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Expression of the *tre* Operon of *Bacillus subtilis* 168 Is Regulated by the Repressor TreR

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The *tre* locus from *Bacillus subtilis* containing the genes *treP*, *treA*, and *treR* has been analyzed for its regulation. We demonstrate that at least *treP* and *treA* form an operon whose expression is regulated at the transcriptional level. TreR activity has been investigated in in vivo and in vitro studies. An insertional inactivation of *treR* led to a constitutive expression of *treP* and *treA*. Upstream of *treP*, we identified a 248-bp DNA fragment containing a potential σ^A-dependent promoter and two palindromes reflecting potential *tre* operators which led to complex formation with TreR-containing protein extracts in DNA retardation experiments. This complex formation is abolished in the presence of trehalose-6-phosphate, which probably acts as an inducer. Therefore, we assume that *treR* encodes the specific Tre repressor involved in regulation of the expression of the *tre* operon.

Bacillus subtilis 168 can use trehalose as its sole carbon and energy source (20, 26). The ability to utilize trehalose is dependent on an intracellular specific phospho-α-(1,1)-glucosidase (TreA) hydrolyzing trehalose-6-phosphate as well as *para*-nitrophenyl α-D-glucopyranoside in vitro (14, 17). The latter property makes *treA* suitable as a reporter gene, which is functional in *Escherichia coli* and *B. subtilis* (14, 41). The *treA* gene was cloned and sequenced. The deduced amino acid sequence exhibits high levels of similarity to oligo-1,6-glucosidases (17, 44, 45) and the trehalose-6-phosphate hydrolase TreC of *E. coli* (35).

TreA activity in *B. subtilis* 168, and presumably the expression of the *tre* operon, is influenced by (i) growth phase dependence, (ii) induction by trehalose in the growth medium, and (iii) carbon catabolite repression (CCR) (17). In *B. subtilis*, CCR is mediated by CcpA (catabolite controlling protein), which either binds alone (21) or binds in conjunction with HPr(Ser-P) (7, 12, 34), to a *cis*-acting catabolite responsive element (*cre*). These *cre*s are located in a range of 200 bp upstream or downstream of the transcriptional start sites of genes which are subject to CCR (19 [for a review, see reference 18]). An inactivation of the central component, CcpA, led to a loss of TreA repression by fructose, whereas repression by glucose is still present, indicating that additional factors or systems may mediate CCR of the trehalose utilization system in *B. subtilis* (17). Factors including salt and glucose regulate the enzymatic activity of TreA in vitro, as demonstrated with purified protein (14).

We have recently cloned and sequenced DNA flanking the *treA* gene from *B. subtilis* (40). The sequence analysis revealed that upstream of *treA*, there is an open reading frame (*treP*) which exhibits high similarities to IIBC enzymes of phosphotransferase systems (38) from several organisms. On the basis of these sequence similarities, it was proposed that TreP acts as the specific enzyme $IIBC^{Tre}$ involved in trehalose transport and phosphorylation as described for trehalose uptake in *E. coli* (2, 23). Downstream of *treA*, another open reading frame (*treR*) was identified whose deduced amino acid sequence revealed similarities to the GntR repressor family (16, 40). In this article, we report that the genes *treP* and *treA* are organized in an operon followed by the *treR* gene. TreR function has been analyzed in vivo and in vitro, showing that it acts as a repressor and is able to interact with a 248-bp DNA fragment in front of *treP*. This interaction is inhibited by increasing trehalose-6-phosphate concentrations in vitro.

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MATERIALS AND METHODS

Reagent and enzymes. All reagents used in this study were of analytical grade. Restriction enzymes, Klenow DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were used as recommended by the manufacturers. Glucose, trehalose, trehalose-6-phosphate, and *para*-nitrophenyl α-D-glucopyranoside were purchased from Sigma (Deisenhofen, Germany).

Media, DNA manipulations, and selection of recombinants. *E. coli* cells were grown in Luria-Bertani (LB) medium (39). Selective media for *E. coli* contained ampicillin (100 μg/ml). *B. subtilis* cells were grown in LB medium, MC competence medium (25), or C minimal medium (31) supplemented with auxotrophic requirements (50 μg/ml) and nutrients as indicated. Selective media for *B. subtilis* contained either chloramphenicol (5 μg/ml) or kanamycin (30 μg/ml).

DNA restriction endonuclease digestions, ligations, and gel electrophoresis were performed according to standard techniques (39). Standard procedures were used to extract plasmids from *E. coli* (39).

