Mutations in the glycerol kinase gene restore the ability of a *ptsGHI* mutant of *Bacillus subtilis* to grow on glycerol

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Although glycerol is not taken up via the phosphotransferase system (PTS) in *Bacillus subtilis*, some mutations that affect the general components of the PTS impair the ability of cells to grow on glycerol. Five revertants of a *pts* deletion mutant that grow on glycerol were analysed. They were shown to carry mutations in the glycerol kinase gene. These are missense mutations located in parts of the *glpK* gene that could encode regions important for the activity of glycerol kinase. The results strongly suggest that the main effect of the PTS on glycerol utilization in *B. subtilis* is mediated via glycerol kinase.

Keywords: *Bacillus subtilis*, glycerol catabolism, PTS components, glycerol kinase

INTRODUCTION

Glycerol catabolism in *Bacillus subtilis* involves a glycerol kinase (encoded by *glpK*) which phosphorylates glycerol to glycerol 3-phosphate (G3P) and a G3P dehydrogenase (encoded by *glpD*) which oxidizes G3P to dihydroxyacetone phosphate, an intermediate in glycolysis. *glpF* encodes a glycerol uptake facilitator and *glpP* encodes a regulatory protein. *glpP*, *glpF*, *glpK* and *glpD* are located at 75° on the *B. subtilis* chromosomal map and they constitute three operons: *glpP*, *glpFK* and *glpD* (Holmberg *et al.*, 1990; Beijer *et al.*, 1993). *B. subtilis* can also grow, although poorly, on G3P as sole carbon and energy source. This requires, in addition to the *glpP* and *glpD* gene products, a G3P permease which is encoded by the *glpT* gene. *glpT* is located at 15° on the chromosomal map and is part of an operon that also contains the *glpQ* gene which encodes a glycerophosphoryl diester phosphodiesterase (Lindgren, 1978; Nilsson *et al.*, 1994). The main control of the *glp* regulon of *B. subtilis* appears to be by termination/antitermination of transcription (Holmberg & Rutberg, 1991, 1992). In the presence of G3P, the GlpP protein is thought to act as an anti-terminator.

Glycerol enters *B. subtilis* via the glycerol uptake facilitator (Beijer *et al.*, 1993), while glucose is taken up via the phosphotransferase system (PTS). Mutations that affect the general components of the PTS, Enzyme I (EI)

encoded by *ptsI* and HPr encoded by *ptsH*, render the cells unable to transport glucose and other PTS sugars. Interestingly, some of them also impair the ability of cells to grow on glycerol (Gay *et al.*, 1973; J. Deutscher, unpublished data). Mutations that affect the glucose-specific component Enzyme II^{Glc} (EII^{Glc}), encoded by *ptsG*, do not interfere with glycerol utilization (Gonzy-Tréboul *et al.*, 1991).

A *ptsGHI* deletion mutant (deficient in EII^{Glc}, HPr and EI), which does not grow on glycerol, can grow on G3P. Furthermore, external G3P, but not glycerol, induces G3P dehydrogenase in this mutant (Beijer & Rutberg, 1992). These findings demonstrate that expression of the *glpD* and *glpP* genes does not require the products of the *ptsG*, *H* and *I* genes and lead to the conclusion that the PTS is not concerned with the overall regulation of the *glp* genes. Revertants of the above *pts* mutant which grow on glycerol but not on glucose have been isolated. The corresponding mutations map at 75° on the *B. subtilis* chromosome, i.e. they are closely linked to or located in the *glpPFKD* region (Beijer & Rutberg, 1992). In this study we have characterized five such revertants. They all carry missense mutations located in parts of the *glpK* gene that could encode regions important for the activity of glycerol kinase. Our results strongly suggest that in *B. subtilis*, the main effect of the PTS on glycerol utilization is mediated via glycerol kinase.

METHODS

Bacterial strains. The bacterial strains and plasmids used in this work are listed in Table 1. LUG9001 was a derivative of wild-type strain BR95, carrying an Em^R gene inserted between the *ptsG* 3' end and the *ptsI* 5' end. The insertion was derived from

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; EI, Enzyme I; EII, Enzyme II; Em, erythromycin; G3P, glycerol 3-phosphate; PTS, phosphotransferase system; Tc, tetracycline.

