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Vectors using the phospho-α-(1,1)-glucosidase-encoding gene *treA* of *Bacillus subtilis* as a reporter

(Integrative vector; recombinant DNA; transcription; trehalose)

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SUMMARY

The intracellular phospho-α-(1,1)-glucosidase, TreA, from *Bacillus subtilis* (*Bs*) hydrolyses trehalose 6-phosphate into glucose and glucose 6-phosphate. The enzyme is also able to cleave *p*-nitrophenyl α-D-glucopyranoside (PNPG). This enzymatic reaction can be easily monitored in a β-galactosidase analogous enzyme assay. The vectors we have constructed can be used to study promoter activity in transcriptional *treA* fusions and may prove especially useful under high-salt conditions due to the halophilic character of TreA. The *treA* gene is useful as a reporter in either *Bs* or *Escherichia coli* (*Ec*). Such fusions can be integrated in the *Bs amyE* locus and selected on either kanamycin or chloramphenicol, or used as plasmids in *Ec*. As an example of the general utility, we demonstrate *treA* expression under *xylA*-operator-promoter control.

INTRODUCTION

Trehalose can be used by *Bacillus subtilis* (*Bs*) 168 as sole carbon and energy source (Kennett and Sueoka, 1971). We have recently identified a putative trehalose operon composed of at least three genes, encoding a proposed phosphoenolpyruvate phosphotransferase-dependent trehalose specific enzyme II^{Tre} (*treP*), a phospho-α-(1,1)-glucosidase (*treA*) and a portion of an open reading frame of unknown function (Helfert et al., 1995). Further experiments using crude protein extracts showed that the *treA* gene product of *Bs* is located intracellularly, hydrolyzing trehalose 6-phosphate and *p*-nitrophenyl α-D-glucopyranoside (PNPG) in vitro (Kennett and Sueoka, 1971; Helfert et al., 1995). TreA has been overproduced in *Escherichia coli* (*Ec*), purified and its enzymatic properties characterized (Gotsche and Dahl, 1995). The molecular mass of TreA was calculated to be 64 kDa and p*I* was 4.3. The enzyme is stable up to 44°C after 15 min treatment and shows a temperature optimum at 37°C and a pH optimum at 4.5 (Gotsche and Dahl, 1995). TreA activity is stimulated by $(NH_4)_2SO_4$, KCl and NaCl up to sixfold, depending on the concentrations used (Gotsche and Dahl, 1995). The enzymatic activity according to PNPG hydrolysis is reduced by one of the cleavage products, namely glucose, but not by the second cleavage product, glucose 6-phosphate (Gotsche and Dahl, 1995). Addition of glucose to concentrations higher than 100 mM completely inhibits TreA-catalyzed PNPG hydrolysis in vitro (Gotsche and Dahl, 1995). The

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Abbreviations: *A*, absorbance (1 cm); *amyE*, gene coding for the amylase of *Bs*; Ap, ampicillin; *Bm*, *Bacillus megaterium*; bp, base pair(s); *Bs*, *Bacillus subtilis*; Cm, chloramphenicol; *Ec*, *Escherichia coli*; kb, kilobase(s) or 1000 bp; Km, kanamycin; *ori*, origin(s) of DNA replication; LB, Luria Bertani (medium); nt, nucleotide(s); PNPG, *p*-nitrophenyl α-D-glucopyranoside; ^{R}, resistance/resistant; *treA*, gene encoding TreA; TreA, phospho-α-(1,1)-glucosidase of *Bs*; *xylA*, gene encoding xylose-isomerase; ::, novel junction (fusion or insertion).

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purified protein or crude protein cell extract could be stored at −20°C for 5 months without any loss of TreA activity.

The overproduction and functionality of TreA in *Ec* (Gotsche and Dahl, 1995) already indicated the possible use of phospho-α-(1,1)-glucosidase as a reporter. The aim of this study was to construct vectors which can be used for promoter analysis in transcriptional *treA* fusions integrated in *amyE* of *Bs* or as plasmids in *Ec*. The functionality of the constructed vectors was verified using the well-characterized *xylA*-operator-promoter region from *Bacillus megaterium* (*Bm*) (Rygus and Hillen, 1991; Dahl et al., 1994; 1995; Henkin et al., 1994; Hueck et al., 1994).