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.) was used as a general cloning host and for large-scale plasmid preparation. In plasmid pSG3 (40), a 254-bp *Xba*I-*Rsr*II internal DNA fragment of *treR* was replaced by a 1.5-kb *Stu*I-*Sma*I fragment of pDG782 (15) carrying the antibiotic resistance gene *aphA3* (kanamycin) after modification of the *Xba*I-*Rsr*II sites to blunt ends by Klenow fill-in reaction (Fig. 1). The resulting plasmid, pFS3, was used to construct the chromosomal *treR* mutation by homologous recombination. A 750-bp *Eco*RI-*Stu*I DNA fragment from pCH1 containing the *tre*-operator-promoter region was ligated to *Eco*RI-*Sna*BI-digested pDH32M, leading to plasmid pFS4. Standard procedures were used for *E. coli* transformation (39). *B. subtilis* 1A1 (168 wild type) was obtained from the *Bacillus* Genetic Stock Center (Ohio State University, Columbus). Strain MD179 was constructed by transformation of plasmid pFS3, previously digested with *Bam*HI-*Apa*I, in the *B. subtilis* wild type and additional selection on LB plates containing 30 μg of kanamycin per ml. MD182 and MD183 were constructed by transformation of pFS4 digested with *Pst*I-*Sca*I in MD179 and the *B. subtilis* wild type, respectively, followed by a selection on LB plates containing 5 μg of chloramphenicol per ml. The insertion in the *amyE* gene was verified by screening for the amylase-negative phenotype on LB starch (1%) plates. Transformation of *B. subtilis* with plasmid DNA was carried out by a one-step procedure (25).

DNA retardation experiments. Wild-type *B. subtilis* 168 without plasmid and transformants with pCH1 and strain MD179 were grown in LB medium to an optical density at 600 nm of 1.0. The cells were then harvested by centrifugation at 4°C for 10 min at 5,000 × *g*, resuspended in 0.05 volume of 50 mM Tris-HCl (pH 7.8)–39 μM dithiothreitol, and broken by sonication 10 times at 45 W for

TABLE 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
168	*trpC2*	BGSC[a]; 1A1
MD179[b]	*trpC2 treR*::*aphA3*	pFS3→168
MD182[b]	*trpC2 amyE*::(*treP′-lacZ cat*) *treR*::*aphA3*	pFS4→MD179
MD183[b]	*trpC2 amyE*::(*treP′-lacZ cat*)	pFS4→168
Plasmids		
pBluescript SK$^-$	Cloning vector, Ap^r	Stratagene
pDH32M	Integrational plasmid for *B. subtilis*	24
pWH1509K	*E. coli-B. subtilis* shuttle plasmid, Ap^r Nm^r	37
pCH1	pWH1509K derivative containing a 5.75-kb DNA fragment from a *B. subtilis* 168 gene library harboring the *tre* operon	17
pDG782	pMTL22 derivative containing the *aphA3* cassette from *S. faecalis*	15
pSG3	pBluescript derivative carrying a 2,018-bp *Nsi*I fragment of pCH1 containing *treR*	40
pFS3[b]	pSG3 derivative carrying *treR*::*aphA3*	This work
pFS4[b]	pDH32M derivative carrying *treP′-lacZ*	This work

[a] BGSC, *Bacillus* Genetic Stock Center.
[b] For description of construction, see Materials and Methods.

20 s. The resulting mixture was cleared by centrifugation for 10 min at 4°C and 15,000 × *g*, and the supernatant was aliquoted and stored at −20°C. The protein concentration was determined with the Bio-Rad assay as described previously (3, 36).

To obtain radioactively labeled DNA fragment for DNA retardation assays, plasmid pCH1 was used as a PCR template with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and the following pair of oligonucleotides: 5′dCATAG AGGCTCACCCTGC and 5′dGCACCGCCGACTGCTTCG in the presence of [α-^{32}P]dATP. The resulting fragment, 248 bp in size, containing the promoter region with the putative operator *treP*$_{O1O2}$ of the *tre* operon, was ethanol precipitated before further use in DNA retardation experiments.

Between 0.04 and 3.84 μg of crude protein extract per microliter was incubated with radioactively labeled *treP*$_{O1O2}$ DNA (8,000 or 16,000 cpm) and 2 μg of competitor DNA (sonicated pWH912) (4) 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–3% Ficoll for 15 min at 21°C. Depending on the experiment, the reaction buffer was used as described or was supplemented with trehalose-6-phosphate or trehalose in several concentrations between 5 μM and 100 mM, as indicated. Electrophoresis of the samples was carried out on 1-mm-thick 5% polyacrylamide gels in 89 mM Tris–89 mM boric acid–2 mM EDTA–10% glycerol (pH adjusted to 8.3) for 2 to 4 h at 180 V. The gels were exposed to Hyperfilm MP (Amersham, Braunschweig, Germany) and analyzed in a PhosphorImager (Fujifilm, BAS-1500).

