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Expression of a novel gene, *gluP*, is essential for normal *Bacillus subtilis* cell division and contributes to glucose export

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Abstract

Background: The *Bacillus subtilis* glucokinase operon was predicted to be comprised of the genes, *yqgP* (now named *gluP*), *yqgQ*, and *glcK*. We have previously established a role for *glcK* in glucose metabolism. In the absence of enzymes that phosphorylate glucose, such as GlcK and/or enzyme II^{Glc}, accumulated cytoplasmic glucose can be transported out of the cell. Genes within the glucokinase operon were not previously known to play a role in glucose transport. Here we describe the expression of *gluP* and its function in glucose transport.

Results: We found that transcription of the glucokinase operon was regulated, putatively, by two promoters: σ^A and σ^H. Putative σ^A and σ^H-recognition sites were located upstream of and within *gluP*, respectively. Transcriptional glucokinase operon – *lacZ* fusions and Northern blotting were used to analyze the expression of *gluP*. GluP was predicted to be an integral membrane protein. Moreover, the prediction of GluP structure revealed interesting signatures: a rhomboid domain and two tetracopeptide repeat (TPR) motifs. Microscopic analysis showed that GluP minus cells were unable to divide completely, resulting in a filamentous phenotype. The cells were grown in either rich or minimal medium. We found GluP may be involved in glucose transport. [^{14}C]-glucose uptake by the GluP minus strain was slightly less than in the wild type. On the other hand, trehalose-derived glucose in the growth medium of the GluP minus strain was detected in very low amounts. Experimental controls comprised of single or multiple genes mutations within the glucose transporting phosphotransferase system.

Conclusions: *gluP* seems to be regulated only by a putative σ^A-dependent promoter. The glucose uptake and export assays suggest that GluP is important for glucose export and may act as an exporter. This also supports the role of the glucokinase operon in glucose utilization.

Background

Bacteria can utilize a variety of sugars for growth. However, glucose, which is one of the most ubiquitous monosaccharides, is used preferentially as a carbon source [1]. Depending upon the physiological state of the cells, assimilated glucose is utilized for cell division, converted into storage compounds, or used for the production of secondary metabolites. Bacteria may exploit glucose

availability ranging from sub-micromolar to millimolar concentrations, by developing and dedicating several transporters composed of multiple protein subunits [2,3]. For example, *Escherichia coli* is known to possess at least seven transporters capable of acquiring glucose and may have additional transporters that have not yet been identified [4]. Bacteria, such as *Bacillus subtilis*, utilize glucose via at least two discrete pathways. The first pathway is the phosphoenolpyruvate-dependent (PEP) phosphotransferase system (PTS) [5,6]. The PTS comprises enzymes such as enzyme I (EI), enzyme II (EII), and histidine-containing phosphocarrier protein of the PTS (HPr). Enzyme II phosphorylates glucose into glucose 6-phosphate upon uptake [6]. In contrast, the phosphorylation will not occur in non-PTS transported glucose. The key molecule involved in the non-PTS pathway is a glucose:H^+ symporter (GlcP) [7]. Consequently, phosphorylation of glucose is carried out by a cytoplasmic enzyme glucose kinase/glucokinase. *B. subtilis* glucokinase (GlcK) has been elucidated by us, and is found within the glucokinase operon [8-10]. The operon was predicted to consist of the genes, *yqgP*, *yqgQ*, and *glcK*, respectively [8]. In contrast to *glcK*, the function of other genes belonging to the operon are not known. Here we report, for the first time, the expression of the upstream gene within the *B. subtilis* glucokinase operon, *yqgP*. Structural prediction of YqgP protein and the role of the protein in glucose transport are also discussed. Based on our findings, described below, we have now named YqgP protein as the glucose transporter protein or GluP.

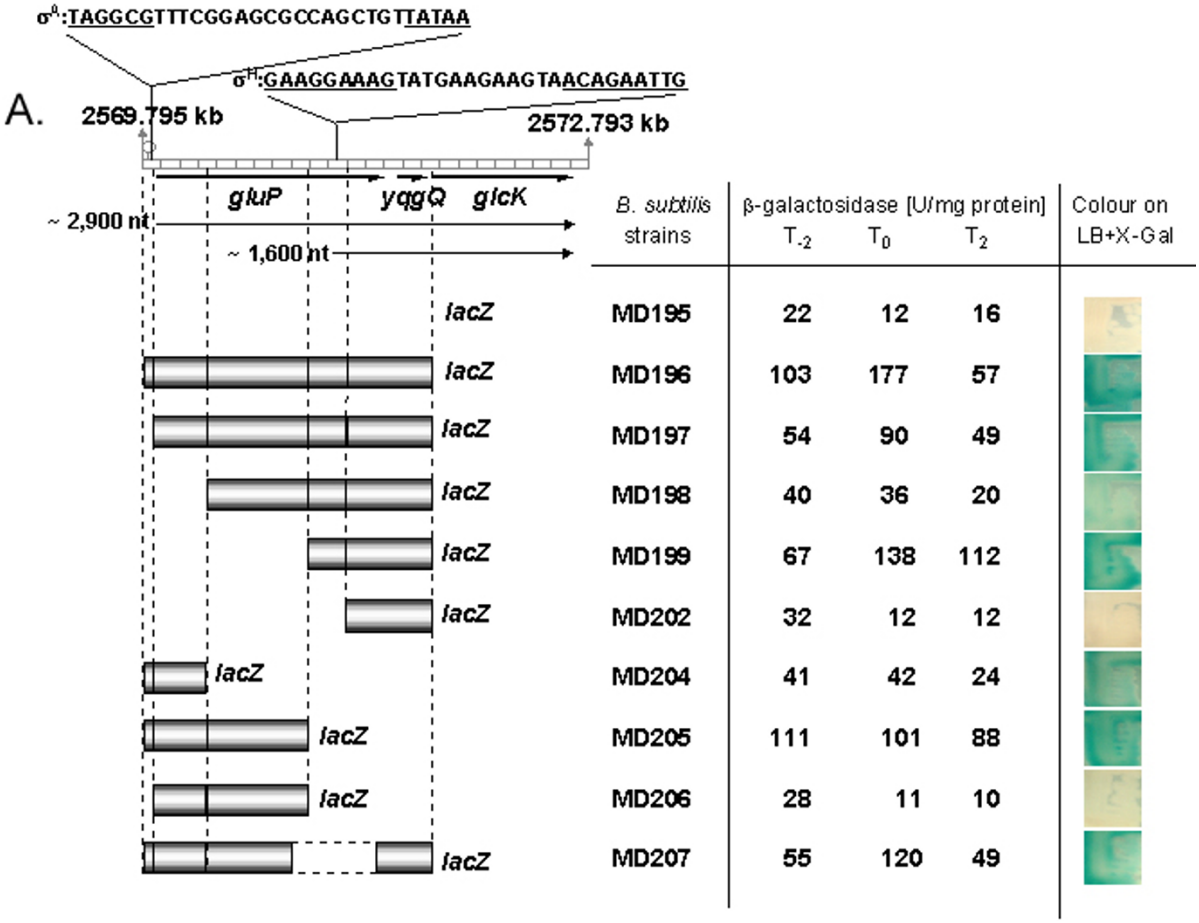
Results

gluP *is located within the glucokinase operon and is putatively regulated by a* σ^A*-dependent promoter*

By examination of the *gluP*'s upstream sequence, we have identified a consensus promoter sequence, TAGGCG$(N)_{17}$TTATAA, recognized by σ^A as proposed by Kunst *et al.* [11,12] and Skarlatos and Dahl [8]. In addition to that, we have found a sequence, GAAGGAAAG$(N)_{12}$ACAGAATTG, within *gluP*, which would fall into a recently described signature of the σ^H binding site (RXAGGAWWW$(N)_{11-12}$RXXGAATWW) [13]. Based on these findings, we constructed several transcriptional glucokinase operon – *lacZ* fusions. The *lacZ* was placed downstream of the constructs replacing *glcK*. We examined the transcription of the glucokinase operon – *lacZ* fusion during exponential (T_{-2}), transition (T_0), and stationary (T_2) phase. *B. subtilis* MD195 harboring a vector containing *lacZ* only, served as a negative control (Fig. 1A). Cells carrying glucokinase operon – *lacZ* fusion lacking both putative σ^A and σ^H recognition sequences (*B. subtilis* MD202 and MD206) displayed similar β-galactosidase activities to *B. subtilis* MD195, suggesting that the corresponding operon fragment was not transcribed (Fig. 1A). In contrast, cells carrying either the putative σ^A-(*B. subtilis* MD204, MD205, and MD207) or σ^H-(*B. subtilis* MD197, MD198, and MD199) recognition sequences alone, or both (*B. subtilis* MD196), were able to transcribe the corresponding operon fragment, resulting in bluish-green colonies on LB agar medium supplemented with X-Gal (Fig. 1A). Based on these transcriptional glucokinase operon fragment – *lacZ* fusions, our results clearly indicated that there were two putative promoters, which were located upstream of and within *gluP*, respectively. Together with *yqgQ* and *glcK*, *gluP* expression is likely under the control of a putative σ^A-dependent promoter. Meanwhile, a putative σ^H-dependent promoter may control only the expression of *yqgQ* and *glcK*.