EXPERIMENTAL AND DISCUSSION

(a) Features of the *treA* reporter vectors

Vectors pMD476 and pMD477 (Fig. 1) are derivatives of plasmids pMD428 and pMD429 (Dahl and Meinhof, 1994), respectively. Both vectors contain a unique *Sma*I

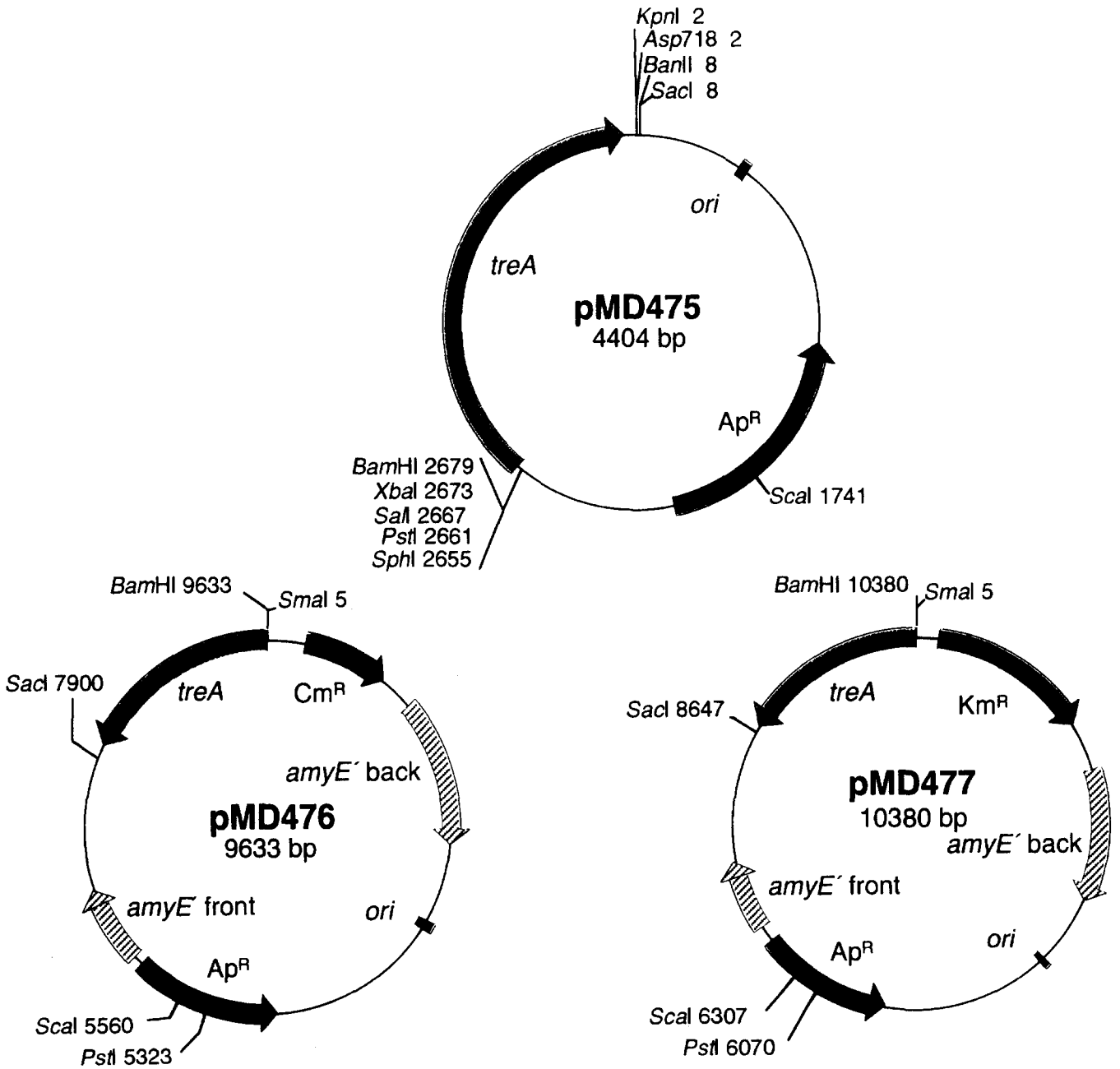

Fig. 1. Maps of vectors pMD475, pMD476 and pMD477. For construction a 1.7-kb *Dra*I-*Ava*II (blunt ended by Klenow fill-in reaction) DNA fragment of pSG2 (Gotsche and Dahl, 1995) containing the Shine-Dalgarno sequence and *treA* was introduced into the *Sma*I site of pUC18 (Yanisch-Perron et al., 1985). The correct orientation was verified by *Eco*RI digestion. The resulting plasmid pMD475 was further digested with *Bam*HI + *Sac*I and the resulting 1.7-kb fragment used to replace the 1.9-kb *Bam*HI-*Sac*I fragment from either pMD428 or pMD429 (Dahl and Meinhof, 1994) to give plasmids pMD476 and pMD477, respectively. Important single sites of restriction enzymes are indicated.