Phospho-α-(1,1)-glucosidase assay. Phospho-α-(1,1)-glucosidase (TreA) activity was determined with wild-type *B. subtilis* or strain MD179 according to the method of Miller (28), with modifications as described previously (17, 31). Enzymatic activities are given in units per milligram of protein as defined previously (17, 31). Cells were grown in C minimal medium (31) containing tryptophan (50 μg/ml), ferric citrate (11 μg/ml), magnesium chloride (2 mM), calcium chloride (1 mM), and 0.3% K-glutamate. To study the induction by trehalose and repression by glucose, the medium was supplemented with sugars in the concentrations and combinations indicated. Cells were harvested at an optical density at 600 nm of 0.5 to 0.6. The data presented are an average of at least three independent experiments.

β-Galactosidase assay. β-Galactosidase activity was determined with strain MD182 or strain MD183 according to the method of Miller (28) with modifications as described previously (31). Enzymatic activities are given in units per milligram of protein as defined previously (31). Cells were grown in the medium described above for the phospho-α-(1,1)-glucosidase assay. To study the induction by trehalose and repression by glucose, the medium was supplemented with sugars in the concentrations and combinations indicated. Cells were harvested at an optical density at 600 nm of 0.5 to 0.6. The data presented are an average of at least three independent experiments.

Determination of transcription start point. RNA isolation (with the Qiagen Total RNA Maxi Kit; Qiagen, Düsseldorf, Germany) and primer extension (1) were done as previously described. RNA (25 μg) was incubated for 5 min at 85°C and then hybridized for 30 min with the 5′-end-labeled primer (5′dGCACCGC CGACTGCTTCG) at 42°C. Reaction mixtures containing 8 U of avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.) were incubated for 30 min at 42°C. After ethanol precipitation, the product was analyzed on a sequencing gel with sequencing reactions with the same primer and pCH1 (17) as the template.

Southern blot analysis. Electrophoresis and Southern blotting of *Hin*dIII-digested chromosomal DNA were performed as previously described (43). As a probe for hybridization, we used plasmid pCH1 (17) labeled with the Gibco BRL Nick Translation System and used PhotoGene (Gibco BRL Life Technologies, Inc.) for detection of hybridized probe.

Northern (RNA) blot analysis. Total RNA was obtained as described above from *B. subtilis* cells grown under trehalose-induced, uninduced, and glucose-repressed growth conditions. Northern hybridization was performed by transfer of denatured (1% agarose–formaldehyde gel) RNA samples (25 μg) to a nylon 66 membrane (Macherey-Nagel, Düren, Germany) and hybridization with a fragment labeled by the random primer extension method (10, 11). The hybridization probe spans the region of *treA* and *treR* from position 2192 to position 3735 of the DNA sequence (EMBL database accession no. Z54245). After hybridization, the membrane was analyzed in a PhosphorImager. The RNA standard was stained with 0.1% methylene blue.

RESULTS AND DISCUSSION

Transcript analysis. *treA* expression in *B. subtilis* is regulated by trehalose induction and catabolite repression (17). It has been shown that the *treA* gene is flanked by two genes, *treP* being located upstream and *treR* being located downstream of *treA* (40). Direct evidence for cotranscription of *treP* and *treA* was obtained by Northern hybridization analysis with total RNA from *B. subtilis* cells grown in medium containing trehalose (Fig. 2). A major hybridizing band with a size of 3.2 kb corresponding exactly to the size expected for a *treP-treA* transcript was detected with a 1.5-kb DNA probe containing regions of *treA* and *treR* (see Materials and Methods). These data strongly support the transcriptional relationship between *treP* and *treA*. Northern blot analysis revealed a weak additional signal above 4 kb, which presumably represents a transcript of all three genes, *treP*, *treA*, and *treR*. This would be similar to the expression of the GntR family regulators regulating pyruvate and L-lactate dehydrogenase expression (9, 33).

