Discovery of a **ptsHl** operon, which includes a third gene (**ptsT**), in the thermophile **Bacillus steaothermophilus**

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The discovery of a **ptsHl** operon in **Bacillus steaothermophilus** XL-65-6 coupled with our previous report of a **cel** operon (Lai & Ingram, *J Bacteriol* 175, 6441-6450, 1993) demonstrates that this thermophilic organism contains all of the genes required for cellobiose uptake by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Genes encoding the two general PTS proteins, HPr (**ptsH**) and enzyme I (**ptsI**), were cloned and sequenced. These form an operon which includes a third small gene (**ptsT**) of unknown function (encoded product Mr 18428). Both **ptsH** and **ptsI** were expressed at high levels from a single plasmid in *Escherichia coli* and complemented corresponding host mutations. Although the translated sequences for these genes were similar to homologues from Gram-positive mesophiles (64-77% identity), the *B. steaothermophilus* gene products were unusual in having a higher predicted pl and fewer negatively charged amino acid residues. Enzyme I also contained more alanine and leucine than mesophilic counterparts. Interestingly, **ptsT** inhibited the growth of *E. coli* ptsI mutants at 37°C. No such inhibition was observed during incubation at a lower temperature (30°C) or in *E. coli* DH5α, which is wild-type for **ptsI**. The predicted translation product from **ptsT** contained a high proportion of basic amino acids (27%) and had a high predicted pl (pH 11.7), properties similar to bacterial histone-like proteins, but did not exhibit homology to any sequences in the current database. Regions upstream and downstream from the **ptsHl** operon contain genes with homology to *Bacillus subtilis* **ptsG** and **wapA** (wall-associated protein), respectively.

**Key words:** *Bacillus steaothermophilus*, HPr, enzyme I, thermophile, phosphoenolpyruvate-dependent phosphotransferase system

**INTRODUCTION**

The phosphoenolpyruvate-dependent phosphotransferase system (PTS) and associated regulatory functions have been extensively characterized in mesophilic bacteria (Postma *et al.*, 1993; Reizer *et al.*, 1993; Saier & Reizer, 1992), though they are essentially unknown in thermophiles. Early evidence for a PTS in a thermophile was provided by Patni & Alexander (1971) with *Clostridium thermocellum*. This was subsequently refuted by Nochur *et al.* (1992), who demonstrated that ATP rather than phosphoenolpyruvate serves as the phosphate donor for a variety of sugars in this organism. Recently, our laboratory cloned and sequenced a cellobiose-specific PTS operon from *Bacillus steaothermophilus* XL-65-6 (Lai & Ingram, 1993). Proteins encoded by this operon are homologous to those from the cryptic PTS **cel** operon in *Escherichia coli* (Parker & Hall, 1990). Recombinant strains of *E. coli* harbouring the *B. steaothermophilus* **cel** operon hydrolysed p-nitrophenyl and methylumbelliferyl analogues of cellobiose and cellotriose provided that the *E. coli* host contained functional genes for the PTS general proteins, HPr (histidine-containing protein; encoded by **ptsH**), and EI (enzyme I; encoded by **ptsI**). However, to our knowledge, no previous studies have demonstrated the presence of these PTS proteins in *B. steaothermophilus* or any other thermophile.
In mesophilic bacteria, HPt and EI serve as essential phosphocarrier proteins linking phosphoenolpyruvate to sugar transport and phosphorylation (Postma et al., 1993; Saier & Reizer, 1992). The \textit{ptsH} and \textit{ptsI} genes are typically found together within an operon which may include an additional gene. Variations have been reported in which general protein functions are supplied as domains within a fructose-specific PTS gene or HPt is encoded on a separate operon (Zhu et al., 1993). Although the amino acid sequences of HPt and EI are highly conserved, distinct differences were noted between Gram-positive and Gram-negative mesophilic bacteria (Postma et al., 1993). The three-dimensional structures of HPt from \textit{E. coli} and from \textit{Bacillus subtilis} have been determined, and both enzymes share a similar pattern of folding (Chen et al., 1989).