B. subtilis MD196 containing both σ^A and σ^H putative binding sites within the glucokinase operon fragment – *lacZ* fusion displayed higher β-galactosidase activity in the T_0 than in the T_{-2} and the T_2. This data suggests that the operon's peak transcription occurs during T_0 (Fig. 1A). However, cells carrying only the putative σ^H-dependent promoter region and *yqgQ* (*B. subtilis* MD199) displayed higher β-galactosidase activity during T_0 and T_2 than during T_{-2}. This indicates that the putative σ^H had higher activity in T_0. On the contrary, cells carrying only the putative σ^A-dependent promoter region and *gluP* fragment, without the σ^H recognition site, (*B. subtilis* MD205) had much higher β-galactosidase activity than the negative control (*B. subtilis* MD195) in all growth phases.

We further verified the glucokinase operon transcripts with Northern blot analysis. An amplified 449-bp DNA fragment from *glcK* was used as a probe and labeled. This analysis showed two bands with sizes of approximately 2900- and 1600-nt, suggesting that two mRNA products were transcribed from the glucokinase operon (Fig. 1B). The band sizes, 2900- and 1600-nt, were in agreement with the distance between the putative σ^A-dependent promoter and the *glcK* stop codon (2878 bp) and between the putative σ^H-dependent promoter and the *glcK* stop codon (1616 bp), respectively. Interestingly, the 2900 nt band was intense at T_{-2} and gradually diminished as the cells shifted to the subsequent growth phases. In contrast, the 1600 nt band was initially fairly small at T_{-2} and peaked at T_0. This result indicates that there was a work-shift between the putative σ^A and σ^H. All genes within the glucokinase operon seemed to be transcribed during the exponential phase. When cells entered the transition phase, *yqgQ* and *glcK* were more abundantly transcribed than *gluP*. So far, it seems that σ^A is the principal sigma factor present in vegetatively growing *B. subtilis*, while σ^H is essential for the early stages of sporulation [14]. Taken together, expression of the transcriptional glucokinase operon – *lacZ* fusions and Northern blot analysis demonstrated that there were two putative promoters regulating the transcription of glucokinase operon.



B. subtilis strains	β-galactosidase [U/mg protein] T_{-2}	T_0	T_2	Colour on LB+X-Gal
MD195	22	12	16	
MD196	103	177	57	
MD197	54	90	49	
MD198	40	36	20	
MD199	67	138	112	
MD202	32	12	12	
MD204	41	42	24	
MD205	111	101	88	
MD206	28	11	10	
MD207	55	120	49	

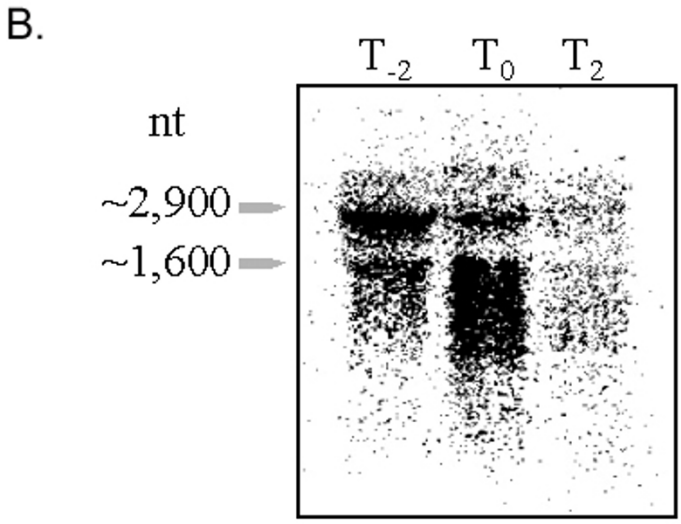


Figure 1
Transcription of *B. subtilis* glucokinase operon. (A) Glucokinase operon is located between chromosomal 2569.795 and 2572.793 kb, as is available in the SubtiList database [12]. β-galactosidase assays of the transcriptional glucokinase operon – *lacZ* fusions were determined quantitatively as described in materials and methods. Average value of β-galactosidase activity (U/mg protein) is presented from two independent experiments. β-galactosidase activity (bluish green colour) was also monitored *in vivo* using LB plates containing X-Gal. (B) Northern blot of glucokinase transcript with a probe localized within *glcK*.

GluP consists of a putative rhomboid domain and two tetracopeptide repeat (TPR) motifs

gluP (1521 bp) encodes a 56 kDa protein (GluP) comprising of 507 amino acid residues. We searched the Prosite database of protein families and domains http://ca.expasy.org/prosite/[15], as well as the protein families database of alignments and HMMs (pfam) http://www.sanger.ac.uk/Software/Pfam/[16] to find GluP domain architectures. It seems that GluP may contain very interesting signatures. These are the rhomboid domain and tetracopeptide repeat (TPR) motif.

The rhomboid domain belongs to a family of integral membrane proteins [17]. Similar to the other rhomboid family members, GluP may contain three strongly conserved histidines at position 232, 237, and 339 (Fig. 2A). In addition, GluP may also consist of a conserved serine at position 288 (Fig. 2A). This Ser-288 is part of a serine protease motif [17,18]. We further checked for the potential transmembrane domain of GluP employing the TMHMM2.0 http://www.cbs.dtu.dk/services/TMHMM/[19] and SOSUI http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html[20] programs. GluP consists of six putative membrane-spanning segments, suggesting that GluP is likely an integral membrane protein (Fig. 2B). Bacterial and archaeal members of the rhomboid family contain six transmembrane helices (TMHs), whereas the eukaryotic members typically have an additional seventh TMH [21].

The second signature, a TPR motif, is a degenerate 34 amino acid sequence that is often arranged in tandem arrays, along with eight conserved consensus residues at position 4(W/L/Y), 7(L/I/M), 8(G/A/S), 11(Y/L/F), 20(A/S/E), 24(F/Y/L), 27(A/S/L), and 32(P/K/E) [22-24]. GluP was predicted to have two TPR motifs, which are positioned at amino acid residues 424–457 and 458–491 (Fig. 3). The prediction of the TPR motif's secondary structure revealed two α-helical domains, named A and B (Fig. 3). The Geno3D program was used http://geno3d-pbil.ibcp.fr/cgi-bin/geno3d_automat.pl?page=/GENO3D/geno3d_home.html[25] to predict the three-dimensional structure of the protein, based on available crystallized proteins. Geno3D found human PEX5 TPR motifs [26], especially the TPR1 and TPR2 motifs, to be a strong template for deducting GluP TPR tertiary structure (Fig. 3). Geno3D recognized three helical turns representing domain B of TPR1 and domains A and B of TPR2 (Fig 3). Domain A of the GluP TPR1 could not be distinguished due to lower identities to the template. No available crystallized proteins could be used as a structural template for the GluP rhomboid domain.