The GenBank accession number for the sequence reported in this paper is M34393.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Reference
<i>B. subtilis</i>		
BR95	<i>trpC2 pheA1 ilvC1</i>	Our collection
LUG0401	<i>trpC2 pheA1 ilvC1</i> Δ <i>glpPFKD ble</i>	Holmberg <i>et al.</i> (1990)
LUG9001	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI</i> Em ^R	Beijer & Rutberg (1992)
LUR9401	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI glpK1</i> Em ^R	This work
LUR9402	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI glpK2</i> Em ^R	This work
LUR9403	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI glpK3</i> Em ^R	This work
LUR9404	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI glpK4</i> Em ^R	This work
LUR9405	<i>trpC2 pheA1 ilvC1</i> Δ <i>ptsGHI glpK5</i> Em ^R	This work
<i>E. coli</i>		
MM294	<i>pro thi endA hsr_k hsr_{m+}</i>	Amann <i>et al.</i> (1983)
Plasmid		
pHV32	Ap ^R Cm ^R Tc ^R	Niaudet <i>et al.</i> (1982)
pHP13	Cm ^R Em ^R	Haima <i>et al.</i> (1987)
pLUM308	Cm ^R Em ^R <i>glpP142</i> <i>glpFK glpD'</i>	This work
pLUM305	Em ^R <i>glpF' glpK glpD'</i>	Our collection
pLUM630	Ap ^R <i>glpK'-ZZ</i>	This work

QB6020 (a kind gift from Dr André Klier, Institut Pasteur, Paris). The insertion of QB6020 is in turn derived from GM329 (Eisermann *et al.*, 1988). For mapping purposes, Beijer & Rutberg (1992) integrated a plasmid containing a *cat* gene immediately downstream of *glpD* in LUG9001. From this derivative of LUG9001, they isolated independent revertants that can grow on glycerol as sole carbon and energy source. Before we continued the analysis of the revertants, their mutations were transferred to LUG9001 to eliminate the integrated plasmid. LUG9001 was transformed with chromosomal DNA from the revertants and transformants were selected on minimal glycerol plates. The transformants were checked for Cm sensitivity (i.e. the plasmid had not been transferred) and Em resistance (marker of LUG9001). The revertant strains were named LUR9401–9405.

Media. Bacteria were kept on Tryptose Blood Agar Base (TBAB) plates. The minimal salts solution was that of Anagnostopoulos & Spizizen (1961) with the addition of a carbon and energy source; glucose (30 mM), casamino acids (0.5%), glycerol (20 mM) or DL- α -G3P (40 mM). Required amino acids were added at 40 mg l⁻¹. Antibiotics were used at the following concentrations (mg l⁻¹): Em, 3; Cm, 5; Ap, 50.

Transformation. Competent *B. subtilis* cells were prepared as described by Arwert & Venema (1973). *Escherichia coli* cells were made competent by the CaCl₂ method described by Mandel & Higa (1970).

DNA techniques. Chromosomal DNA was prepared as described by Marmur (1961). Plasmid DNA from *E. coli* was prepared using Qiagen (Diagen; maxi preparations) and plasmid

DNA from *B. subtilis* was prepared using Magic Maxipreps (Promega). DNA amplification was carried out by PCR with a GeneAmp kit (Perkin-Elmer Cetus). The nucleotide sequence was determined by the dideoxy chain-termination method of Sanger *et al.* (1977), using Sequenase version 2.0 (USB) and [α -³²S]dATP. Both strands were sequenced using synthesized primers. The primers were located at 250-bp intervals, giving overlaps in both directions. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim and used as recommended by the manufacturer.

Preparation of cell extracts and enzyme assays. Cell-free extracts were prepared as described by Lindgren & Rutberg (1974). Cells were grown at 37 °C in minimal salts solution with 0.5% casamino acids until OD₆₀₀ 0.5. The culture was then divided into three parts. Glycerol was added to one part and G3P to a second part. No addition was made to the third part. After 2 h incubation, cells were harvested and lysed. Glycerol kinase activity was measured as described by Freedberg & Lin (1973), but with the buffer at pH 9.0 instead of 9.5. G3P dehydrogenase activity was measured as described by Lin *et al.* (1962). The amount of protein was determined by the Pierce BCA Protein Assay.