No signal was observed in Northern blot analyses with RNA from strains grown under uninduced conditions. However, with RNA obtained from cells grown under glucose-repressed conditions, a small amount of the 3.2-kb transcript can be observed (Fig. 2, lane 3). This result indicates that CCR of the *tre* operon effected by glucose is acting at the transcriptional level but is not leading to complete repression. It supports the proposal of additional mechanisms involved in CCR of the trehalose uti-

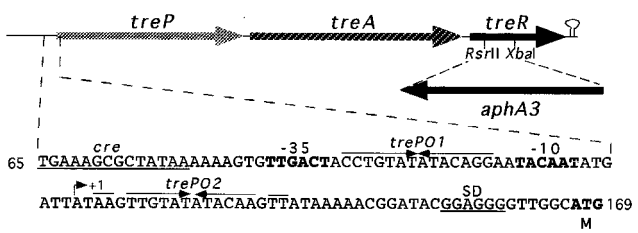

FIG. 1. Organization of the *tre* locus of *B. subtilis*. Genetic organization of the *tre* operon, showing *treP* (encoding the putative enzyme IIBC for trehalose uptake), *treA* [encoding phospho-α-(1,1)-glucosidase], and the distally located gene *treR* (encoding the Tre repressor). Sequences located upstream of *treP*, containing the suggested σ^A-dependent promoter and potential regulatory elements, are shown in the enlarged section. *cre*, catabolite responsive element; SD, Shine-Dalgarno ribosome binding site. Two palindromes, *treP*$_{O1}$ and *treP*$_{O2}$, which represent potential operators, and the ATG start codon (M) of *treP* are indicated. The numbers flanking the sequence refer to the corresponding DNA sequence available from the EMBL database under accession no. Z54245. The *treR*::*aphA3* construct and the restriction enzymes used are depicted below *treR*, leading to TreR$^-$.

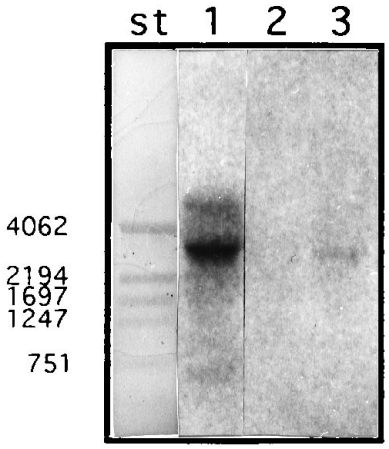

FIG. 2. Northern hybridization analysis of transcripts expressed from the *tre* operon. Total RNA from *B. subtilis* grown to an optical density at 600 nm of 0.8 in minimal medium C containing 0.3% K-glutamate with 5.3 mM trehalose (lane 1), with no supplement (lane 2), and with 5.3 mM trehalose and 10 mM glucose (lane 3) was blotted and hybridized to a specific DNA probe as described in Materials and Methods. The positions of standard RNA markers (st) are indicated in nucleotides to the left.

lization system, which may include regulation at the enzymatic level as suggested previously (14, 17).

Mapping of the 5′ end of *treP* mRNA and potential promoter elements. Upstream of the start codon (ATG) of *treP*, a potential promoter, *trePp*, with −10 and −35 sequences matching those of σ^A-dependent promoters (29, 30), is present (Fig. 1).

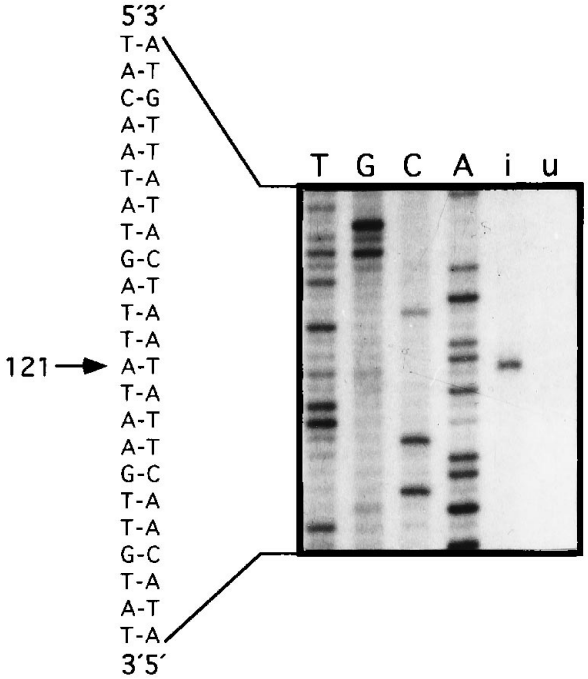

FIG. 3. Primer extension analysis of the *trePA* transcript. The lanes designated T, G, C, and A show the results of sequencing experiments (sequence shown on the left side) carried out with pCH1 as template and the same primer used for extension analysis of the mRNAs. Lanes i and u contain results with total RNAs prepared from *B. subtilis* 168 wild-type cells harvested from cultures grown under trehalose-induced (i) and uninduced (u) growth conditions. The transcriptional start point is indicated by an arrow. The number represents the position in the sequence of the *tre* operon (EMBL database accession no. Z54245).