In this paper, we report the cloning and sequencing of the \textit{ptsH}, \textit{ptsI} and \textit{ptsT} genes from a thermophilic bacterium, \textit{B. stearothermophilus} XL-65-6, and the hyperexpression of \textit{ptsHI} in \textit{E. coli}.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used in this study are listed in Table 1. Luria broth (or agar) was used for growth and maintenance of \textit{E. coli} (Sambrook et al., 1989). Ampicillin (50 \mu g ml\(^{-1}\)) was added as appropriate for selection. DiCo MacConkey agar base plus 1% (w/v) carbohydrate was used to test carbohydrate utilization. Complementation of \textit{E. coli} ptsI mutations was evaluated using MacConkey agar containing each of five PTS carbohydrates (fructose, glucose, mannose, mannitol or sorbitol). Sorbitol-MacConkey agar was used to test functional complementation of the \textit{ptsH} mutation in \textit{E. coli} 1101.

**DNA manipulation.** Standard procedures were used for the construction, isolation and analysis of plasmids (Sambrook et al., 1989). Commercial restriction enzymes were used as recommended by each manufacturer.

**Cloning of the \textit{B. stearothermophilus} \textit{ptsHI} operon.** Clones containing \textit{B. stearothermophilus} XL-65-6 \textit{ptsH} and \textit{ptsI} genes were isolated by transforming an \textit{E. coli} \textit{ptsI} mutant strain MM6 (Fraenkel et al., 1964), with a previously constructed pUC18-based genomic library (Lai & Ingram, 1993). Transformants were screened for the utilization of PTS sugar by plating on fructose-MacConkey agar. Deep red colonies were selected after overnight incubation at 30 °C and purified for further investigation. \textit{E. coli} DH5\(\alpha\) was used as the host for subcloning experiments.

**Southern hybridization.** Restriction endonuclease digests of genomic DNA from \textit{B. stearothermophilus} XL-65-6 were prepared using \textit{ClaI}, \textit{EcoRI}, \textit{SalI} and \textit{SmaI}. Fragments were separated by agarose gel electrophoresis, blotted with Zeta-Probe GT membranes (Bio-Rad), and probed. The probe was labelled with digoxigenin (Genius System; Boehringer Mannheim), hybridized at 55 °C, and developed as recommended by the manufacturer. Genomic DNA from \textit{E. coli} DH5\(\alpha\) was digested with \textit{PstI} and served as a negative control.

**DNA sequencing and sequence analysis.** Plasmid DNA was purified using Wizard Miniprep columns (Promega). Sequencing was performed by the dideoxy method of Sanger et al. (1977) using fluorescent primers (forward, 5'-CACGACGT-TGTTAAAACGAC-3'; reverse, 5'-ATAACAATTTCACACAGG-3') from LI-COR and Sequenase with 7-deaza-dGTP nucleotides (USB). Primers were annealed to template DNA at 42 °C for 30 min. Extension reactions were incubated at 37 °C for 3 min and the products of the reactions were separated and analysed using a LI-COR model 4000 DNA sequencer. Regions of compression were resolved by using the TaqTrack Sequencing System, Deaza (Promega). Nucleotide and deduced amino acid sequences were analysed using the Wisconsin Genetics Computer Group sequence analysis software package, (Devereux et al., 1984) and the National Center for Biotechnology Information with the BLAST network service (Benson et al., 1993).

**SDS-PAGE.** Cultures of \textit{E. coli} JLT2 harbouring recombinant plasmids were grown overnight in Luria broth. Cells were harvested by centrifugation and washed twice with 10 mM