TABLE I

Expression of *treA* in *Bs* 1A167 (*tre*-12) under *xylA*-operator-promoter control[a]

Relevant fusion integrated in *amyE*[b]	Uninduced	Xylose induced[c]	Glucose repressed[c]
treA (pMD476)	5.2	4.5	ND
xylA::*treA* (pFS10)	4	16.1 (4)	2.4 (6.7)
treA (pMD477)	5.2	4.9	ND
xylA::*treA* (pFS11)	4.6	20.5 (4.5)	3.1 (6.6)

[a] TreA activities are presented in units/mg protein (Miller, 1972) with modifications published previously (Msadek et al., 1990; Helfert et al., 1995). Each value represents the average of three independent measurements.

[b] Linearized plasmids used for transformation are depicted in parenthesis. Competent cells were obtained by a one-step procedure (Kunst et al., 1994) and transformants selected on 20 μg Km/ml or 5 μg Cm/ml, respectively. The resulting integrants were further tested on LB 1% starch plates to confirm the expected AmyE$^-$ phenotype. Such strains were grown in either minimal medium C (Msadek et al., 1990) containing 0.4% K-glutamate, or in addition xylose, or xylose and glucose, respectively, with 10 mM each. The most suitable assay conditions proved to be 28°C and buffer Z (Miller, 1972) pH 6.

[c] Induction or repression factors, respectively, are depicted in parenthesis. Induction factors are presented as ratio of units obtained under induced and uninduced growth conditions. Repression factors are presented as ratio of units obtained under induced and repressed growth conditions.

and *Bam*HI site suitable for cloning DNA sequences immediately upstream from the Shine-Dalgarno sequence of *treA* (Fig. 1). The vectors contain an *ori* from pBR322 (Bolivar et al., 1977). These vectors can replicate in *Ec* but not in *Bs*. For use in the latter organism the vectors can integrate into *amyE* after linearization, transformation and selection on Cm (5 μg/ml) or Km (20 μg/ml), respectively. Integrants can be easily verified by scoring AmyE$^-$ phenotype on LB plates containing 1% starch.

(b) Influence of salt on TreA activity in vivo

As previously demonstrated using purified TreA (Gotsche and Dahl, 1995), the enzymatic activity is not diminished under high salt concentrations in in vitro reactions up to 800 mM KCl or NaCl. We have also investigated the influence of NaCl up to 800 mM in the growth medium on TreA activity in *Bs* wild-type cells. The TreA activity remained unchanged within a standard deviation of 5% (data not shown). This result indicates that TreA activity is not influenced in vivo by high salt concentrations in the growth medium.

(c) Determination of K_m and V_{max} of TreA using PNPG

The increase of $A_{420\ nm}$ in a TreA activity, the test was monitored spectrophotometrically over the time at different PNPG concentrations (650, 325, 160, 80 and 40 μM) using purified TreA protein (Gotsche and Dahl, 1995). The V_0 was determined and used in a Lineweaver-Burk plot for calculation of K_m and V_{max} by regression analysis. From these data the K_m of TreA for the PNPG substrate was calculated to be 1.4 mM and the V_{max} was 5.5 μmol/min per mg protein.

(d) Application of the vectors for measurement of *Bs* promoter activity

To test the vector constructs, a 902-bp *Bam*HI-*Stu*I fragment of plasmid pWH1520 (Rygus and Hillen, 1991) containing the well-characterized *xylA* promoter of *Bm* was cloned in front of the *treA* gene of pMD476 and pMD477 (see Fig. 1), creating plasmids pFS10 and pFS11, respectively. To study *treA* expression in *Bs*, the plasmids pMD476, pMD477, pFS10 and pFS11 were linearized with *Pst*I prior to transformation in *Bs* 1A167 (*tre*-12) (Kennett and Sueoka, 1971). The results are shown in Table I. TreA activity can only be observed in cells grown under inducible conditions (10 mM xylose) and the *treA* expression is repressed in the presence of xylose and glucose as observed with *xylA*::*lacZ* fusions (Rygus and Hillen, 1991). However, factors of induction and repression between *lacZ* and *treA* fusions are not comparable. This could be due to the use of a *Bm* promoter in *Bs* or effected by different efficiencies of gene expression.