regulators	potential DNA recognition sites
TreR	CCTGTATATACAGG (*treP*$_{O1}$)
	TTGTATATACAA (*treP*$_{O2}$)
$HutC_{Pp}$	**CTTGTATATACA**TA
GntR	ATA**CTTGTATACA**AGTAT
FadR	AT**CTGGTA**CGAC**CAG**AT
FarR	**TGTAT**TGTAT
	TGTATTATTT
PdhR	AATT**GGTA**AGAC**CA**ATT

FIG. 4. Comparison of DNA recognition sites of the GntR family with putative operators of the *tre* operon promoter region. The potential operators, designated *treP*$_{O1}$ and *treP*$_{O2}$ as depicted in Fig. 1, correspond to nucleotide positions 93 to 106 and 126 to 137, respectively, in the sequence published previously (40). The interaction sites presented for $HutC_{Pp}$ and GntR were taken from Haydon and Guest (16), those for FadR were taken from DiRusso et al. (8), those for FarR were taken from Quail et al. (32), and those for PdhR were taken from Quail et al. (33). Nucleotides identical to either *treP*$_{O1}$ or *treP*$_{O2}$ are presented in boldface letters.

Primer extension analysis (Fig. 3) revealed the transcriptional start point of *treP* to be 7 nucleotides downstream of the potential −10 region at position 121 (corresponding to the sequence with EMBL database accession no. Z54245); this transcription start was only detected with RNA from cells grown in trehalose, similar to the data obtained from Northern blot analysis. Examination of the DNA sequence upstream of the promoter region revealed two palindromes with the consensus sequence TGTATATACA (Fig. 1 and 4). Both palindromes are almost identical to each other and to the binding sites of the GntR transcriptional regulator family (15) (Fig. 4), to which TreR belongs (40).

Functional analysis of the *trePp-trePo* region. To verify the functionality of the *tre* promoter, we have constructed a transcriptional *lacZ* fusion to *trePp* (see Materials and Methods and Table 1) integrated in *amyE*, leading to strain MD183. β-Galactosidase expression from this fusion increased 13.5-fold in cells grown with trehalose, and expression in trehalose-grown cells is repressed 2.8-fold by glucose (Table 2). These data demonstrate that the DNA fragment fused to *lacZ* contains the promoter and *cis* elements involved in trehalose induction and glucose repression of the *tre* operon. A potential *cis*-acting catabolite responsive element (*cre*) is located in the promoter region with one deviation from the proposed consensus sequence (position 13, A instead of C) (19, 40). Mutational analysis of position 13 in *cre* in which C was changed to G or T led to a significant reduction in amylase repression (46), and a change to A resulted in a 2.7-fold reduction of the glucose repression of the *lev* operon (27). Thus, the *cre* of the *tre* operon does not have an optimized consensus sequence for interaction with CcpA but could well confer CCR, as indicated by the results described above (Fig. 2).

TreR acts as a repressor. To assess the function of TreR, we

TABLE 2. Analysis of *trePp* transcriptional *lacZ* fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (U/mg of protein)		
		No sugar added	5.3 mM trehalose added	5.3 mM trehalose and 10 mM glucose added
MD183	*treP′-lacZ*	35 ± 6	473 ± 48	167 ± 3
MD182	*treR*::*aphA3 treP′-lacZ*	1,210 ± 39	682 ± 7	395 ± 37

[a] Cultures were grown in minimal medium C containing 0.3% K-glutamate alone or with sugars in the concentrations and combinations indicated (see Materials and Methods).

TABLE 3. Constitutive TreA activity in a *treR*-negative strain[a]

Strain	Relevant genotype	TreA activity (U/mg of protein)		
		No sugar added	5.3 mM trehalose added	5.3 mM trehalose and 10 mM glucose added
B. subtilis 168	Wild type	9 ± 2	46 ± 6	11 ± 2
MD179	*treR*::*aphA3*	58 ± 4	21 ± 1	8 ± 1

[a] Cultures were grown in minimal medium C containing 0.3% K-glutamate alone or with sugars in the concentrations and combinations indicated (see Materials and Methods).

