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Regulation of the *Bacillus subtilis* Phosphotransacetylase Gene[1]

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The enzyme, phosphotransacetylase (Pta), catalyzes the conversion of acetyl coenzyme A to acetyl phosphate. The putative *pta* gene of *Bacillus subtilis*, which had been sequenced as part of the Genome Project, was cloned and overexpressed in *Escherichia coli*. We confirmed that the gene encodes Pta by measuring the enzymatic activity of the purified protein. Insertional mutagenesis of the *pta* gene resulted in complete loss of the Pta activity, indicating that *B. subtilis* contains only one kind of *pta* gene. Expression of a *pta-lacZ* fusion was induced in the presence of excess glucose in the growth medium, and the intact *ccpA* gene was required for this activation. The transcriptional start site of the *pta* gene was located at 37 nucleotides upstream of the *pta* start codon, and a cre (catabolite responsive element) sequence, a *cis*-acting element that is responsible for the catabolite repression of a number of carbon utilization genes in *B. subtilis*, was identified upstream of the tentative promoter site. Experiments involving oligonucleotide-directed mutagenesis showed that the cre sequence is involved in glucose-mediated transcriptional activation.

Key words: *Bacillus subtilis*, CcpA, phosphotransacetylase.

The Gram-positive spore-forming bacterium, *Bacillus subtilis*, can grow on various carbon sources. In a rich medium containing an excess amount of a carbohydrate such as glucose, *B. subtilis* typically secretes acids such as pyruvate and acetate, which results in a decrease in the culture pH (*1*). After all the glucose has been exhausted, the acids secreted into the medium are oxidized again, the culture pH increasing again (*1*). *Escherichia coli* also excretes acetate during aerobic growth on glucose, which is inhibitory for exponential growth (*2*). In both *B. subtilis* and *E. coli*, acetate is formed from acetyl CoA mainly *via* the phosphotransacetylase (Pta) [EC 2.3.1.8] and acetate kinase (Ack) [EC 2.7.2.1] pathway (Pta-Ack pathway), with the production of ATP (Fig. 1).

E. coli cells can utilize acetate as the sole carbon source. It is believed that two independent pathways are involved in the activation of acetate. One pathway is the Pta-Ack pathway, which is also used in acetate excretion, and the second one is catalyzed by acetyl CoA synthetase (Acs) [EC 6.2.1.1] (Fig. 1). It was proposed that the Pta-Ack pathway is used with high concentrations of acetate, and Acs is used with low concentrations of acetate (*3*). In *B. subtilis*, on the other hand, it appears that only AcsA is involved in the utilization of acetate (*4*), suggesting that different modes of regulation of acetate metabolism may be involved in *B. subtilis*. In addition to the role of the Pta-Ack pathway in acetate metabolism, acetyl phosphate has been implicated in the regulation of two-component signal transduction systems as a phosphoryl donor for response regulators, including CheY, PhoB, NtrC, and OmpR (*5-7*).

Despite the importance of the Pta-Ack pathway in metabolism and the signal transduction pathway, only limited information is available on the regulation of the Pta-Ack pathway. In *B. subtilis*, it has been found that the *ackA* and *acsA* genes are affected by a CcpA protein which was shown to be involved in the catabolite repression of many genes and operons (*8-10*). Pta has also been purified and characterized in *B. subtilis* (*11*), but no genetic approach has been taken as to regulation of the *pta* gene. Recently, the complete genome sequence of *B. subtilis* was reported (*12*), and now a sequence for *pta* is available in the genome database. But this *pta* gene was only defined by sequence homology and there is no evidence that this *pta*-like gene really encodes a Pta.

In this study, we report the cloning of a *pta* gene and the purification of its protein product from over-expressing *E. coli* cells. On analysis of the *pta* mutant, we concluded that the *pta* gene encodes a Pta. From the results of *pta-lacZ* fusion studies, we found that the transcription of the gene is activated by glucose, and that this activation is dependent on an intact *ccpA* gene and a cre sequence located in the *pta* upstream region.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The bacterial strains used in this study are described in Table I. *E. coli* DH5α was used for plasmid construction and for overexpression of the *pta* gene for purification of Pta. All *B. subtilis* strains were derivatives of JH642.

Media and Growth—LB medium (*13*) was routinely used

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

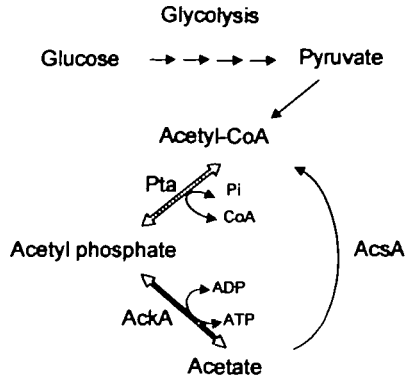

Fig. 1. **Pathways of acetate metabolism in *B. subtilis*.** The Pta-Ack pathway is shown by open arrows. Abbreviations: Pta, phosphotransacetylase; AckA, acetate kinase; AcsA; acetyl CoA synthetase.