A protein containing combined rhomboid domain – TPR motifs seemed rare. BLAST searching [27] showed that, besides GluP, there were seven other rhomboid-TPR proteins found in Gram-positive bacteria. Their sequences can be accessed from GenBank with the IDs NP_242287 (*B. halodurans*), NP_692844 (*Oceanobacillus iheyensis*), NP_464862 (*Listeria monocytogenes*), NP_470710 (*L. innocua*), NP_646318 (*Staphylococcus aureus*), NP_764790 (*S. epidermidis*), and ZP_00060595 (*Clostridium thermocellum*). With respect to GluP, their amino acid identities are 33.1%, 29.6%, 26.8%, 27.0%, 17.9%, 18.9%, and 26.8%, respectively.

GluP is important for sporulation and normal cell division

GluP minus cells were still able to sporulate (Fig 4). However, the sporulation frequency was two-fold lower (33 %) than the wild type's. Microscopic analysis showed that GluP minus cells were defective in their ability to separate during cell division, resulting in a filamentous phenotype. The GluP minus cells were grown in either rich or minimal medium (Fig. 4). We then visualized the DNA chromosome and the cell membrane of GluP minus cells with bisbenzimide and FM5-95 dye, respectively. Bisbenzimide would generate blue fluorescence of the chromosomal DNA and FM5-95 dye would produce red fluorescence of the cell membrane when visualized using the proper filter lenses (Fig. 5). Every GluP minus cell still contained chromosomal DNA, although in a filamentous phenotype (Fig. 5A). Wild type cells used as a comparison are shown in Fig. 5B. Taken together, the decrease in the GluP minus strain's ability to sporulate may be due to the inability of the cells to divide completely and normally. This may indicate a role for GluP in cell division. The contribution of the rhomboid domain and/or the two TPR motifs to the failure of the GluP minus strain to divide normally warrants further studies.

The absence of GluP slightly affects glucose uptake

Since *gluP* is located within the glucokinase operon and the gene product, GluP, is predicted to be a transmembrane protein, we tested whether GluP plays a role in glucose uptake. We performed a time course study to observe the ability of GluP minus cells to take up [^{14}C]-glucose. Several experimental controls were employed, such as the wild type cells (positive control), enzyme II^{Glc} (*ptsG::cat*) minus cells (negative control), and GluP (*gluP::aphA3*)-enzyme II^{Glc} minus cells (negative control) (Fig. 6). Enzyme II^{Glc} is a key molecule involved in PTS – dependent glucose uptake. The gene that encodes enzyme II^{Glc}, *ptsG*, is highly regulated by glucose [28]. Consequently, cells were initially grown in medium containing 50 mM glucose, before adding the [^{14}C]-glucose, in order to induce the production of enzyme II^{Glc}. Our experiment showed that the ability of GluP minus cells to take up [^{14}C]-glucose was generally lower, i.e. ~20% at 120 s, than the wild type's (Fig. 6). Nevertheless, the increase of [^{14}C]-glucose uptake for both wild type and GluP minus cells

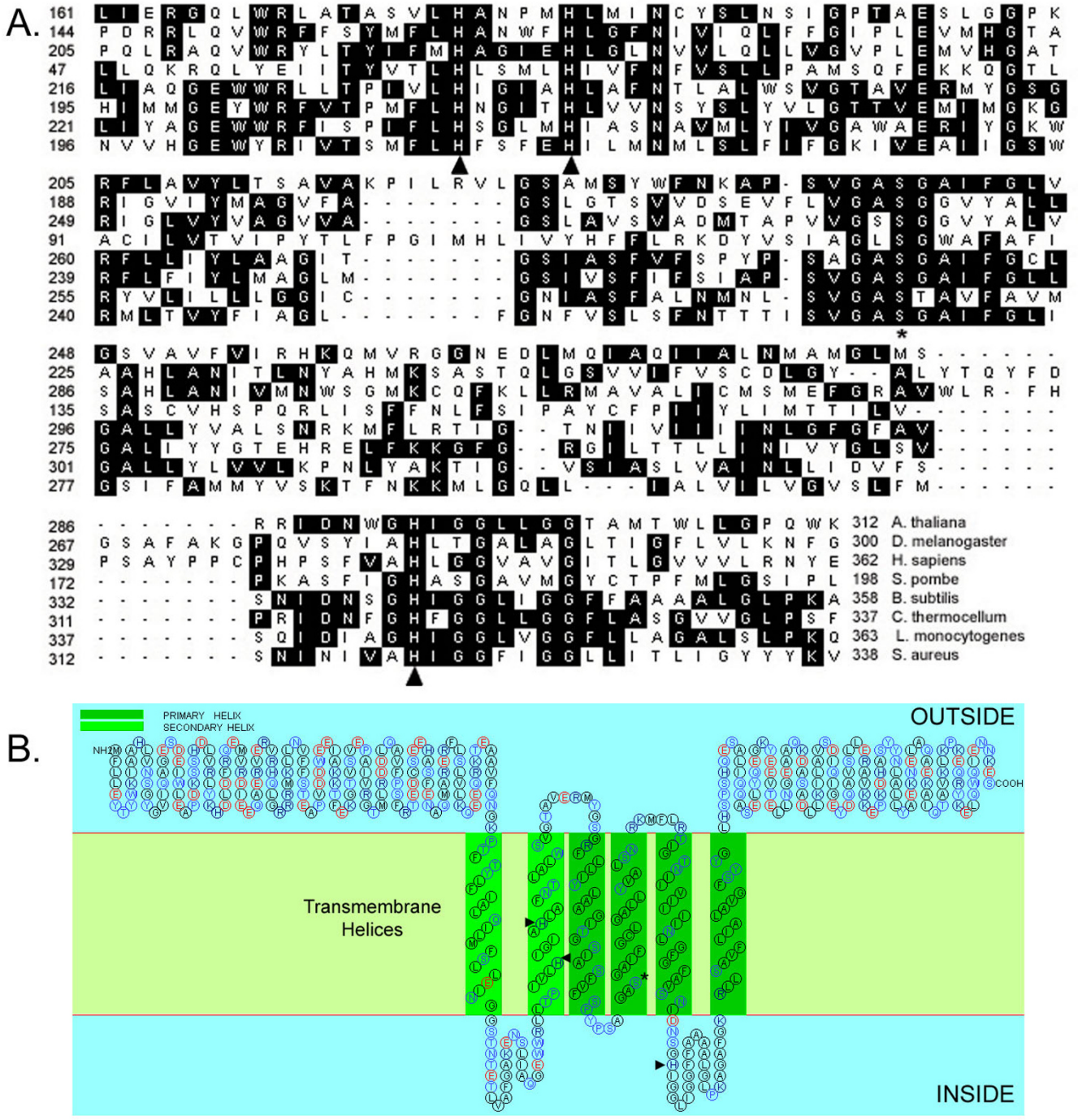


Figure 2
GluP is predicted to have a rhomboid domain, which is located within transmembrane helices. (A) Multiple alignments of GluP and other rhomboid proteins. (B) Predicted 2-D structure of GluP (SOSUI program [20]). Arrowheads are conserved histidine residue. Predicted conserved Ser-288 of serine protease motif is indicated with asterisk.