constructed a *treR*-negative mutant by insertion of a 1.5-kb *Stu*I-*Sma*I fragment from pDG782 (15) harboring the *aphA3* gene (Km^r) of *Streptococcus faecalis* in *treR* of plasmid pSG3. This construct lacks a 254-bp fragment coding for the N-terminal part of TreR (see Fig. 1 and Materials and Methods). The resulting plasmid, pFS3, is able to replicate in *E. coli* but not in *B. subtilis*. *B. subtilis* transformants obtained by chromosomal integration were selected on kanamycin (see Materials and Methods). The *treR*::*aphA3* integration in MD179 (see Table 1) has been verified by Southern blot analysis showing that the *aphA3* cassette is integrated by double crossover (data not shown), and MD179 was used for further in vivo and in vitro investigations to analyze the possible role of TreR. TreA activity was monitored under uninduced (growth on K-glutamate) and induced (growth in the presence of 5.3 mM trehalose) conditions (Table 3). In *B. subtilis* 168, TreA activity above the basal level can only be observed in induced cells (Table 3) (17), whereas in strain MD179 (*treR*::*aphA3*), TreA activity is high under uninduced conditions with a specific activity similar to that in induced wild-type cells. Qualitatively, the same results were obtained by monitoring the β-galactosidase expression of the *treP′-lacZ* fusion in the $TreR^-$ background (Table 2). The constitutive *treA* and *treP′-lacZ* expression in $TreR^-$ strains under uninduced growth conditions indicates that TreR is a repressor. The TreA and β-galactosidase activities in the *treR* mutant grown with trehalose were 2.8- and 1.8-fold lower, respectively, than those in cultures grown under uninduced conditions. The 1.8-fold-lower β-galactosidase expression was similar to the result of the xylose system of *Staphylococcus xylosus* (42) and could result from a change in the internal glucose/glucose-6-phosphate ratio after trehalose-6-phosphate hydrolysis. The even lower TreA activity could be partially caused by product inhibition due to the accumulation of glucose as shown in vitro (14).