TABLE I. **Bacterial strains used in this study.**

Strain	Genotype[a]	Source
JH642	*trpC2 pheA1*	BGSC
BS9844	*trpC2 pheA1 Δpta::erm*	This work
BS9853	*trpC2 pheA1 amyE::(pta-lacZ spc)*	This work
BS9854	*trpC2 pheA1 pta::(pta-lacZ spc)*	This work
BS9901	*trpC2 pheA1 ΔccpA::erm*	This work
BS9902	*trpC2 pheA1 ΔccpA::erm amyE::(pta-lacZ spc)*	This work
BS9903	*trpC2 pheA1 spo0A::erm amyE::(pta-lacZ spc)*	This work
BS9904	*trpC2 pheA1 spo0H::erm amyE::(pta-lacZ spc)*	This work
BS9905	*trpC2 pheA1 amyE::(pta*(PT1)*-lacZ spc)*	This work
BS9906	*trpC2 pheA1 amyE::(pta*(PT2)*-lacZ spc)*	This work
BS9907	*trpC2 pheA1 amyE::(pta*(PT3)*-lacZ spc)*	This work
BS9908	*trpC2 pheA1 Δpta::erm amyE::(pta-lacZ spc)*	This work

[a]In BS9903 and BS9904, the alleles of *spo0A::erm* and *spo0H::erm* came from strains MO699 and MO1614, respectively, which were obtained from P. Stragier.

TABLE II. **Oligonucleotide primers used in this study.**

Primers	Sequence (5′→3′)
CC1	GCGTCTAGATCGTACGAGCCCATACGA (*Xba*I)[a]
CC2	CGCGGATCCGAAAGAGGTCAGATCAAG (*Bam*HI)
CC3	CGCTTCTGTAGAAGAGCA
ER1	CGCGAATTCGTCGTTAAACCGTGTGCTCT (*Eco*RI)
ER2	CGCGGATCCTAGGTGTCACAAGACACTCT (*Bam*HI)
ER3	GCGCTGCAGGTCGTTAAACCGTGTGCTCT (*Pst*I)
LZ1	TTCCACAGTAGTTCACCA
PC1	TATGAAAGCTCTATAATGAA
PC2	GAAAGCGCAATAATGAAAG
PC3	GAAAGCGCTTTAATGAAAGT
PC4	AAGCGCTATCATGAAAGTTG
PT1	CGCTTCATTCGTCATTTGCT
PT2	TTCAGCACGTCGTAATACAA
PT3	ACATTGCACCTGACAGCC
PT4	TGCAGCATCAAATTGGAA
PT5	CGCGGATCCGGCAGATTTATTTTCAACAGTG (*Bam*HI)
PT6	GCGCTGCAGTTACAGTGCTTGCGCCGC (*Pst*I)
PT7	AGCAACCCGGTGAATTCA (*Eco*RI)
PT8	CGCGGATCCTGCAAGCTTGCTGACCGC (*Bam*HI)
PT11	TTTAACGTCTTTTCCAGCTAC
YW1	GACGAGGGAAAAGTGCTG

[a]Restriction sites used for subcloning are underlined.

for cultivating *E. coli* and *B. subtilis*. Difco sporulation medium (DSM) (*14*) was used for growth and maintenance of *B. subtilis*. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, spectinomycin at 100 μg/ml, and erythromycin and lincomycin at 1.0 and 25 μg/ml, respectively, for selection of the *erm* gene. Competent cells were obtained in Spizizen's medium as described by Albano *et al.* (*15*). To make DSMG, sterile 50% glucose was added, to a final concentration of 1.0%, to sterile DSM. TSS minimal medium (*16*) was made with 0.2% NH_4Cl as the nitrogen source. Casamino acids (Difco) were added to the TSS medium to 1.0% as a 10% sterile stock solution as indicated.

The growth rates of strains were measured using Bioscreen C (Labsystems, Finland). An inoculum was prepared by growing the cells on TSS (containing 1% casamino acids) agar plates at 30°C overnight with appropriate antibiotics. Then the cells were harvested by washing the plate surface with 1.0 ml of the same broth, and then liquid medium (without antibiotics) was inoculated to an initial absorbance level at 600 nm of <0.001. The increase in turbidity was monitored automatically every 10 min at 600 nm at 37°C. Data for determination of the growth rate were taken between A_{600} of 0.01 and 0.1. Analysis of data was performed using the SigmaPlot 4.0 program.