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**Table 1. \textit{E. coli} strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genetic characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli} DH5(\alpha)</td>
<td>F(^-) ΔlacZM15 recA</td>
<td>BRL*</td>
</tr>
<tr>
<td>MM6</td>
<td>lacI22 delBJ ptsl2 relA1 thi-1 spoT1</td>
<td>Fraenkel et al. (1964)</td>
</tr>
<tr>
<td>1101</td>
<td>bist-4 ptsh1 relA1 spoT1 thi-1 bgIR11</td>
<td>Fox &amp; Wilson (1968)</td>
</tr>
<tr>
<td>JLT2</td>
<td>F(^-) recA13 ptsl</td>
<td>Titgemeyer (1986)</td>
</tr>
<tr>
<td>BL21</td>
<td>ΔptsI</td>
<td>F. Chauvin &amp; S. Roseman†</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>bla amp lacI(^Z)*</td>
<td>BRL*</td>
</tr>
<tr>
<td>pLOI1801</td>
<td>pUC18 containing \textit{ptsG} \textit{ptsHI} \textit{wupA}</td>
<td>This study</td>
</tr>
<tr>
<td>pLOI1803</td>
<td>pUC18 containing \textit{ptsI}</td>
<td>This study</td>
</tr>
<tr>
<td>pLOI1804</td>
<td>pUC18 containing \textit{ptsH}</td>
<td>This study</td>
</tr>
<tr>
<td>pLOI1805</td>
<td>pUC18 containing \textit{ptsG} \textit{ptsHI}</td>
<td>This study</td>
</tr>
<tr>
<td>pLOI1806</td>
<td>pUC18 containing \textit{ptsHI} \textit{wupA}</td>
<td>This study</td>
</tr>
<tr>
<td>pLOI1807</td>
<td>pLOI1806 with mutated \textit{ptsH}</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Bethesda Research Laboratories.
† Department of Biology, Johns Hopkins University, Baltimore, MD, USA.
‡ Incomplete lacI and incomplete lacZ.
sodium phosphate buffer (pH 7.0). Pellets were resuspended in an equal volume of this buffer and disrupted by two passes through a French pressure cell at 20000 p.s.i. (138 MPa). Cell debris was removed by centrifugation (13000 g, 10 min). Nucleic acid was digested by adding CaCl₂ (to 2 mM) and 50 µg ml⁻¹ DNase (1 h, 22 °C). Denatured proteins were separated by SDS-PAGE (Schägger & von Jagow, 1987) and stained with Coomassie Blue.

RESULTS AND DISCUSSION

Cloning of the ptsHI operon from B. stearothermophilus XL-65-6

Clones containing the XL-65-6 ptsI gene were readily isolated by functional complementation of a ptsI mutation in E. coli MM6 provided that the selection plates were incubated at 30 °C. Initial attempts using MacConkey agar with ampicillin and glucose or fructose were not successful when incubated at 37 °C. Six positive clones were examined and all restored the ability of MM6 to utilize glucose, fructose, mannose, mannitol and sorbitol at 30 °C. These recombinants also formed red colonies at 37 °C, indicating complementation, but growth was severely limited. Based upon an analysis of restriction sites, plasmids in five clones appeared identical (6.4 kbp fragment) and one of these, denoted pLOI1800, was selected for further study (Fig. 1). Plasmid pLOI1800 also contains a ptsH gene. Transformation of an E. coli ptsH mutant (strain 1101) with pLOI1800 restored PTS function.

A series of subclones was constructed to localize the pts genes (Fig. 1). Plasmid pLOI1801 was constructed by deleting the BamHI to SaeI (polylinker region) fragment and retained both EI and HPr functions. Both functions were lost after deletion of the PstI (polylinker region) to Spbi fragment (pLOI1802). Plasmid pLOI1803, an exonuclease III deletion of pLOI1800, retained both functions, and both were also retained after deletion of the terminal SaI (one SaI site in the polylinker region) fragment (pLOI1806). By making a similar SaI deletion in pLOI1803 to produce pLOI1804, the coding regions for HPr and EI were localized to a 2.3 kbp region. Only the HPr function was retained after deleting 5 kbp from pLOI1800 using exonuclease III (pLOI1805).

Southern analysis confirmed that the cloned DNA was derived from B. stearothermophilus XL-65-6. A probe prepared from the internal 1.3 kbp EcoRI fragment spanning ptsHI (Fig. 1) hybridized to a single band in each digestion of XL-65-6 genomic DNA but did not hybridize to E. coli DNA (data not shown). These results confirmed XL-65-6 as the source of the cloned gene and are consistent with the presence of a single chromosomal copy.