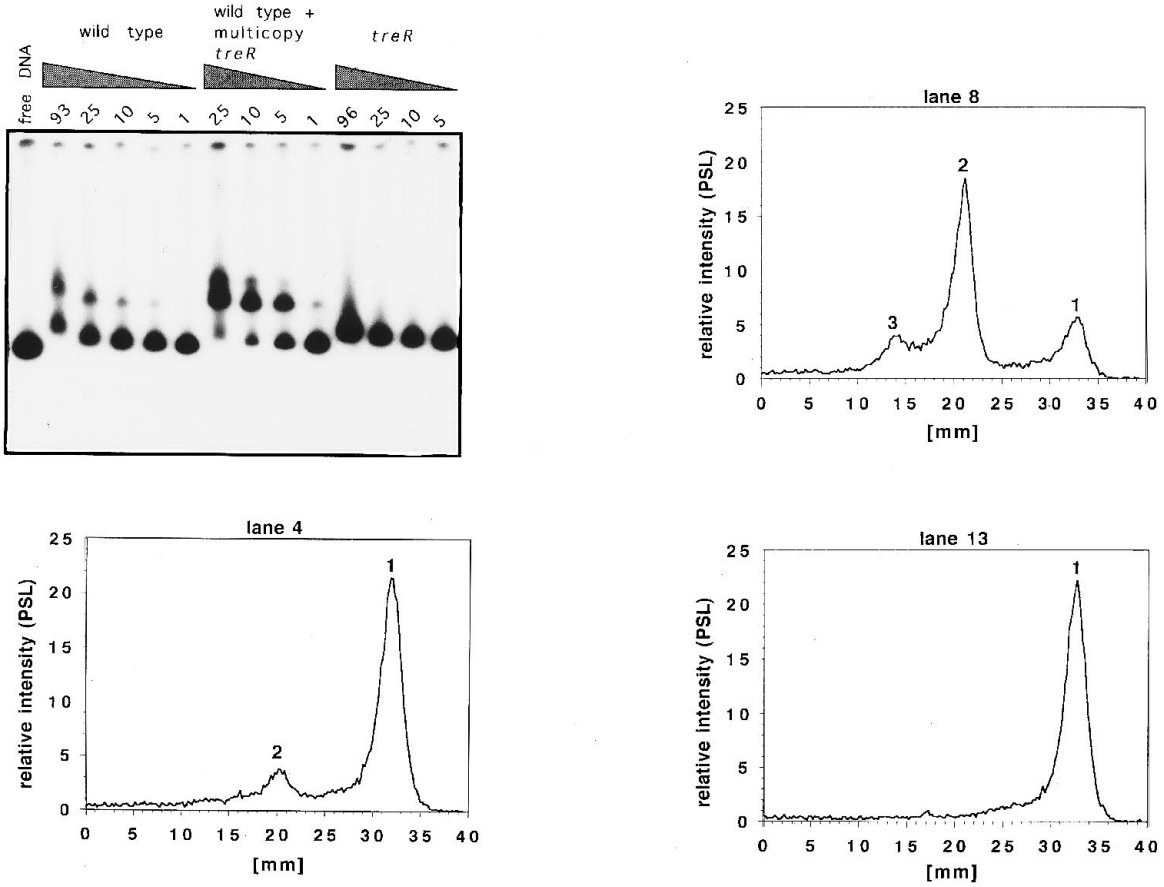

FIG. 5. Autoradiograph and analysis of the DNA retardation experiment with crude protein extracts from wild-type ($TreR^+$), *treR* in multicopy (pCH1), and $TreR^-$ *Bacillus* strains. The total amount (in micrograms) of protein crude cell extract loaded on the gel is indicated above each slot. The upper left panel shows the autoradiograph. Lane 1, 248-bp DNA fragment without protein; lanes 2 to 6, 248-bp DNA fragment plus crude protein extract from the *B. subtilis* wild type with amounts of protein indicated; lanes 7 to 10, 248-bp DNA fragment plus crude protein extract from *B. subtilis* containing plasmid pCH1 with amounts of protein indicated; lanes 11 to 14, 248-bp DNA fragment plus crude protein extract from *B. subtilis* MD179 (*treR*::*aphA3*) with amounts of protein indicated. Analyses of lanes 4, 8 and 13, respectively, of the autoradiograph in a PhosphorImager (Fujifilm, BAS-1500) are presented as indicated. Relative intensities (photo-stimulated luminescence [PSL]) of the signals as measured by the PhosphorImager are presented in correlation with their migration distance (in millimeters) in the gel. Free DNA is denoted by 1, and shifted DNA is denoted by 2 and 3 (higher- and lower-mobility bands, respectively).

treA expression, and probably the expression of the entire *tre* operon, is subject to CCR (17). We have therefore analyzed *treA* expression under glucose-repressive growth conditions in the *treR* mutant. The results (Table 3) show that TreA activity is glucose repressed in MD179, as observed in the wild-type strain. Analysis of the transcriptional *treP′-lacZ* fusion (MD182 and MD183) also shows glucose repression; however, this is to a slightly lower (2.4-fold) extent in the *treR* mutant (MD182) than that in the wild type (MD183). This suggests that TreR itself may contribute to CCR, as has also been proposed for the xylose system (5, 6, 24). Furthermore, glucose repression is lower in the *treP′-lacZ* fusion (MD183) than in *B. subtilis* 168. This might indicate a posttranscriptional mechanism of repression. Previous data showed that CcpA alone is not responsible for glucose repression of TreA activity (17), and TreA activity itself is probably glucose repressed (14).

TreR-*tre* operator interaction. As shown above, the potential *tre* regulatory DNA from *B. subtilis* contains two palindromic sequence elements (referred to as operators $treP_{O1}$ and $treP_{O2}$ in Fig. 1). To determine if TreR interacts with this DNA region, we analyzed a 248-bp DNA fragment harboring the respective region (see Materials and Methods) in DNA retardation experiments. It has been shown previously, by analysis of XylR-*xyl* operator interaction, that such experiments can be carried out with crude protein extracts of wild-type strains in comparison with crude extract of a repressor-negative strain (13). The results depicted in Fig. 5 show that increasing concentrations of crude protein extract from the *B. subtilis* wild type and *B. subtilis* transformed with pCH1 (multicopy *treR*) led to increased complex formation with the 248-bp DNA fragment. When an extract from cells containing multicopy *treR* was used, sixfold less total protein was necessary to obtain retarded DNA fragments compared with that when the wild-type extract was used (calculated from the quantitative analysis shown in Fig. 5). Additionally it was observed that a second complex with lower mobility occurred (see Fig. 5 autoradiograph, lane 8, and analysis of this lane) with 10 μg of protein extract of the *treR* multicopy strain. When 10 μg of crude extract from the wild type was used, 14% of the labeled DNA fragment was shifted (referred to as 2 in Fig. 5, analysis of lane 4). With extract containing TreR in multicopy, 66 and 13% of labeled DNA shifted to two complexes with higher and lower mobility (referred to as 2 and 3, respectively, in Fig. 5, analysis of lane 8). No retardation was observed with crude extract of strain MD179, not even with 96 μg of protein extract, showing that TreR in the crude extract is necessary for the retardation of the 248-bp DNA fragment.

To verify the specificity of the TreR-DNA interaction, we analyzed formation of complexes with the radioactively labeled 248-bp fragment in the presence of increasing concentrations of unlabeled fragment. With increasing amounts of unlabeled DNA (from 17 to 117 ng/μl), complex formation was nearly completely abolished at 117 ng/μl (data not shown). Taken together, these data clearly show that TreR is responsible for the complex formation with a 248-bp DNA fragment containing $treP_{O1O2}$. These results are comparable to those obtained with the Xyl repressor-*xyl* operator of *B. subtilis*, which is composed of two overlapping operators spaced by 4 bp (4, 13). However, whether TreR binds to the potential operators $treP_{O1O2}$ remains to be determined.