Chemicals—Restriction enzymes, T4 polynucleotide kinase, Expand™ reverse transcriptase, DNase-free RNase, malate dehydrogenase, and citrate synthase were purchased from Boehringer Mannheim. The substrates used in the Pta assay and molecular weight markers for gel filtration were purchased from Sigma. [γ-^{32}P]ATP was purchased from Amersham.

DNA Manipulations—The oligonucleotide primers used for PCR and site-directed mutagenesis were purchased from Genotech (Taejon, Korea) and are shown in Table II. Recombinant techniques, site-directed mutagenesis and DNA sequencing were performed by standard methods (*17*). *B. subtilis* chromosomal DNA was isolated as described by Cutting and Horn (*18*). Preparation and transformation of competent *B. subtilis* cells were performed as described by Albano *et al.* (*15*).

Purification of Pta—The open reading frame of a *pta* gene fragment was amplified by PCR using primers PT5 and PT6, and a 1.07-kb *Bam*HI-*Pst*I fragment obtained from the PCR product was cloned into plasmid pQE31 (Qiagen) so that six histidine codons were fused into the 5′ end of the *pta* gene, resulting in pQEpta. *E. coli* DH5α transformed with pQEpta was grown in 1 liter of LB medium containing 50 μg/ml of ampicillin. Then expression of *pta* was induced by the addition of 1 mM IPTG at the mid-exponential phase, and growth was continued for 14 h. Cells were harvested by centrifugation and resuspended in lysis buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF]. After incubation with lysozyme (final, 1 mg/ml) on ice for 1 h, the cells were disrupted by sonication, followed by clarification by centrifugation at 20,000×*g* for 30 min. The supernatant was mixed with 5 ml of a 50% suspension of Ni-NTA resin (Qiagen) previously equilibrated with the same buffer, followed by gentle stirring for 1 h at 4°C. The suspension was poured into a Spectra/Chrom™ disposable minicolumn (Spectrum) and washed with 10 ml of lysis buffer containing 20 mM imidazole. The protein was eluted with 76 mM imidazole in the lysis buffer. All steps were performed at room temperature unless otherwise indicated. Fractions were analyzed by SDS-PAGE. The purified protein was aliquoted and stored at −65°C. When crude lysates were

required, cells were harvested by centrifugation, washed with 0.1 M potassium phosphate (pH 7.6), and lysed by sonication. Cell debris was removed by centrifugation at 12,000 × *g* for 10 min.

Determination of the Native Molecular Weight—The native molecular weight of the purified protein was determined by gel filtration on a Sephacryl S-300-HR column (Pharmacia; 1.5 × 60 cm). The molecular mass markers used were as follows: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Enzyme Assays—The activity of Pta in the forward reaction (acetyl phosphate→acetyl CoA) was estimated by using the coupled reaction (*19*). The assay mixture contained 225 mM Tris-Cl (pH 7.8), 15 mM malic acid, 4.5 mM $MgCl_2$, 3.75 mM CoA, 22.5 mM NAD^+, 12 units of malate dehydrogenase, 1.1 units of citrate synthase, and 10 mM acetyl phosphate. The reaction was started by the addition of the enzyme solution, and the initial velocity was determined at 340 nm. The specific activity of Pta was expressed in U/mg. Protein concentrations in cell extracts were determined by the Bradford method (*20*), using a kit from BioRad, with bovine serum albumin as the standard.

Primer Extension Analysis—Total RNA isolated from JH642 cells, which had been grown in DSMG (DSM +1% glucose) until T_1, was subjected to primer extension. RNA was prepared using a High Pure RNA Isolation Kit (Boehringer Mannheim). A 21-mer primer (PT11), which is complementary to nucleotides +71 to +91, was used for cDNA synthesis. The primer was labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. 50 pmol of the labeled primer was mixed with 48 μg of RNA in 13 μl of distilled water. The mixture was heated at 65°C for 10 min and then immediately cooled on ice. Then the annealing mixture was mixed with 5 μl of 5 × reverse transcriptase buffer, 1 μl of a four deoxynucleotide mixture (20 nmol/μl of each deoxynucleotide), 2 μl of actinomycin D (0.5 mg/ml) (Sigma), 2 μl of DTT (100 mM), and 25 units of Expand™ reverse transcriptase. The reaction mixture was incubated at 42°C for 100 min. After the RNA had been digested with RNase, 8 μl of formamide stop buffer was added to the reaction mixture. Following that, the extended cDNA was analyzed by electrophoresis on a 6% sequencing gel. The DNA sequencing reaction was performed using the same oligonucleotide as the primer as the size standard.

