducer or co-inducer. All in all, these observations strongly indicate that several effectors regulate the TreR-*tre* operator interaction, which could be involved in mediating signals that cause induction or CCR of the trehalose system.

However, at this point, the question of biological significance arises. Earlier research has indicated that additional, partly repressor-mediated, mechanisms are involved in CCR of the trehalose system (Helfert et al. 1995, Dahl 1997). The results presented in this paper show that different sugars have different effects on the TreR-*tre* operator interaction. The in vitro experiments performed here cannot and do not claim to determine the physiological concentrations of effectors present in the cell. Earlier work has shown that glucose-6-phosphate can be present in the cell in concentrations of the order of approximately 2 mM relative to total cytoplasm volume (Thompson 1978). One must bear in mind, though, that a cell is not a bag of water, but is filled with a great variety of molecules: DNA, RNA, proteins – the latter in an intercellular concentration of 350 mg/ml (Methews 1993). Thus, a large proportion of the water in the cell is bound and no longer available as a solvent for other molecules, such as the effectors discussed here (Welch and Easterby 1994). Seen in this light, it is quite reasonable to conclude that the concentrations used in our in vitro experiments indeed reflect possible physiological conditions, because a homogenous distribution of the substances in the cell cannot be assumed (Welch and Easterby 1994). This, however, is the case in the dilute aqueous solutions used in in vitro experiments.

We have provided evidence that the trehalose repressor TreR acts as an oligomer, at least as a dimer. In solution, no oligomers larger than dimers could be observed; however, this does not exclude the possibility of the formation of unstable higher oligomers. The titration experiments using a DNA fragment containing the wild-type operator revealed formation of two different complexes that differ in mobility. Therefore, we assume that one TreR dimer binds each of the two putative operators. This observation is supported by the results obtained with DNA fragments containing either one of the two operators in a similar set of experiments. In that case, formation of only one complex can be observed even at high protein concentrations. However, operators *O1* and *O2* are bound by TreR with different efficiencies, as indicated by the approximately 25-fold higher protein:DNA ratio necessary for half-maximal binding to

operator *O1* compared to *O2*. Furthermore, when both operators are present in the same DNA fragment, total binding efficiency is 2.5-fold than for *O2* alone, and 63-fold higher than for *O1* alone. These data strongly suggest cooperative binding of TreR to the *tre* operators. The trehalose repressor presumably binds each operator as a dimer. Thus, full occupation of the wild-type operator would be achieved by two TreR dimers. Whether TreR forms a tetrameric complex in the presence of wild-type operator DNA remains to be analyzed. But it should be noted that the operators are 32 bp apart, which corresponds to three turns of the DNA helix. Therefore one would assume that binding of both operators take place at the same side, facilitating an interaction of two dimers. The negative influence of the molecular inducer, as well as increasing sodium chloride concentrations and low pH, indicates an inactivation of TreR, resulting in an inability to bind *tre* operators. This inactivation could be due to loss of the ability to form oligomers. It has been demonstrated previously for the tetracycline repressor that increasing concentrations of sodium chloride lead to an increase in the dissociation rate constant (Kleinschmidt et al. 1991). Therefore, we suspect that the effectors tested may abolish TreR oligomerization, correlated with the loss of the ability to bind *tre* operators. Nevertheless, for a detailed biochemical description further studies are needed to determine the affinities of TreR for its specific recognition sequences *O1* and *O2* and for the potential effectors, as well for the inducer trehalose-6-phosphate.

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