Generation of antibodies against glycerol kinase. The plasmid pTrcZZ (E. Holmgren, unpublished results) was used for constructing a GlpK-ZZ translational fusion. pTrcZZ is a derivative of pTrc99A (Pharmacia) with an insertion of a reading frame encoding a two-domain analogue of the IgG-binding staphylococcal protein A, designated ZZ. The ZZ cassette is preceded by a promoter (inducible by IPTG) and a cloning cassette. A 1.4 kb *EcoRI*–*Bam*HI fragment covering most of *glpK* (starting 63 bp upstream of the start codon and ending 133 bp upstream of the stop codon) was amplified from pLUM305 (containing the complete *glpK* gene; the vector part is pHP13) using PCR. The sequences for the *EcoRI* and the *Bam*HI restriction sites were introduced into the primers. The oligonucleotides used for PCR were 5' TGTTTATAC GAATTCACATTCTGC 3' (the *EcoRI* site is underlined) and 5' CCAGGATCCACAGCGATACCC 3' (the *Bam*HI site is underlined). The amplified fragment was purified using the GeneClean kit (Bio101). The fragment and pTrcZZ were cut with *EcoRI* and *Bam*HI and ligated. *E. coli* MM294 was transformed with the ligate and transformants were selected on TBAB plates containing Ap. A plasmid carrying the amplified *glpK* fragment was isolated from one transformant and designated pLUM630. The identity of the plasmid was verified by restriction cleavage and DNA sequencing. *E. coli* MM294(pLUM630) was grown in 250 ml Luria broth containing Ap. Expression of the fusion protein was induced by addition of IPTG (final concentration 0.5 mM). After incubation for another 2.5 h, the cells were harvested, washed with 0.9% NaCl and suspended in 2 ml 10 mM Tris/HCl, pH 8.0. After sonication and centrifugation (25000 g, 15 min), the supernatant was diluted to 40 ml and applied to an IgG Sepharose 6 Fast Flow column (Pharmacia). The column was first washed with 50 bed volumes of TST buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20), then with 20 bed volumes of 10 mM ammonium acetate, pH 4.6. The bound protein was eluted with 5 bed volumes of 200 mM acetic acid, pH 3.2, lyophilized and suspended in 50 mM sodium phosphate buffer, pH 7.0. A rabbit was injected subcutaneously with 50 μ g fusion protein in Freund's complete adjuvant. Two booster injections using 2 $\times$ 50 μ g fusion protein in Freund's incomplete adjuvant were given over a period of 6 weeks. Antiserum was collected and used without further purification.

Western blot. Glycerol kinase protein in cell extracts was detected by antibodies against glycerol kinase. The proteins

were separated on an SDS polyacrylamide gel (10%, w/v). Western blot analysis was done using a Western Exposure Chemiluminiscent Detection system (Clontech).

RESULTS

Localization of suppressor mutations that allow a *ptsGHI* mutant to grow on glycerol

Beijer & Rutberg (1992) isolated independent revertants of a *pts* deletion mutant, LUG9001, that can grow on glycerol as sole carbon and energy source. In transformation crosses they showed that the corresponding mutations are linked to the *glpPFDK* region. To get the approximate location of the suppressor mutations, we cloned different parts of this region from the revertants using PCR. The PCR fragments were ligated to plasmid pHP13 and the resulting recombinant plasmids were introduced into LUG9001. Cm^R transformants were selected and checked for growth on glycerol. From all five revertants, we derived a 1.4 kb fragment covering most of *glpK* (starting 63 bp upstream of the start codon and ending 133 bp upstream of the stop codon) that enabled LUG9001 to grow on glycerol. The corresponding fragment from the wild-type strain BR95 did not rescue LUG9001.