Construction of a pta-lacZ Fusion—The *pta* upstream region, nucleotides −372 to +148 relative to the *pta* transcriptional start site, with flanking *Eco*RI and *Bam*HI restriction sites, was amplified with PCR using primers PT7 and PT8, and then cloned into the *spoVG-lacZ* fusion vector, pDG1728 (*21*), to generate pBS9853. The DNA sequence was determined to verify that no changes had occurred during the PCR amplification. The fusion construct was linearized with *Sca*I and then integrated into the *amyE* locus in a single copy by means of a double crossover, selecting for spectinomycin resistance. Integration into the *amyE* locus was confirmed by examining the amylase activity on an LB agar plate containing 1% soluble starch. The amylase activity was visualized by staining the plate with a staining solution comprising 0.2% potassium iodide and 0.1% iodine. For construction of isotopic integration of a *pta-lacZ* fusion, a 6.14-kb *Nsi*I-*Sma*I fragment bearing the *pta-lacZ* fusion and the *spc* gene conferring spectinomycin resistance was eluted from pBS9853 and then cloned into pGEM-7Zf(+) (Promega), generating pBS9854. The resulting plasmid was recombined into the chromosome by means of a single crossover, selecting for spectinomycin resistance, giving strain BS9854. Integration into the *pta* locus was confirmed by PCR using primers YW1 (complementary to downstream of the *ywfl* gene, which is located upstream of the *pta* gene, Fig. 3) and LZ1 (complementary to the 5′ end of the *spoVG-lacZ* fusion).

β-Galactosidase Assay—The inoculum for the β-galactosidase assay was prepared by growing cells on a DSM agar plate at 30°C overnight with appropriate antibiotics. The cells were then harvested by washing the plate surface with 1.0 ml of prewarmed DSM, and liquid medium was inoculated to an initial absorbance level at 600 nm of about 0.01. Cultures were grown at 37°C and 300 rpm, and growth was monitored by measuring A_{600}. When the absorbance (A_{600}) of the culture reached about 0.1 (approximately 1.5 h), samples were taken at appropriate intervals (30 min to 1 h) for the β-galactosidase assay. T_0 was defined as the end of exponential growth. The assays were performed with toluenized cells, as described by Nicholson and Setlow (*22*). The specific activity was expressed as Miller units (*13*).

Construction of a pta Null Mutant—To construct a *pta* mutant, a 1.44-kb DNA fragment containing the *pta* gene was amplified by PCR using primers PT1 and PT2, and then cloned into pGEM-T (Promega), giving pBSpta. Then, a deletion-insertion mutation of the *pta* gene was constructed by replacing the 0.18-kb *Bgl*II-*Eco*RI fragment of pBSpta with a 1.2-kb *Bam*HI-*Eco*RI fragment of the *erm* gene, cloned from pDG1728 (*21*) by PCR with primers ER1 and ER2. The resulting plasmid, pBS9844 (Fig. 3), was integrated into the chromosome by means of a double crossover, creating strain BS9844. The orientation of the *erm* gene in the resulting construct is opposite to that of the *pta* gene. Mutation of the *pta* gene was confirmed by PCR using PT3 and PT4, which were designed to detect the integration of the *erm* gene into *pta*. Chromosomal DNA isolated from the *pta* mutant (BS9844) was used as a template DNA for PCR.

Construction of a ccpA Null Mutant—To construct a *ccpA* mutant, a 2.08-kb DNA fragment containing the *ccpA* gene was amplified by PCR using primers CC1 and CC2, with flanking *Xba*I and *Bam*HI restriction sites, and then cloned into pGEM-7Zf(+), giving pBSccpA. Then, a deletion-insertion mutation of the *ccpA* gene was constructed by replacing the 0.21-kb *Cla*I-*Pst*I fragment of pBSccpA with a 1.1-kb *Cla*I-*Pst*I fragment of the *erm* gene, cloned from pDG1728 by PCR with primers ER2 and ER3. The resulting plasmid, pBS9901 (Fig. 3), was integrated into the chromosome by means of a double crossover, creating strain BS9901. Mutation of the *ccpA* gene was confirmed by PCR using CC3 (complementary to just upstream of the *Cla*I site located in the *ccpA* gene) and ER3 (complementary to the 5′ end of the *erm* gene), which were designed to detect the integration of the *erm* gene into *ccpA*. Chromosomal DNA isolated from a *ccpA* mutant (BS9901) was used as a template DNA for PCR.

Sporulation Assay—Cells were cultured in DSM at 37°C until approximately 20 h after the end of exponential growth. Serial dilutions were made in 10 mM potassium phosphate buffer (pH 7.4) containing 50 mM KCl and 1 mM $MgSO_4$, and then plated on LB agar (for JH642) or DSM

(for *pta* mutant BS9844) before and after heat treatment (80°C for 10 min). We used DSM agar plates for counting colonies in the case of the *pta* mutant (BS9844) because the growth rate of this strain in LB medium was extremely low. The sporulation frequency is the ratio of spores per milliliter to viable cells per milliliter.