(e) Conclusions

(*1*) Two vectors for transcriptional *treA* fusions were constructed which can integrate into the *amyE* locus of *Bs* and be selected either as Km^R or Cm^R. The enzymatic activity of TreA can be monitored as easily as β-galactosidase activity.

(*2*) To confirm the vectors utility, *treA* expression under *xylA*-operator-promoter control was analyzed. Both vectors allowed about fourfold induction by xylose and about sevenfold repression upon addition of glucose.

(*3*) The advantage of transcriptional *treA* fusions is the stability of TreA under high salt conditions in *Bs* in vivo. Furthermore, with a suitable promoter::*lacZ* fusion, two promoters can be studied simultaneously.

(*4*) As we have shown previously, the *treA* gene product is also functional in *Ec* DH5α (Gotsche and Dahl, 1995) and, therefore, can also be used in this strain as a reporter gene. However, we recommend using an *Ec* strain in which the chromosomal phosphotrehalase gene (*treC*) is inactivated (Boos et al., 1990; Rimmele and Boos, 1994).

For the convenience of the user we have constructed two plasmids carrying either Cm^R or Km^R. The plasmids are available on request.

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REFERENCES

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S.: Construction and characterization of new cloning vehicles, II. A multipurpose cloning system. Gene 2 (1977) 95–113.

Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmele, M. and Postma, P.: Trehalose transport and metabolism in *Escherichia coli*. J. Bacteriol. 172 (1990) 3450–3461.

Dahl, M.K., Degenkolb, J. and Hillen, W.: Transcription of the *xyl* operon is controlled in *Bacillus subtilis* by tandem overlapping operators spaced by four base pairs. J. Mol. Biol. 243 (1994) 423–424.

Dahl, M.K. and Meinhof, C.-G.: A set of integrative plasmids for *Bacillus subtilis* with unique cloning sites in all three open reading frames for translational *lacZ* fusions. Gene 145 (1994) 151–152.

Dahl, M.K., Schmiedel, D. and Hillen, W.: Glucose and glucose-6-phosphate interaction with Xyl repressor proteins from *Bacillus* sp. may contribute to regulation of xylose utlization. J. Bacteriol. 177 (1995) 5467–5472.

Gotsche, S. and Dahl, M.K.: Purification and characterization of the phospho-α-(1,1)-glucosidase (TreA) of *Bacillus subtilis* 168. J. Bacteriol. 177 (1995) 2721–2726.

Helfert, C., Gotsche, S. and Dahl, M.K.: Cleavage of trehalose-phosphate in *Bacillus subtilis* is catalyzed by a phospho-α-(1,1)-glucosidase encoded by the *treA* gene. Mol. Microbiol. 16 (1995) 111–120.

Henkin, T.M., Grundy, F.J., Nicholson, W.L. and Chambliss, G.H.: Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a *trans*-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. 5 (1991) 575–584.

Hueck, C., Hillen, W. and Saier, M.H.: Analysis of a *cis*-acting sequence mediating catabolite repression in gram positive bacteria. Res. Microbiol. 145 (1994) 503–518.

Kennett, R.H. and Sueoka, N.: Gene expression during outgrowth of *Bacillus subtilis* spores. J. Mol. Biol. 60 (1971) 31–41.

Kunst, F., Msadek, T. and Rapoport, G.: Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*. In: Piggot, P.J., Moran Jr., C.P. and Youngman, P. (Eds.), Regulation of Bacterial Differentiation. American Society for Microbiology, Washington, DC, 1994, pp. 1–20.

Miller, J.H.: Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972.

Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G. and Dedonder, R.: Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS* and *degU*. J. Bacteriol. 172 (1990) 824–834.

Rimmele, M. and Boos, W.: Trehalose-6-phosphate hydrolase of *Escherichia coli*. J. Bacteriol. 176 (1994) 5654–5664.

Rygus, T. and Hillen, W.: Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose utilization operon. Appl. Microbiol. Biotechnol. 55 (1991) 594–599.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103–109.