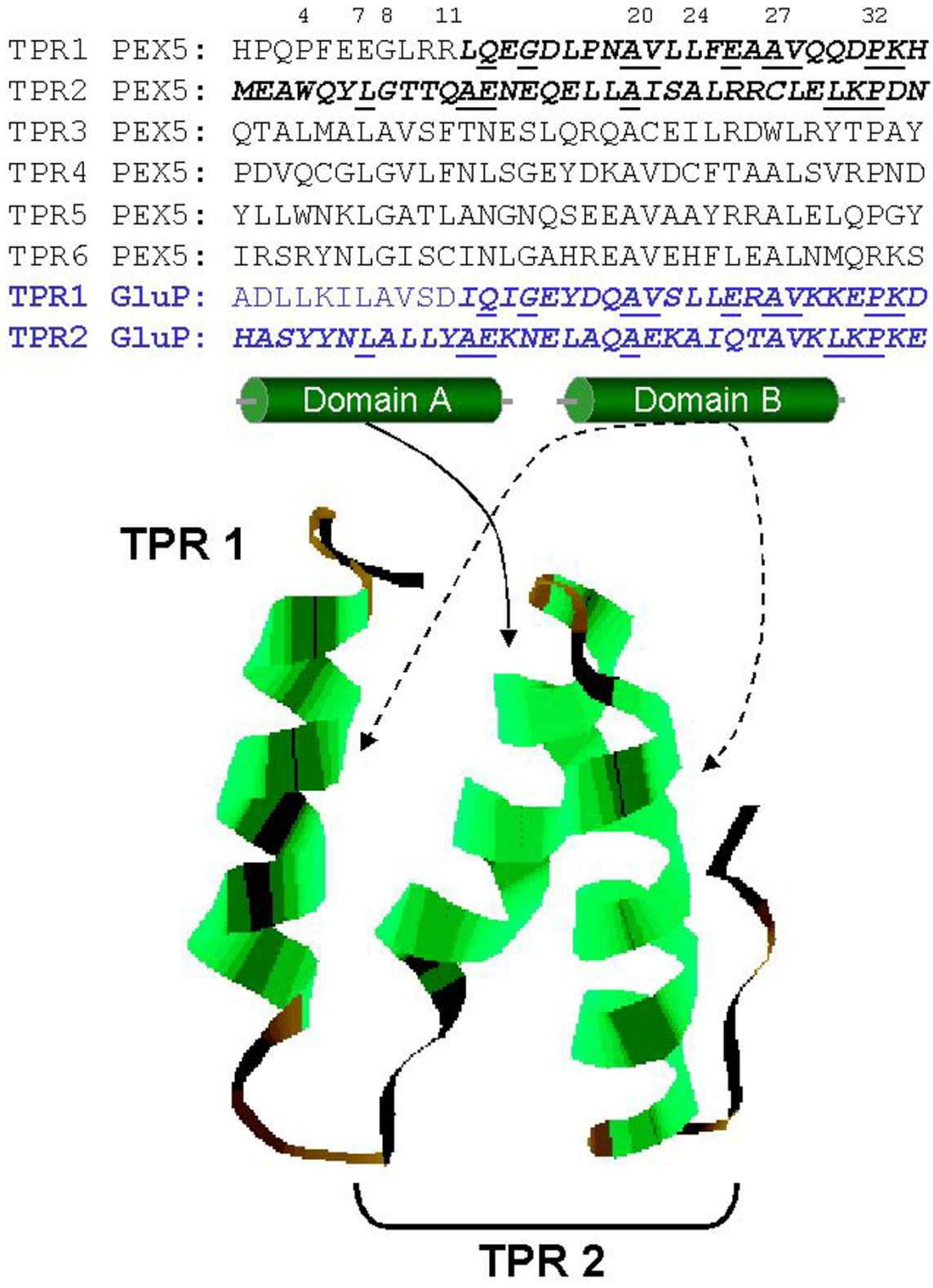


Figure 3
Human protein PEX5 [26] based deduction of three-dimensional structure of GluP TPR motif (Geno3D program [25]). Amino acid residues that were used to build the GluP TPR structure are italicized. The conserved residues are underlined.

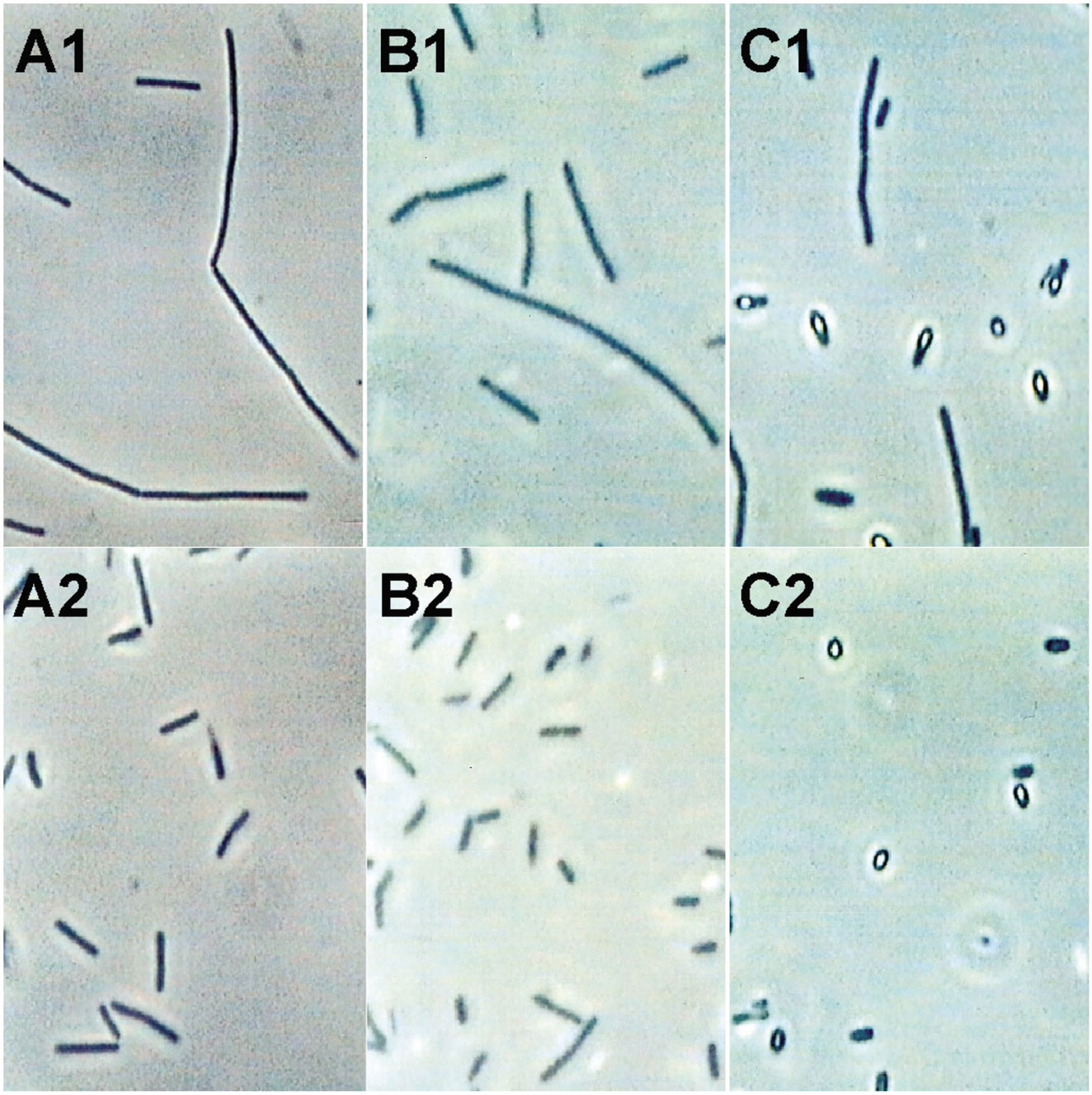


Figure 4
GluP minus cells (1) phenotype as shown by phase contrast microscopy, in comparison with the wild type cells (2). Cells were grown either in rich (A) or minimal medium (B). The ability of the cells to sporulate is shown in panel C. Magnification used was 400 ×.

was positively correlated with time, although there was an expanding gap in their ability to take up [^{14}C]-glucose (Fig. 6). In contrast, the uptake of [^{14}C]-glucose by enzyme II^{Glc} minus cells was nearly abolished (Fig. 6). This suggests that both wild type and GluP minus cells rely on the presence of enzyme II^{Glc} to take up [^{14}C]-glucose. Surprisingly, cells lacking both GluP and enzyme II^{Glc} were able to take up [^{14}C]-glucose, although at a