Site-Directed Mutagenesis—For *in vitro* site-directed mutagenesis of the cre region, an Altered Sites® II *in vitro* Mutagenesis System (Promega) was used. A 0.52-kb *Eco*RI-*Bam*HI fragment from pBS9853, which was used for construction of the *lacZ* fusion, was inserted into the pALTER-1 vector and then mutagenesis was performed following the instructions of the manufacturer. All mutations were confirmed by DNA sequencing. The oligonucleotides directing mutations are shown in Table II.

RESULTS AND DISCUSSION

Purification of Pta—The putative *pta* gene in *B. subtilis* encodes a protein of 323 amino acid residues with a calculated molecular mass of 34,758 Da. To determine whether or not the putative *pta* open reading frame actually encodes a protein that has Pta activity, we cloned the *pta* gene by PCR and purified the protein from over-expressing *E. coli* cells as an N-terminal His_6 tag. The purified protein gave a single protein band when analyzed by SDS-PAGE, corresponding to a molecular mass of 36 kDa, which is close to the calculated molecular mass of 34,758 Da (Fig. 2A). The activity of the protein was determined in the forward reaction, *i.e.* towards acetyl CoA formation, using a coupled reaction, as described under "MATERIALS AND METHODS." The protein showed specific activity of 1,150 units/mg, indicating that this open reading frame actually encodes a Pta. The Pta of *B. subtilis* was previously isolated and characterized by Rado and Hoch (*11*), and the molecular mass of the purified protein was found to be 90 kDa. Since the molecular mass of the subunit was found to be 36 kDa, it was not easy to determine the oligomeric state of the intact enzyme. Thus we performed gel filtration chromatography again with the purified protein, the estimated molecular mass being 76,000 Da (Fig. 2B), indicating that the Pta of *B. subtilis* probably exists as a dimer under our experimental conditions.

Characterization of the pta Mutant—In an effort to determine the *in vivo* function of the *pta* in *B. subtilis*, we constructed a mutant in which a 0.18-kb internal fragment of the gene was replaced by the *erm* gene (Fig. 3), resulting in strain BS9844 (Table I). To test for Pta activity, we grew the wild-type (JH642) and *pta* mutant (BS9844) at 37°C in DSMG until 1 h after the end of the exponential phase (T_1). Crude lysates were prepared by sonication of cells and centrifugation to remove cellular debris, and then assayed for the ability to convert acetyl phosphate to acetyl CoA through coupled reactions. While the wild-type strain (JH642) showed 16 units of enzymatic activity per mg of protein, the *pta* mutant (BS9844) exhibited no detectable activity, indicating that strain BS9844 is deficient in the Pta. This result also suggests that only one copy of the gene

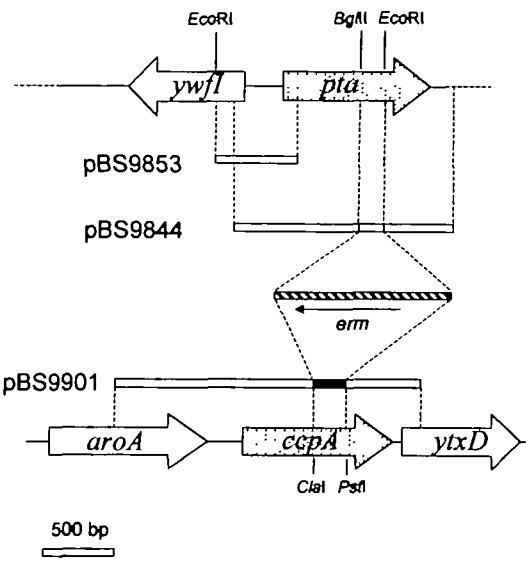

Fig. 3. **Genetic map of the *pta* (top) and *ccpA* (bottom) region, and plasmids carrying different parts of this region.** The arrows indicate the coding regions of the genes. Only relevant restriction sites used in some constructions are indicated. The construction of plasmids is described under "MATERIALS AND METHODS." The location and direction of the *erm* gene are also indicated.