The suppressor mutations are point mutations in *glpK*

To map the above mutations precisely, *glpK* was cloned from the revertants and LUG9001. This was done by integrating plasmid pHV32 carrying the *glpD* control region which is located immediately downstream of *glpK* (see Fig. 1), into the chromosome of each strain. Plasmids containing *glpK* and part of *glpF* were regenerated in *E. coli* after cleavage of the chromosomal DNA with *Nco*I and ligation (Niaudet *et al.*, 1982). From these plasmids, the DNA sequence of the entire *glpK* with flanking DNA (about 40 bp upstream of the start codon and about 70 bp downstream of the stop codon) was determined. The five revertants were all found to have point mutations within the 5'-proximal half of *glpK*. The mutations all lead to amino acid substitutions in glycerol kinase (Table 2). Two of the revertants, LUR9403 and LUR9404, had the same nucleotide change, a T to C transition at position 1101. Two other revertants, LUR9401 and LUR9405, had nucleotide changes close to each other: LUR9401 had an A to G transition at position 1386; and LUR9405 had a T to C transition at position 1392. The suppressor mutation

Table 2. Nucleotide exchanges in *glpK* of the revertants and the corresponding amino acid substitutions in glycerol kinase

The positions of the nucleotide exchanges refer to the reported DNA sequence (GenBank accession number M34393).

Strain	Nucleotide exchange, position	Amino acid exchange, position
LUR9401	A to G, 1386	His to Arg, 230
LUR9402	A to G, 898	Glu to Gly, 65
LUR9403	T to C, 1101	Phe to Ser, 135
LUR9404	T to C, 1101	Phe to Ser, 135
LUR9405	T to C, 1392	Phe to Ser, 232

of the fifth revertant, LUR9402, was an A to G transition at position 898. The DNA sequence of *glpK* from LUG9001 corresponds to the previously reported wild-type sequence (Holmberg *et al.*, 1990, GenBank accession number M34393) except for a C nucleotide at position 1298 which should be a G.

Enzyme activities in the *pts* mutant, the revertants and the *pts* mutant carrying pLUM308

Activities of glycerol kinase and G3P dehydrogenase were measured in extracts from the various strains grown with and without glycerol and G3P, respectively. The results are presented in Table 3. BR95, the wild-type, contained low, basal levels of the two enzymes when grown in minimal medium without glycerol and G3P. Addition of either substance induced both enzymes. Glycerol kinase activity increased two- to fivefold and G3P dehydrogenase activity five- to tenfold. The true inducer is G3P which means that a basal activity of glycerol kinase is necessary for G3P to be formed from glycerol. This can be seen in LUG9001. This strain cannot grow on glycerol and addition of glycerol induced neither glycerol kinase nor G3P dehydrogenase. However, addition of G3P induced both enzymes. The inability of LUG9001 to grow on glycerol must relate to an effect on the glycerol facilitator and/or the glycerol kinase which renders the cells unable to form the inducer, G3P. In all revertants, addition of glycerol as well as G3P induced glycerol kinase and G3P dehydrogenase activities. This demonstrates that the glycerol kinase of the revertants can form inducing amounts of G3P from glycerol, independently of the PTS.

Serendipitously, we have found that increasing the copy number of the *glpFK* operon also restores growth of LUG9001 on glycerol. Plasmid pLUM308 is pHP13 carrying *glpFK*, a defective *glpP* and a truncated *glpD*. When LUG9001 was transformed with pLUM308 it gained the ability to grow on glycerol. pHP13 containing only the *glpP* or the *glpD* operon, respectively, did not enable LUG9001 to grow on glycerol. LUG9001 carrying pLUM308 showed a somewhat lower glycerol kinase activity than BR95. It produced highly increased amounts of glycerol kinase protein compared to the wild type (or

Fig. 1. Schematic representation of the *glpPFDK* region with pHV32 integrated between *glpK* and *glpD*. Inverted repeats are shown as stem-loops and the *glpD* control region, which is duplicated upon integration, is indicated by bold lines. The restriction sites for *Nco*I, which were used in regeneration of pHV32 containing *glpK*, are indicated.

Table 3. Enzyme activities in the wild type, the *pts* mutant, the revertants and the *pts* mutant carrying pLUM308

The values represent the mean of at least two different measurements. The deviation from the mean is less than 30 %. Activities are expressed as nmol substrate converted min⁻¹ (mg protein)⁻¹.