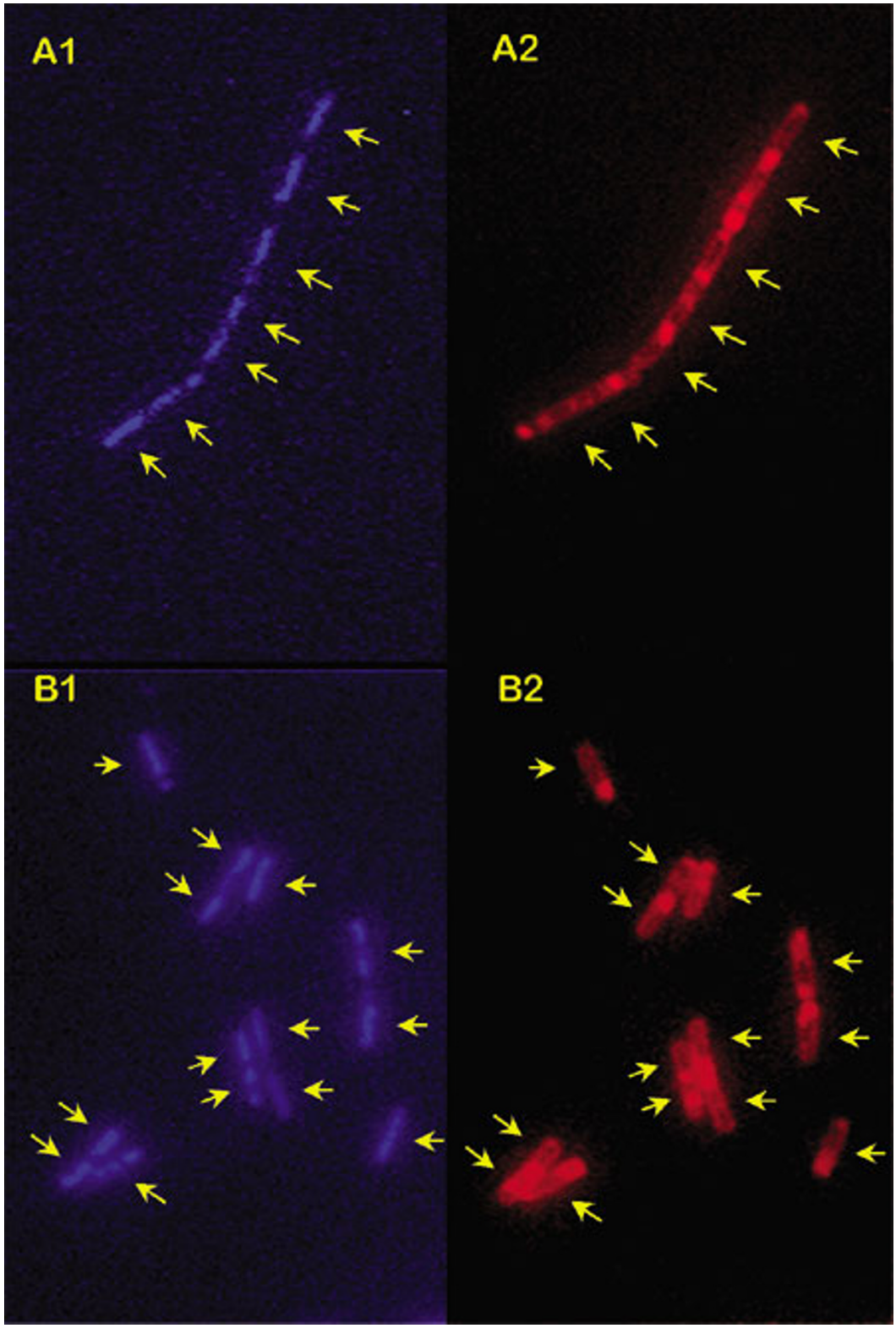


Figure 5
GluP minus cells (A) phenotype as shown by fluorescence microscopy, in comparison with the wild type cells (B). Chromosomal DNA (1) was stained with bisbenzimide (blue fluorescence). Septa and cell membrane (2) was stained with FM5-95 (red fluorescence). Arrows indicate single cell. Magnification used was 1000 ×.

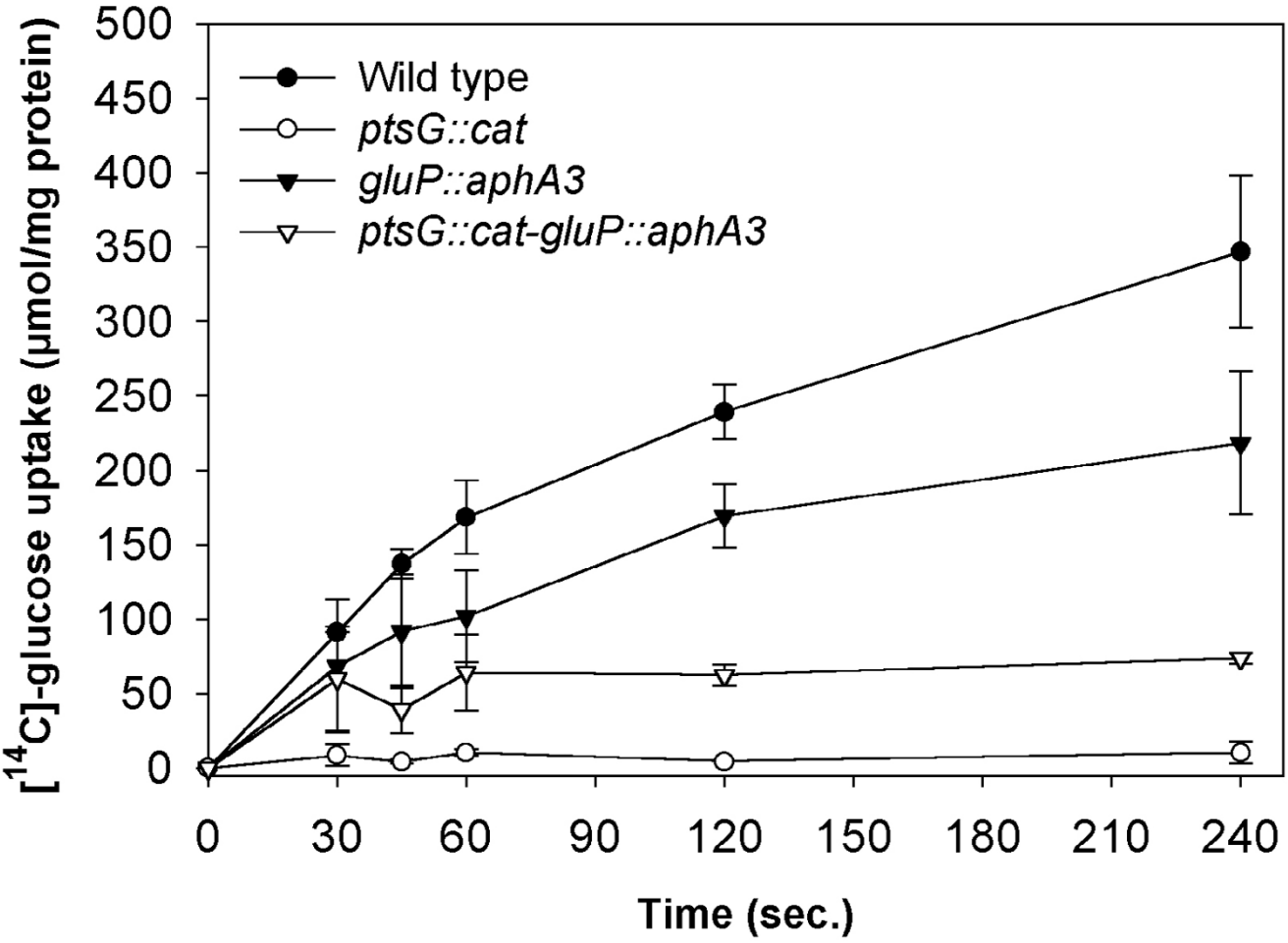


Figure 6
[^{14}C]-glucose uptake by GluP minus cells was slightly lower than the wild type's. Cells were grown in LB contraining 50 mM glucose. The *B. subtilis* strains used are indicated. Standard deviation of two independent experiments is indicated.

much lower [^{14}C]-glucose concentration (i.e. ~80% at 120 s) than the wild type cells (Fig. 6). These mutant cells were importing glucose only at a limited [^{14}C]-glucose concentration, i.e. ~50 µmol/mg protein, across the time points started at 30 s (Fig. 6). The phenomenon here may be due to the role of non-PTS dependent glucose uptake as suggested by Paulsen *et al.* (1998) [7].