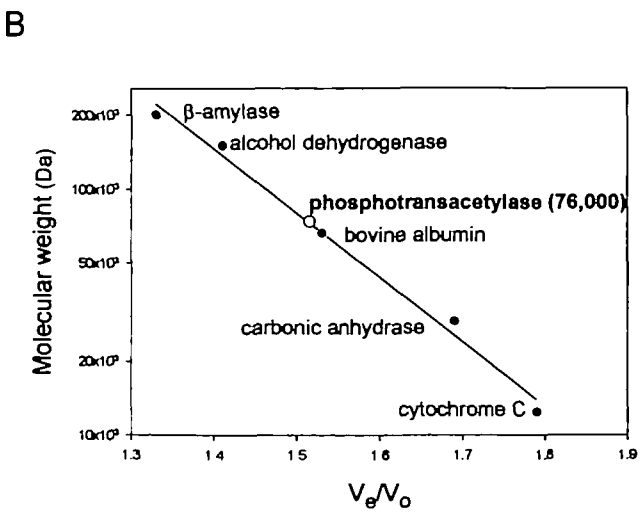

Fig. 2. **Purification of Pta and gel permeation chromatography.** (A) Purification of Pta was performed by Ni-NTA column chromatography. The SDS-PAGE results are shown for a soluble extract (lane 1), the flowthrough fraction from the column (lane 2), and the fraction eluted with 76 mM imidazole (lane 3), which was used for further work. The molecular mass standards (lane 4), in kilodaltons, were, from top to bottom, 66, 55, 36, 29, 24, 20, and 14.2. (B) The native molecular weight of the purified Pta was determined by gel filtration on a Sephacryl S-300-HR column.

showing Pta activity is present on the chromosome of *B. subtilis*.

The ability of the *pta* mutant cells to produce spores was also examined. Both the wild-type (JH642) and mutant (BS9844) were grown in DSM until approximately T_{20}, and then the sporulation frequency was determined from the percentage of colonies that survived heat treatment. Strain BS9844 sporulated at a frequency of 92% while the wild-type cells sporulated at a frequency of 76%, indicating that insertional inactivation of the *pta* did not have a negative effect on sporulation in DSM. In *E. coli*, it was shown that loss of the *pta* gene function results in defective survival on glucose starvation, and it was speculated that this impaired ability might be due to the absence of acetyl phosphate, because the *ackA* mutant is normal as to the ability to survive glucose starvation (*23*). If we consider sporulation as a kind of survival process caused by nutrient deprivation, it is interesting that the *pta* mutant of *B. subtilis* exhibits a normal (or even higher) sporulation frequency.

Generally, the *pta* mutant (BS9844) exhibited good growth in DSM and TSS (containing 1% casamino acids) minimal medium, although the growth rates were somewhat lower than those of JH642. A previous study by Grundy *et al.* (*8*) showed that the addition of 1% glucose to TSS minimal medium (with 1% casamino acids) greatly inhibited growth of the *ackA* mutant. In the case of the *pta* mutant, however, the addition of 1% glucose (or fructose) to the TSS minimal medium increased the growth rate of strain BS9844, although the growth stimulation was not as remarkable as for the wild type (Table III). Interestingly, LB medium could not support the vegetative growth of strain BS9844.

Transcript Mapping—Primer extension analysis was performed using primer PT11, which is complementary to nucleotides +71 to +91 (Fig. 5), to determine the putative transcription start point of the *pta*. The major 5′ end of the mRNA was identified at 37 nucleotides upstream of the *pta* start codon (Fig. 4). Sequences similar to those of the −35 and −10 regions of a sigma-A-dependent promoter could be identified just upstream of the *pta* transcription start point (Fig. 5). The TG motif of the −16 region, which is found in a large number of Gram-positive bacterial promoters (*24*), was absent in the *pta* promoter.

Regulation of pta Transcription—In many bacterial species, the genes for *pta* and *ack* are close to each other on the chromosome, and it seems that these two genes are co-transcribed. The close arrangement of the *pta* and *ack* genes has been detected in *Clostridium acetobutylicum* (*25*), *E. coli* (*26*), *Methanosarcina thermophila* (*27*), and *Sinorhizobium meliloti* (*28*). However, in *B. subtilis*, the *pta* and *ackA* genes are separated by about 848-kb on the chromosome, suggesting that a different mode of regulation may be involved for each gene. To study *pta* gene expression, the upstream region of the *pta* gene bearing the 5′ end of the *pta* gene and the promoter region was cloned in front of the promoterless *spoVG-lacZ* fusion in pDG1728. The resulting plasmid, pBS9853, was linearized and integrated

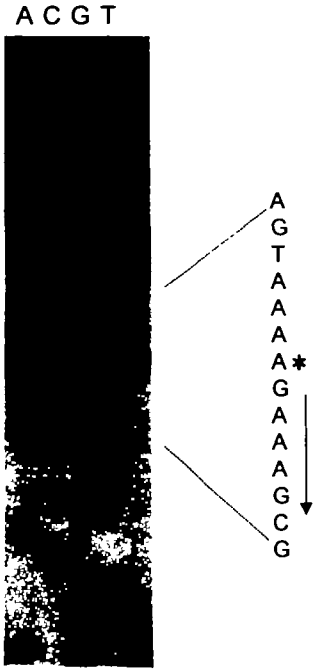

Fig. 4. **Determination of the transcriptional start site for the *pta* gene by primer extension analysis.** Total RNA was purified from *B. subtilis* JH642 cells grown in DSM containing 1% glucose. The DNA sequence of the complementary strand obtained through the sequencing reaction with PT11 as a primer is shown on the right. The direction of transcription is shown by the arrow, and the position of a start site is indicated by an asterisk.