Trehalose-6-phosphate is the potential inducer. Previous studies have demonstrated that trehalose-6-phosphate and not trehalose is the substrate for the internal phospho-α-(1,1)-glucosidase TreA (14, 17) and that the activity can only be observed in cells grown under induced conditions (medium containing trehalose). As proposed previously (40), trehalose

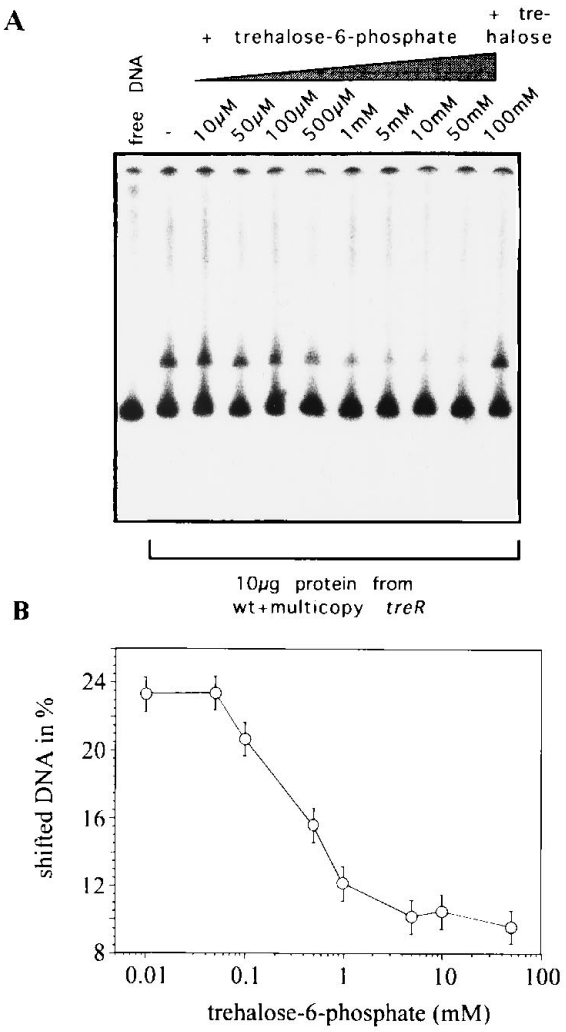

FIG. 6. Autoradiograph and analysis of the DNA retardation experiment with crude protein extracts from *B. subtilis* containing pCH1. (A) Autoradiograph. Lane 1, 248-bp DNA fragment without protein; lanes 2 to 11, 248-bp DNA fragment in the presence of 0.4 μg of crude protein extract per μl. Lanes 3 to 10 contain, in addition, trehalose-6-phosphate in the concentrations indicated. Lane 11 contains 100 mM trehalose. wt, wild type. (B) Analysis of lanes 3 through 10 of Fig. 6A in a PhosphorImager (Fujifilm, BAS-1500). The percentage of shifted DNA in correlation with the concentration of trehalose-6-phosphate is shown.

is probably phosphorylated during transport by a phosphotransferase-dependent enzyme, II^{Tre}. In order to distinguish if trehalose or trehalose-6-phosphate is the inducer in vivo, an in vitro experiment was performed. The DNA retardation experiment was carried out in the presence of trehalose and trehalose-6-phosphate (Fig. 6). TreR-DNA complex formation (Fig. 6A) was prevented at increasing concentrations of trehalose-6-phosphate. The efficiency of trehalose-6-phosphate induction in vitro has been quantified with a PhosphorImager. Figure 6B presents the analysis of each concentration, showing the amount of shifted DNA as a percentage of the total. Full induction was achieved at 5 mM trehalose-6-phosphate, and a minimum of 100 μM was necessary to obtain a reduction of complex formation. Trehalose showed no influence on complex formation up to 200 mM (shown only for 100 mM in Fig. 6). These properties would be expected for the Tre repressor, because trehalose is not present in the cytoplasm, since it is

presumably phosphorylated by the enzyme II^{Tre} during transport. The role of trehalose-6-phosphate as an inducer has been previously concluded for the *E. coli* trehalose repressor, with a trehalose-6-phosphate phosphatase involved in the regulation of the level of inducer at high osmolarity (22, 23). Thus, the in vivo and in vitro experiments show that TreR of *B. subtilis* is a trehalose-6-phosphate-dependent repressor involved in the regulation of the *tre* operon.

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