GluP plays a role in the export of glucose

Once we had found out that GluP was affecting glucose uptake, we wondered whether GluP might contribute to glucose export. In order to understand this mechanism, we set up a more complex experiment involving multiple mutations in the PTS pathway and glucose metabolism. We added trehalose in all culture media, so that we could measure the glucose content in the growth medium if there was some export of cytoplasmic glucose.

As we reported previously, trehalose was imported via enzyme II^{Tre} of the PTS and was phosphorylated into trehalose 6-phosphate [29-31] (Fig 7A). Phosphorylation of trehalose was subsequently followed by hydrolysis of trehalose 6-phosphate into glucose and glucose 6-phosphate [29-31]. The hydrolysis of trehalose 6-phosphate was catalyzed by a cytoplasmic phospho-α-1, 1-glucosidase (TreA) [29-31]. The glucose was then phosphorylated by glucokinase (GlcK) [8,10]. Interestingly, we observed that there was some glucose detected in the growth medium [9]. This glucose could be transported back into the cytoplasm and phosphorylated by *ptsG*-encoded enzyme II^{Glc} or via non-PTS' GlcP. A summary of the glucose

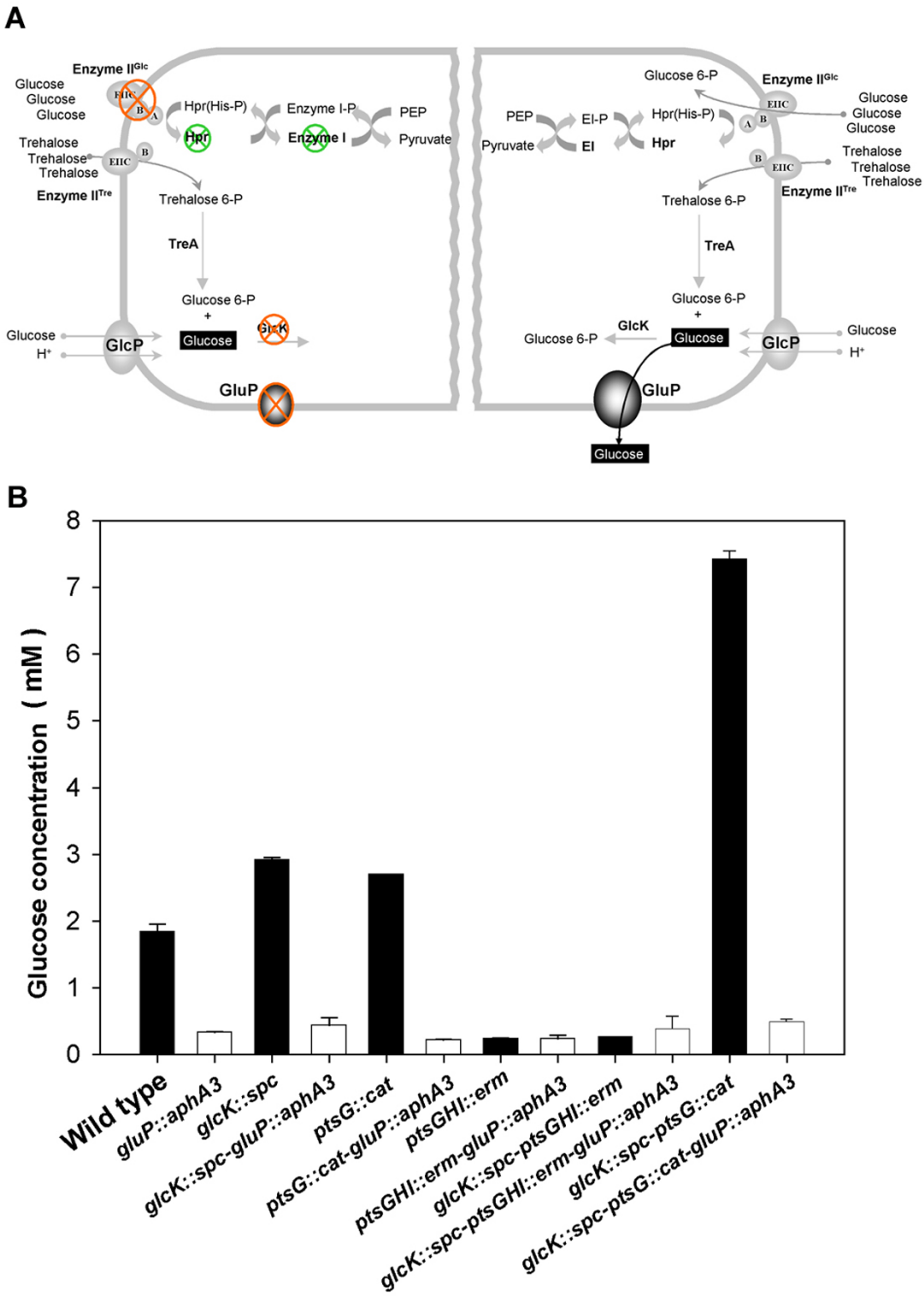


Figure 7
GluP contributes to glucose export. (A) A model of import-export of glucose by *B. subtilis* depicts the role of GluP as glucose exporter, as shown by right panel. Left panel indicates single or multiple eliminations (red or green cross) of proteins used in this study. Green crosses were part *ptsGHI::erm* mutation. (B) Glucose content in 50 mM trehalose supplemented LB medium of the wild type cells or various genotypes of *B. subtilis*. Standard deviation of two independent experiments is indicated.

metabolism and transport is depicted on the right panel of Fig. 7A.

Cells were grown for six hours on trehalose supplemented medium, before we were able to detect a considerable amount of glucose in the growth media of wild type, GlcK (*glcK*::*spc*) minus, and enzyme II^{Glc} (*ptsG*::*cat*) minus cells (Fig. 7B). The latter mutant cells exported about the same amount of glucose as the wild type, suggesting a balance between phosphorylation of glucose and hydrolysis of trehalose 6-phosphate. The glucose content was even greater, i.e. ~7 mM, in the growth medium of cells lacking both GlcK and enzyme II^{Glc} (Fig. 7B). This was probably due to the accumulation of the glucose byproduct from trehalose 6-phosphate hydrolysis plus the recycling of glucose that was imported by GlcP (Fig. 7A, left panel). These observations hinted us to hypothesize that GluP probably carried out the export of glucose. This was likely to be the case, since the abrogation of GluP resulted in a very low amount of glucose in the growth medium of GluP minus cells (Fig. 7B). As a negative control, we used *B. subtilis* QB6020, which lacked enzyme II^{Glc} (encoded by *ptsG*), enzyme I (*ptsI*), and HPr (*ptsH*). Cells carrying the *ptsGHI*::*erm* mutation or PTS minus cells were unable to take up trehalose, and therefore their growth medium contained very little glucose (Fig. 7B). Cells carrying multiple mutations, i.e. *glcK*::*spc-gluP*::*aphA3*, *ptsG*::*cat-gluP*::*aphA3*, *ptsGHI*::*erm-gluP*::*aphA3*, *glcK*::*spc-ptsGHI*::*erm*, *glcK*::*spc-ptsGHI*::*erm-gluP*::*aphA3*, or *glcK*::*spc-ptsG*::*cat-gluP*::*aphA3*, had only very small amounts of glucose in their growth media (Fig. 7B). These results are strong evidence for the contribution of GluP in the export of glucose. It is possible that GluP may act as a glucose exporter.