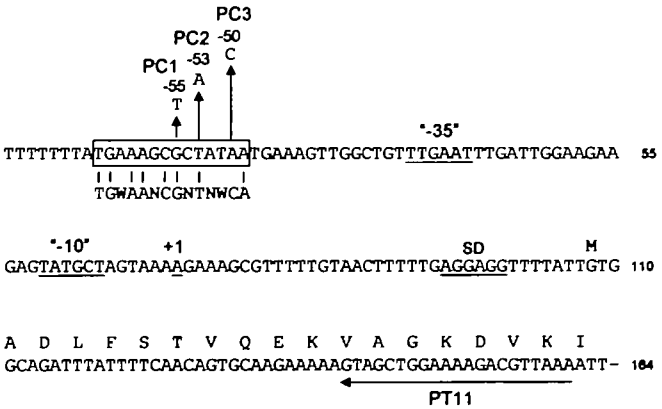

Fig. 5. **DNA sequence of the *pta* upstream region.** The putative ribosome binding site (SD), "−35" and "−10" regions, and transcriptional start site (+1) are underlined. The positions corresponding to PT11 used for primer extension are indicated by an arrow. The putative cre sequence is boxed. Vertical lines indicate matches between the *pta* cre sequence and the *amyO* consensus sequence (*31*). The positions of mutations are also shown on the cre sequence.

TABLE III. **Growth of *B. subtilis* strains in TSS minimal medium.**

	Generation time[a] of strains in[b]		
	TSS	TSSG	TSSF
JH642	33.5	25.9	23.1
BS9844 (JH642 *Δpta::erm*)	40.8	33.8	37.4

[a]The generation time was calculated from the specific growth rate measured with an automatic turbidometer, Bioscreen C (see "MATERIALS AND METHODS"). The data for each medium were from a representative experiment, and similar results could be obtained in at least three independent experiments. [b]TSS, TSS minimal medium containing 0.2% NH_4Cl and 1% casamino acids; TSSG, TSS plus 1% glucose; TSSF, TSS plus 1% fructose.

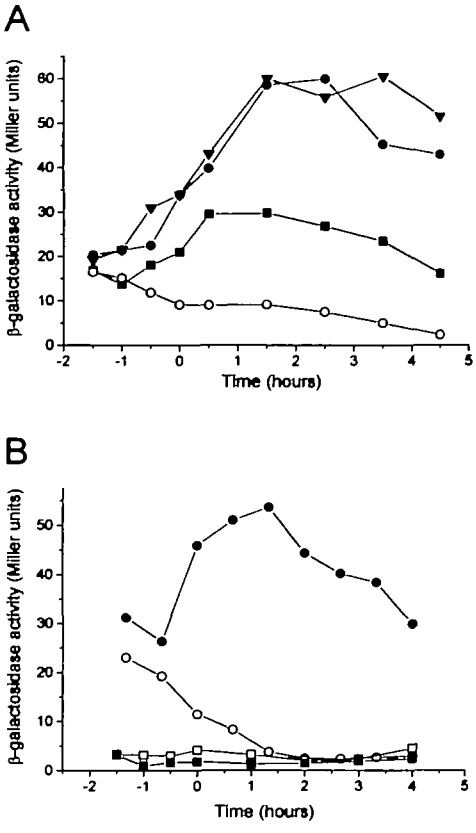

Fig. 6. **Expression of β-galactosidase from the *pta-lacZ* fusion in cells grown in DSM.** (A) The wild type (BS9853) strain was grown in DSM (open circles), and DSM containing 1% glucose (solid circles), 1% fructose (solid triangles), or 2% glycerol (solid squares). (B) Effect of the *ccpA* mutation (squares) compared to the wild type strain (circles). Cells were grown in DSM (open symbols), and DSM containing 1% glucose (solid symbols). (C) Effect of the cre mutations on the expression of the *pta-lacZ* fusion. The mutant strains, bearing PC1 (squares), PC2 (triangles), or PC3 (circles), were grown in DSM (open symbols), and DSM containing 1% glucose (solid symbols). Wild type controls are not shown for clarity of presentation. Time zero is the onset of the stationary phase.