DNA sequence analysis and gene identification

The 5211 bp region in pLOI1801 was sequenced in both directions by a combination of nested deletions and overlapping subclones (Fig. 2). Five ORFs, four of which

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**Fig. 1.** Restriction map of B. stearothermophilus DNA fragment in pLOI1800 and subclones. Plasmid pLOI1800 contains an MboI fragment which has been cloned into the BamHI site of pUC18. Polylinker sites on either side are not shown. The arrows indicate the direction (but not the location) of transcription from the lacZ (within vector) and native B. stearothermophilus promoters. The hatched area corresponds to the ptsHI operon. Subclones are aligned beneath the corresponding sites in pLOI1800. The position of the frame-shift mutation in pLOI1807 is indicated by an arrowhead. The ability of constructs to complement the ptsH mutation in E. coli 1101 and the ptsI mutation in E. coli MM6 are indicated on the right: +, positive reaction (complementation); -/+ weak positive reaction (light pink colonies); -, negative reaction (lack of complementation).
were complete, were found in the sense strand. All appeared to be transcribed in the opposite direction to that of the lac promoter on the vector (Fig. 1). The central region in pLO1801 contained three genes, two of which were readily identified as ptsH and ptsI by comparisons of translated sequences. The flanking ORFs were provisionally identified by amino acid homology to known genes from B. subtilis and corresponding gene designations were retained. The translated upstream incomplete ORF (324 amino acids) is similar to the carboxy-terminus of glucose-
Table 2. Characteristics of the ptsHI operon from B. stearothermophilus

<table>
<thead>
<tr>
<th></th>
<th>Nucleotides</th>
<th>G + C (mol%)</th>
<th>Total amino acids</th>
<th>Mr</th>
<th>Charge</th>
<th>Predicted PI</th>
</tr>
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<tbody>
<tr>
<td>HPr</td>
<td>267</td>
<td>50.2</td>
<td>88</td>
<td>9166</td>
<td>-1</td>
<td>5.9</td>
</tr>
<tr>
<td>EI</td>
<td>1737</td>
<td>54.7</td>
<td>578</td>
<td>63470</td>
<td>-23</td>
<td>4.9</td>
</tr>
<tr>
<td>PTST</td>
<td>468</td>
<td>55.8</td>
<td>155</td>
<td>18428</td>
<td>15</td>
<td>11.7</td>
</tr>
</tbody>
</table>

The ptsHI operon

The three central ORFs in pLOI1800 appear to form an operon. A putative promoter is located 36 bp upstream of the start codon for ptsH. This region resembles the consensus sequence for B. subtilis σ^70, which is primarily associated with constitutive housekeeping genes (Moran, 1993). The sequence 11 bp upstream of the ptsH coding region is similar to the 3' end of 16S RNA from B. stearothermophilus (Van Chardorp et al., 1981) and is nearly identical to the RBSs for B. subtilis ptsH (Gonzy-Treboul et al., 1989) and bgI (Murphy et al., 1984). The Shine-Dalgarno region for ptsI is located within the carboxy-terminus of ptsH. The ATG start codon for ptsT overlaps the TAA stop codon for ptsH as previously reported for the ptsHI operon in B. subtilis (Gonzy-Treboul et al., 1989). A third ORF, designated ptsT, begins immediately adjacent to the stop codon of ptsI but it lacks an obvious RBS.

The ptsH gene encodes a peptide containing 88 amino acids with a calculated Mr of 9166. Based on sequence comparisons in the GenBank database, this translated sequence is more similar to HPrs from other Gram-positive bacteria (65–71% identity) than to HPrs from other Gram-negative bacteria (32–42%).

The ptsI gene encodes 578 amino acids (Mr, 63470). Although this gene encodes a protein which is very similar to EI from B. subtilis (76.5% identity), the translated sequence for B. stearothermophilus ptsI contains considerably less serine and threonine, with correspondingly higher levels of alanine and leucine. This shift in amino acid composition may be related to thermal stability (Mozhaev & Martinek, 1984). As with ptsH, the translated amino acid sequence of ptsI from B. stearothermophilus is more similar to enzyme I proteins from other Gram-positive bacteria (64–77% identity) than to those from Gram-negative bacteria (40–50% identity).

The ptsT gene potentially encodes 155 amino acids. The translated sequence for this gene was not similar to any entries in the GenBank database, although the size and the high proportion of basic amino acids (Arg, Lys and His comprise 27%) resemble bacterial histone-like proteins (Drlica & Rouviere-Yaniv, 1987). The ptsT gene was not essential for functional complementation of E. coli strains containing mutations in either ptsH or ptsI. No third gene is present immediately adjacent to ptsI in other Gram-positive bacteria although a third ORF which is much further downstream in the B. subtilis ptsHI operon has been reported (Reizer et al., 1993).