Discussion

B. subtilis GluP is clearly an interesting molecule. Firstly, the protein consists of a putative rhomboid domain (probably located within transmembrane helices) and two C-terminus TPR motifs. Based on protein structure-function relationships, the rhomboid family may control many aspects of growth and development [32-35]. Rhomboids are thought to have evolved in bacteria and were later acquired by archaea and eukaryotes through several independent horizontal gene transfers [21]. Subsequent evolution of the rhomboid family in eukaryotes took place through multiple duplications and functional diversification, i.e. by addition of extra transmembrane helices [21]. It is intriguing that eukaryotic rhomboid protein, evolved from the bacterial membrane transporter, gained intracellular membrane protease properties. The first rhomboid family domain was actually identified in *Drosophila melanogaster* roughoid (Rho), and is involved in the epidermal growth factor (EGF)-dependent signaling pathways [18,36,37]. Inactivation of both Rho-1 and Rho-3 was lethal due to loss of the EGF receptor [32]. The loss of EGFR caused the absence of MAP Kinase pathway, which led to failure in cell differentiation [32]. In simpler organisms such as bacteria, the inactivation of rhomboid protein AarA of *Providencia stuartii* resulted in abnormal cell division [34]. Here we also provide evidence that GluP minus cells were defective in their ability to separate during cell division, resulting in a filamentous phenotype on both rich and minimal medium (Fig. 4, Fig. 5). Therefore, it is thought that this phenotype may be related to the role of the rhomboid domain.

On the other hand, the TPR motif was originally identified as a protein interaction module in yeast cell cycle proteins [38,39]. However, TPR motifs have since been shown to occur in proteins with diverse functions mediating a variety of different protein-protein interactions [24]. Four major complexes involving TPR proteins have been identified: (a) molecular chaperone complex, (b) anaphase promoting complex, (c) transcription repression complex, and (d) protein import complex [24]. Based on this functional deduction, the C-terminus containing TPR motif of GluP may interact with other unknown proteins.

As we have mentioned earlier, GluP is among the first combined rhomboid-TPR proteins reported. Interestingly, the seven other bacterial rhomboid-TPR proteins are restricted to Gram-positives. Although we have shown the possible function of GluP in glucose export, the implication of combined rhomboid domain and TPR motifs toward a protein function is still not understood.

The second interesting aspect of GluP is its role in glucose transport, particularly as an exporter. We now know that at least two genes, *gluP* and *glcK*, within glucokinase operon contribute to glucose utilization. Whether GluP and GlcK are able to have direct interaction remains unclear. The balance of transport reactions involves the export of metabolic end products and the import of exogenous substrates [4]. Glucose can re-enter the cell via the glucose PTS' enzyme II^{Glc}, which in turn triggers a regulatory cascade involving CcpA, HPr, and the HPr kinase leading to carbon catabolite repression [9]. The glucose-transporting PTS, which is bacterial specific, plays important roles in transport (import), metabolism, and transcriptional regulation [2]. Another method of re-importing glucose into the cytoplasm is via a non-PTS pathway, which involves GlcP (Fig. 7A). The expression of *glcP* may be induced by the presence of glucose 6-phosphate as a result of glucose phosphorylation either by enzyme II^{Glc} or GlcK [5,7,8]. However, the GlcP minus cells imported only about 30 % less glucose than the wild type [7]. There was no obvious detectable effect on the glucose uptake between cells harboring the *ptsGHI* mutation and cells carrying double mutations in *ptsGHI* and *glcP* [7]. This suggests that the major player in glucose

uptake was the PTS rather than the non-PTS. Therefore it is not surprising that glucose was found in much higher concentrations in the growth medium of enzyme II^{Glc}-GlcK minus *B. subtilis* cells than in enzyme II^{Glc} minus, GlcK minus, or the wild type cells alone (Fig. 7B). Single or multiple mutations in the glucose metabolism and transport pathways provided strong evidence for the possible role of GluP in exporting glucose. Since GluP itself may acts as an exporter, we therefore, would like to propose GluP as a new member of the sugar transporter family.

Conclusions

Our results confirmed that GluP is located within *B. subtilis* glucokinase operon and is putatively regulated by σ^A-dependent promoter. Together with two other genes within the operon, *yqgQ* and *glcK*, the transcription of *gluP* was prominent during the exponential growth phase (T_{-2}) of *B. subtilis*. In the course of transition into stationary growth phase ($T_0 - T_2$), the transcription of *gluP* was down regulated. However, another putative transcription factor, σ^H, may bind its recognition site within *gluP*, and therefore, maintain the transcript levels of *yqgQ* and *glcK* during the transition growth phase (T_0). Transcription of the glucokinase operon seems to be completely down regulated once the cells enter the stationary growth phase (T_2). The importance of *gluP* expression during the exponential growth phase may be linked to the role of the product, GluP, in cell division. GluP may contain very interesting structural signatures: rhomboid domain and two TPR motifs. It would be intriguing to elucidate the role of these signatures in the bacterial cell cycle. Nevertheless, this is among the first reports of a protein with combined rhomboid-TPR signatures. Microscopic analysis showed that GluP minus cells were unable to divide normally and had lower sporulation frequencies than the wild type. GluP, which is likely located within the transmembrane helices, may be important for normal cell division. Another possible role of GluP is in glucose transport. We obtained evidence that the import of glucose was slightly reduced in the absence of GluP. However, whether this was due to incomplete cell division is yet to be confirmed. More striking evidence of GluP function was a direct correlation with glucose export. Cells lacking GluP were unable to export glucose. Therefore, GluP can be categorized as one of the glucose transporters. Our results also showed that *gluP* is the second gene within the glucokinase operon, besides *glcK*, which has a possible role in glucose utilization.

Methods

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. *B. subtilis* and *E. coli* strains were grown at 37°C on LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) and supplemented with antibiotics whenever required, e.g. 5 μg ml^{-1} chloramphenicol, 100 μg ml^{-1} ampicilin, 6 μg ml^{-1} phleomycin, 50 μg ml^{-1} spectinomycin, 25 μg ml^{-1} kanamycin, 1 μg ml^{-1} erythromycin, 25 μg ml^{-1} lincomycin or 10 μg ml^{-1} tetracycline. Minimal medium C [40] containing 50 μg ml^{-1} tryptophan, 11 μg ml^{-1} ferric citrate, 2 mM magnesium chloride, 1 mM calcium chloride, and 20 mM potassium glutamate was used. Sporulation was observed in NB medium (Oxoid, England, UK) which contained 0.1 % Lab-Lemco powder, 0.2 % yeast extract and 0.5 % NaCl.

Construction of transcriptional glucokinase operon – lacZ fusions

Primers used to clone different glucokinase operon fragments for transcriptional *lacZ* fusions are listed in Table 2. We replaced *glcK* with *lacZ*. *B. subtilis* chromosomal DNA was used as a PCR template. The amplified DNA was digested with *Eco*RI and *Bam*HI and ligated into the corresponding cloning site of plasmid pAC6, which is flanked by partial *amyE* gene fragments [41]. The resulting plasmid construct was linearized and then transformed into *B. subtilis* using the method as described by Kunst *et al.* (1994) [42]. Chromosomal integration of the linearized plasmid containing gene fusion into *amyE* of *B. subtilis* was achieved by homologous recombination. Therefore, transformants selected on the appropriate antibiotic were tested for the loss of α-amylase activity on LB starch (1%) plates (Table 1).

β-galactosidase assay

β-galactosidase activity was assayed qualitatively on LB plates containing 200 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). β-galactosidase activity was also determined as described by Miller (1972) [43] with modification as described by Msadek (1990) [44]. This quantitative assay used *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The enzyme activity is described as units per milligram total protein.