Strain	Addition	Glycerol kinase	G3P dehydrogenase
BR95	–	7	3
	G3P	35	32
	Glycerol	17	62
LUG9001	–	6	7
	G3P	18	31
	Glycerol	6	8
LUR9401	–	1	2
	G3P	11	32
	Glycerol	6	60
LUR9402	–	5	4
	G3P	27	24
	Glycerol	27	52
LUR9403	–	4	5
	G3P	14	33
	Glycerol	24	78
LUR9404	–	7	5
	G3P	33	29
	Glycerol	10	44
LUR9405	–	1	4
	G3P	8	37
	Glycerol	34	14
LUG9001(pLUM308)	–	4	2
	G3P	10	8
	Glycerol	20	5

the revertants, data not shown), as determined by Western blots using antibodies against glycerol kinase (Fig. 2). It thus appears that the need for PTS in glycerol utilization can be overcome by an increased intracellular amount of glycerol kinase protein, possibly in combination with an increased amount of the glycerol uptake facilitator. The fact that the glycerol kinase activity was not increased shows that the need for a PTS-mediated activation is not overcome and the mutant can grow on glycerol only because glycerol kinase (and the glycerol facilitator) is overexpressed. The reason for the low G3P dehydrogenase activity may be that GlpP is titrated in the presence of the target region in multiple copies, giving a low expression of the intact chromosomal *glpD* operon.

DISCUSSION

The PTS affects glycerol utilization in both *B. subtilis* and *E. coli*. The activity of the *E. coli* glycerol kinase is controlled by one of the PTS components, EIIA^{Glc}. This soluble enzyme, which is involved in transport of glucose, has a central regulatory function. It exists in a

phosphorylated and an unphosphorylated form. When glucose is present, EIIA^{Glc} is predominantly in its unphosphorylated form since the phosphate group is transferred to glucose. The unphosphorylated EIIA^{Glc} has been shown to bind to and thereby inhibit the activity of proteins and enzymes involved in uptake/utilization of non-PTS sugars. One of these enzymes is glycerol kinase (Novotny *et al.*, 1985). This inhibition of glycerol kinase activity in the presence of glucose will minimize unnecessary expression of the *glp* regulon since formation of inducer, G3P, is prevented.

B. subtilis does not have a soluble EIIA^{Glc}. Instead, EIIA^{Glc} is a domain in a membrane-bound polypeptide, EIICBA^{Glc}. A control mechanism similar to the one found in *E. coli* is therefore unlikely. A *B. subtilis* *ptsGHI* mutant defective in the genes encoding EII^{Glc}, HPr and EI cannot grow on glycerol (Gonzy-Tréboul *et al.*, 1991). This suggests a positively acting regulatory mechanism in which one or more of the central PTS components is needed for growth on glycerol. Beijer & Rutberg (1992) showed that the *ptsGHI* mutant can grow on G3P and that its *glp* genes are induced by G3P but not by glycerol. This shows that one or more PTS components is needed for the utilization of glycerol but not of G3P and that the PTS-mediated control is separate from the specific control of the *glp* regulon. It was concluded that the glycerol uptake facilitator or the glycerol kinase are the most probable targets for PTS-mediated control. This control may involve phosphorylation of the proteins (see below).

Revertants of the *pts* mutant LUG9001 that can grow on glycerol as sole carbon and energy source, carry suppressor mutations that are linked to the *glp* region at 75° (Beijer & Rutberg, 1992). We have now characterized five such mutations. They are all translation missense mutations within the 5'-proximal half of the glycerol kinase gene (Table 2). In addition, we have found that increasing the amount of glycerol kinase protein (and the glycerol uptake facilitator) by increasing the number of gene copies also suppresses the glycerol-negative phenotype of LUG9001.

The crystal structure of the *E. coli* glycerol kinase in different complexes with glycerol, EIIA^{Glc} of the PTS and ADP has been determined (Hurley *et al.*, 1993). The glycerol kinases of *B. subtilis* and *E. coli* show high sequence identity (62%). They are also equal in size; the *B. subtilis* enzyme consists of 501 amino acids (Holmberg *et al.*, 1990) and the *E. coli* enzyme consists of 496 amino acids (Pettigrew *et al.*, 1988). Furthermore, amino acid residues important for binding of glycerol in the *E. coli* enzyme are all conserved in *B. subtilis*. When discussing our results we will make the reasonable assumption that the glycerol kinases from these two bacteria are structurally similar.

In *E. coli*, catalytically active glycerol kinase exists *in vitro* (and probably also *in vivo*) as a mixture of dimers and tetramers. The proportion of tetramers increases at increasing concentrations of the enzyme. The latter form is also the form that is sensitive to allosteric control by fructose 1,6-diphosphate (de Riel & Paulus, 1978).