The general characteristics of the ptsI, ptsI and ptsT products are listed in Table 2. The predicted pIs for HPr and EI were 5.9 and 4.9, respectively, higher than those of mesophilic Gram-positive homologues, which had pI values ranging from pI 4.15 to pH 4.6 for HPrs, and from pH 4.40 to pH 4.63 for Els. Both B. stearothermophilus HPr and EI had fewer negatively charged amino acid residues than other Gram-positive homologues. The predicted pI for the ptsT product was unusually high, pH 11.7, due to the high proportion of basic amino acids.

The discovery of a ptsHI operon in B. stearothermophilus coupled with our previous report of a PTS cel operon (Lai & Ingram, 1993) demonstrates that this organism contains the essential genes for cellobiose uptake by the PTS and for intracellular cleavage of the phosphorylated product.

Temperature-inducible hyperexpression of ptsHI in E. coli

Expression of the ptsHI operon in E. coli JLT2 (ptsI mutant) was examined in cells grown in Luria broth at 30 °C and 37 °C using denaturing gels (Fig. 3). Two new protein bands were observed in lanes containing extracts of JLT2(pLOI1801) and JLT2(pLOI1804) which correspond in size to HPr and EI, apparent Mr 10000 and 6400, respectively (Fig. 3). No new bands were observed which corresponded to the ptsT gene product. However, expression of this gene at a modest level may be obscured by native proteins of similar size. With both pLOI1801 and pLOI1804, HPr and EI levels were estimated to be at least fivefold higher in cells grown at 37 °C than in cells grown at 30 °C. Since ptsH and ptsI are expressed from a native promoter which has evolved to function at elevated conditions, the expression of the operon is subjected to temperature regulatory mechanisms within the cell. The expression of the operon at 37 °C is likely to be sufficient to mediate the increased uptake of cellobiose.
temperatures, the increase in expression at 37 °C may reflect increased transcriptional initiation by *E. coli* RNA polymerase at this higher temperature, or simply a more rapid cell growth.

**Effects of a frame-shift mutation in ptsH on expression in *E. coli***

A frame-shift mutation in *ptsH* was constructed by inserting a *SacI* linker (pCGAGCTCG) into the *HpaI* site of *ptsH* in plasmid pLOI1806 (Fig. 1). In the resulting plasmid (pLOI1807), the carboxy-terminal 12 amino acids of HPt were replaced with a new 45 amino acid segment which terminated within the *pts* coding region. After transformation into *E. coli* strains 1101 (*ptsH* mutant) and MM6 (*ptsI* mutant), recombinants were tested for PTS function on carbohydrate-MacConkey agar plates. Strain 1101(pLOI1807) was negative, as expected. MM6(pLOI1807) exhibited weak EI activity at 37 °C (light pink colonies) but was negative at 30 °C, indicating that functional expression of *ptsH* from the initiation site of *ptsI* may result from a decrease in stability of the *SacI*-modified message.

**Growth inhibition of *E. coli* ptsI mutants by the *B. stearothermophilus* ptsHI operon**

The basis for the initial failure to isolate clones which complemented *E. coli* MM6 (*ptsI* mutant) at 37 °C was further investigated. MM6 recombinants (pLOI1800, pLOI1801) harbouring this operon grew well on PTS sugars at 30 °C, but they formed small red colonies at 37 °C. No inhibition was observed with recombinants of *E. coli* DH5α (*ptsI*) at either temperature. Two additional *E. coli* ptsI mutants (JLT2 and BL21) were tested and were strongly inhibited by the *ptsHI* operon at 37 °C. High temperature-dependent toxicity of this operon in *E. coli* ptsI mutants was not limited to a particular medium and did not require the presence of added carbohydrate in rich medium. Toxicity of this operon was eliminated by deleting *ptsT* (pLOI1803 and pLOI1804) without affecting the temperature-dependent hyperexpression of functional EI and HPt at 30 °C and 37 °C (Fig. 3). Toxicity was also eliminated by insertion of a frame-shift mutation in *ptsH* (pLOI1807), which also decreased the expression of *ptsI* and presumably the downstream gene, *ptsT* (Fig. 3). These results indicate that the toxicity of pLOI1800 and pLOI1801 is due to the function of the *ptsT* gene.

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**REFERENCES**


Foster, S. I. (1993). Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing...