at the *amyE* locus by double-crossover recombination, giving strain BS9853. Expression of the *pta-lacZ* fusion in strain BS9853 was monitored during growth in DSM. As shown in Fig. 6A, *pta-lacZ* expression decreased continuously during the entire growth stage, decreasing to about 5 Miller units between T_3 and T_4. However, the addition of glucose to a final concentration of 1% as to DSM significantly induced the *pta-lacZ* expression as the cells entered the stationary phase (T_0), a maximum level (about 60 Miller units) being reached around T_1. A similar induction pattern was observed when 1% fructose or 2% glycerol was added to DSM, although the addition of glycerol resulted in weaker induction than that of fructose or glucose (Fig. 6A). The same *pta-lacZ* fusion was also integrated isotopically into the original *pta* locus (see "MATERIALS AND METHODS"), and a similar induction pattern of transcription was observed. Mutations in *spo0A* or *spo0H* also had little or no effect on the induction pattern of *pta-lacZ* expression indicating that Spo0A or Spo0H does not appear to play a role in the regulation of *pta* expression (data not shown).

In order to examine the possibility that CcpA, which has been shown to act as either a repressor or an activator of gene expression (*29*), also might be responsible for the activation of *pta-lacZ* expression, a fusion construct was introduced into a *ccpA* mutant strain (BS9901), giving strain BS9902. Expression of the *pta-lacZ* fusion in strain BS9902 was not activated by the addition of glucose unlike in the case of the wild type (Fig. 6B), suggesting that the CcpA protein is critically involved in the induction of the *pta-lacZ* fusion by glucose.

Identification and Mutagenesis of the cre-Like Sequence—Since it appeared that glucose-induced *pta-lacZ* expression required the CcpA protein, we searched for *cis*-acting sequences called catabolite-responsive element (cre), which has been functionally identified in a number of genes or operons affected by a CcpA (*30*). A putative cre sequence, located upstream of the tentative promoter site, at positions −49 to −62, was identified (Fig. 5). This cre-like sequence is very similar to the consensus sequence originally proposed by Weickert and Chambliss (*31*) based on the results of mutational analyses of the catabolite-repression-mediating sequence in *amyE*. To examine the functional role of the cre-like sequence located in the *pta* upstream region, site-directed mutagenesis was performed. We made three different single-base changes in the cre-like sequence: PC1 (−55G to T), PC2 (−53T to A), and PC3 (−50A to C) (Fig. 5). To analyze the effects of the mutations on *pta* expression, *pta-lacZ* fusions containing a mutagenized cre-like sequence were constructed and integrated into the chromosome as a single copy at the *amyE* locus. As shown in Fig. 6C, the *pta-lacZ* expression of strain BS9905 bearing the PC1 mutation was not activated by the addition of glucose to DSM. Glucose induction of the *pta-lacZ* fusion in strain BS9905 was comparable to that in the *ccpA* mutant (BS9902) (Fig. 6B). The PC2 mutation also abolished the *pta-lacZ* activation by glucose, though the basal level of β-galactosidase activity was higher than that of strain BS9905 bearing the PC1 mutation (Fig. 6C). On the other hand, the *pta-lacZ* expression was still activated by the addition of glucose in strain BS9906 bearing the PC3 mutation. This result was expected because the PC3 mutation was designed to increase the homology to the consensus sequence of the cre sequence (*31*) (Fig. 5). All of these results suggest that the cre sequence in the *pta* upstream region functions as an active *cis*-element for glucose-induced activation of the *pta* gene, which is mediated by CcpA.

Although the *pta* and *ackA* genes are located separately on *B. subtilis* chromosomal DNA, it seems that they share a common mechanism for transcriptional regulation. The following observations support this conclusion: (i) The transcription of both the *pta* and *ackA* genes is activated in the presence of excess glucose (*8*). (ii) The cre sequences are present in the upstream region of the promoter, and this element was found to be indispensible for transcriptional activation mediated by glucose (*9*). (iii) The transcriptional

activation of both genes was abolished in the *ccpA* mutant (*8*). All of these results indicate that these two genes are positively regulated by the CcpA protein with excess glucose. Since the catabolite repression of the *acsA* gene is also mediated by CcpA (*10*), it is very interesting that all three genes, *i.e. pta, ackA*, and *acsA*, for interconversion between acetyl CoA and acetate are regulated by the CcpA protein. It has been shown that a number of genes encoding carbon catabolic enzymes and some of the genes encoding enzymes of the central metabolic pathway contain the cre sequence (*32*), suggesting that global regulation of carbon flux is mediated by the CcpA protein in *B. subtilis*. Like the CcpA protein, *E. coli* Cra is also a member of the LacI-GalR family of transcription factors, and represses the carbon catabolic pathway and activates the carbon anabolic pathway (*33*). Thus the protein structure and physiological role of the Cra protein may be homologous to those of CcpA, although the mechanism of sensory transduction for activation or repression is different in each case (*33*).

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