Fig. 2. Western blot analysis of glycerol kinase protein in cell extracts from BR95, LUG9001(pLUM308) and LUG0401, uninduced and induced with glycerol. LUG0401 is deleted for *glpK*. Total protein (12 µg) was loaded on lanes 1–4 and 7–8. Lanes: 1, uninduced BR95; 2, induced BR95; 3, uninduced LUG9001(pLUM308); 4, induced LUG9001(pLUM308); 5, induced LUG9001(pLUM308) diluted 1:10; 6, induced LUG9001(pLUM308) diluted 1:50; 7, uninduced LUG0401; 8, induced LUG0401. The position of glycerol kinase (top band in double band) is indicated.

Voegelé *et al.* (1993) have shown that *in vivo*, the *E. coli* glycerol kinase needs to associate with the glycerol uptake facilitator to be fully active.

In *B. subtilis*, components of the PTS exert positive control of glycerol utilization. A main function for PTS proteins is the transfer of phosphate groups, e.g. to drive the transport of various sugars into the cell or to control the expression of genes in carbohydrate catabolism, as in the *E. coli bgl* system (Amster-Choder & Wright, 1993). In the Gram-positive *Enterococcus faecalis*, glycerol utilization is positively controlled by the PTS and it has been shown that glycerol kinase is activated by phosphorylation by the HPr (Deutscher & Sauerwald, 1986; Romano *et al.*, 1990). HPr of *B. subtilis* is phosphorylated at two different sites, at histidyl residue 15 by EI and at seryl residue 46 by an ATP-dependent HPr kinase. A recent report from Deutscher *et al.* (1994) suggests that phosphorylation at Ser-46 is not important for glycerol kinase activity.

We speculate that the PTS activates *B. subtilis* glycerol kinase in a manner that favours tetramerization of the enzyme and/or increases its affinity for the glycerol uptake facilitator. Possibly, this is achieved by phosphorylation of the kinase by P-his-HPr. The turnover of glycerol would then increase as a consequence of a more active glycerol kinase. Alternatively, an association of the glycerol kinase with the glycerol uptake facilitator would increase the concentration of glycerol near the kinase as glycerol enters the cell through the facilitator. Applying these speculations to the revertants of LUG9001 leads to the following interpretation of our results. The Phe to Ser substitution at position 135 in LUR9403 and LUR9404 is near the glycerol binding site in the similar *E. coli* enzyme (Fig. 3). The introduction of a more polar amino acid in

Fig. 3. A α trace of the 3-dimensional structure of *E. coli* glycerol kinase in complex with glycerol. Within the encircled domain, the bound glycerol is indicated with a small circle. The amino acid residues predicted to correspond to those exchanged in the *B. subtilis* revertants are indicated. The protein structure was imported from the Brookhaven Protein Databank (Bernstein *et al.*, 1977).

this region could increase the affinity of the enzyme for its substrate and bypass the requirement for increased tetramer formation or association to the facilitator. The other three amino acid changes (Glu to Gly at position 65, His to Arg at position 230 and Phe to Ser at position 232) are all located at the surface, in a part of the enzyme monomer which may be involved in protein–protein interactions (in the *E. coli* glycerol kinase, this part is involved in dimer–dimer contacts). We propose that these mutations promote formation of tetramers or increase the affinity of the kinase for the facilitator or make glycerol kinase activity independent of interaction with the facilitator. The changed glycerol kinase would then trap sufficient amounts of glycerol (by forming G3P) to allow growth.

Introduction of pLUM308 into LUG9001 leads to suppression of the glycerol-negative phenotype and a highly increased amount of glycerol kinase protein. The glycerol kinase activity is, however, quite low which could reflect the lack of PTS-mediated activation. It is not clear why a moderate increase in *glpK* (and *glpF*) gene dosage results in such a large amount of glycerol kinase protein, but we propose that this high concentration of kinase protein allows some tetramerization and/or association with the glycerol facilitator in the absence of activation by the PTS.

The above speculations lead to several testable predictions concerning regulation of glycerol catabolism in *B. subtilis* and its relation to the PTS. Our data establish that glycerol kinase is the major target for PTS-mediated control.

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