Northern blotting

Total RNA of *B. subtilis* 168 was isolated and purified using RNeasy total RNA kit (Qiagen, Hilden, Germany). The hybridization probe was obtained by amplifying a 449-bp DNA fragment of *glcK*, using plasmid pMD496 as DNA template. The following set of primers used was 5'-gctgcgctcggggaaatgtg and 5'-gatacgccgccgccaagaac. An 11 μg denatured total RNA sample was run in 1.3 % agarose-formaldehyde gel and was transferred to nylon membrane. The membrane was pre-hybridized for 2 h. Afterward, the membrane was incubated in hybridization solution containing denatured ^{32}P-labeled DNA probe for 24 h at 42°C [45]. The membrane was then washed once in washing buffer I for 15 min at RT, twice in washing buffer II for 45 min at 65°C, and finally in washing buffer

Table 1: Bacterial strains used in this study

Strain	Relevant genotype/phenotype	Reference/source
B. subtilis		
168	*trpC2* (wild type)	BGSC[a], 1A1
MD153	*trpC2 ptsG::cat*	[40]
MD186	*trpC2 glcK::spc*	[9]
MD189	*trpC2 glcK::spc ptsGHI::erm*	[9]
MD191	*trpC2 glcK::spc ptsG::cat*	[9]
MD195	*trpC2 amyE::(cat-lacZ)*	pAC6 tf> 168[b]
MD196	*trpC2 amyE::*(2112 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P01 tf> 168[b]
MD197	*trpC2 amyE::*(1994 bp *Eco*RI-*Bam* HI fragment-*lacZ*, *cat*)	pLR-P011 tf> 168[b]
MD198	*trpC2 amyE::*(1673 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P012 tf> 168[b]
MD199	*trpC2 amyE::*(974 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P013 tf> 168[b]
MD202	*trpC2 amyE::*(659 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P014 tf> 168[b]
MD204	*trpC2 amyE::*(464 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P021 tf> 168[b]
MD205	*trpC2 amyE::*(1163 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P031 tf> 168[b]
MD206	*trpC2 amyE::*(1046 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P032 tf> 168[b]
MD207	*trpC2 amyE::*(1308 bp *Eco*RI-*Bam*HI fragment-*lacZ*, *cat*)	pLR-P015 tf> 168[b]
MD229	*trpC2 gluP::aphA3*	pLM-YqgP-Km1 tf> 168[b]
MD230	*trpC2 glcK::spc gluP::aphA3*	pLM-YqgP-Km1 tf> MD186[b]
MD231	*trpC2 glcK::spc ptsGHI::erm gluP::aphA3*	pLM-YqgP-Km1 tf> MD189[b]
MD232	*trpC2 ptsG::cat gluP::aphA3*	pLM-YqgP-Km1 tf> MD153[b]
MD233	*trpC2 glcK::spc ptsG::cat gluP::aphA3*	pLM-YqgP-Km1 tf> MD191[b]
MD234	*trpC2 ptsGHI::erm gluP::aphA3*	Chr. DNA QB6020 tf> MD229[b]
QB6020	*trpC2 ptsGHI::erm*	[51]
E. coli		
TG1	*supE hsd*△ 5 *thi*△ (*lac-proAB*) F'(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*△ M15	[52]

[a]BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio [b]tf> denotes transformation of linear plasmid DNA in indicated strain.

III for 15 min at RT [45]. Autoradiograph was captured on Phosphoimager BAS-1500 (Raytest, Straubenhardt, Germany), after exposing the membrane for 1 h on a film image plate BAS-IIIS (Fuji, Japan).

Construction of B. subtilis gluP minus cells

Cloning of the complete *gluP* gene was only successful when a low copy number plasmid, pRK415, was used in *E. coli*. *B. subtilis* chromosomal DNA was used as a PCR amplification template. The primers used were 5'-cgcggatcgcgcggtttctgccgtcatgt and 5'-cggggtacctttcctcaccatttcttg. These primers recognized sequences between 235-bp upstream and 109-bp downstream of the *gluP* open reading frame. The amplified 1877-bp DNA fragment was then ligated into *Bam*HI and *Kpn*I sites of pRK415, resulting in plasmid pLR-YqgP [46]. A suicide plasmid carrying *gluP* mutation (pLM-YqgP-Km1) was created by inserting a 1497-bp *aphA3* cassette, obtained from *Sma*I-*Stu*I digestion of plasmid pDG792 [47], into the *Bst*1107I site of *gluP*. Using the same set of primers and the pLM-YqgP-Km1 as template, a 3374-bp PCR fragment was then obtained. The orientation of the *aphA3* cassette in direction with the *gluP* was determined by *Eae*I digestion resulting in 1244-bp and 2130-bp DNA fragments.

Construction of double or multiple mutations within B. subtilis gluP, glcK, ptsG, and/or ptsGHI

B. subtilis 168 (BGSC, Ohio State University, Columbus, Ohio, US) was transformed with a linearized 3374-bp pLM-YqgP-Km1 to generate the strain MD229 carrying the *gluP::aphA3*. The GlcK and GluP minus strain MD230 was obtained by transformation of strain MD186 [9] with the linearized pLM-YqgP-Km1. The enzyme II^{Glc}-GluP minus strain MD232 was obtained by transformation of strain MD153 [9] with the pLM-YqgP-Km1. The enzyme II^{Glc}, GlcK, and GluP minus strain MD233 was obtained by transformation of strain MD191 [9] with the pLM-YqgP-Km1. The enzyme II^{Glc}, enzyme I, HPr, GlcK, and GluP minus strain MD231 was obtained by transformation of strain MD189 [9] with the pLM-YqgP-Km1. All GluP minus transformants were selected on LB medium containing 0.5 % glucose and 25 μg ml^{-1} kanamycin. The enzyme II^{Glc}, enzyme I, HPr, and GluP minus strain MD234 was obtained by transformation of strain MD229 with total chromosomal DNA of *B. subtilis* QB6020 [9]. The MD234 was selected on LB medium containing 0.5 % glucose, 25 μg ml^{-1} kanamycin, 1 μg ml^{-1} erythromycin, and 25 μg ml^{-1} lincomycin. The validation of each transgenic *B. subtilis* strains was done by PCR analysis of the corresponding chromosomal DNA.

Table 2: Plasmids and primers used in this study

Plasmids	Relevant characteristics	Primers	Reference/source
pAC6	Vector for transcriptional *lacZ* fusions		[41]
pLR-P01	pAC6 *Eco*RI *Bam*HI; 2117 bp *Eco*RI-*Bam*HI fragment[a]	5'atgccattttcgcggtttct3'[c] 5'cgcggatccgtcgccggttttatctgtcg3'[d]	this study
pLR-P011	pAC6 *Eco*RI *Bam*HI; 2000 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgcggtaaacatgtttttgc3' 5'cgcggatccgtcgccggttttatctgtcg3'[d]	this study
pLR-P012	pAC6 *Eco*RI *Bam*HI; 1679 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgagaagggaactgtgtcag3'[c] 5'cgcggatccgtcgccggttttatctgtcg3'[d]	this study
pLR-P013	pAC6 *Eco*RI *Bam*HI; 980 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcggacatatcggcggcttga3'[c] 5'cgcggatccgtcgccggttttatctgtcg3'[d]	this study
pLR-P014	pAC6 *Eco*RI *Bam*HI; 674 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgcgggtttccctttttggaac3'[c] 5'cgcggatccgtcgccggttttatctgtcg3'[d]	this study
pLR-P015	pLR-PO1 derivative *Bst*BI; 1313 bp fragment[b]	-	this study
pLR-P02	pAC6 *Eco*RI *Bam*HI; 352 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgcggtaaacatgtttttgc3'[c] 5'cgcggatccctgacacagttcccttctc3'[d]	this study
pLR-P03	pAC6 *Eco*RI *Bam*HI; 730 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgagaagggaactgtgtcag3'[c] 5'cgcggatcctcaagccgccgatatgtcc3'[d]	this study
pLR-P04	pAC6 *Eco*RI *Bam*HI; 346 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcggacatatcggcggcttga3'[c] 5'cgcggatccgttccaaaagggaaaccgc3'[d]	this study
pLR-P021	pAC6 *Eco*RI *Bam*HI; 469 bp *Eco*RI-*Bam*HI fragment[a]	5'atgccattttcgcggtttct3'[c] 5'cgcggatccctgacacagttcccttctc3'[d]	this study
pLR-P031	pAC6 *Eco*RI *Bam*HI; 1168 bp *Eco*RI-*Bam*HI fragment[a]	5'atgccattttcgcggtttct3'[c] 5'cgcggatcctcaagccgccgatatgtcc3'[d]	this study
pLR-P032	pAC6 *Eco*RI *Bam*HI; 1051 bp *Eco*RI-*Bam*HI fragment[a]	5'ccggaattcgcggtaaacatgtttttgc3'[c] 5'cgcggatcctcaagccgccgatatgtcc3'[d]	this study
pLR-YqgP	pRK415 *Bam*HI *Kpn*I; 1877-bp*Bam*HI *Kpn*I fragment[a]	5'cgcggatcgcgcggtttctgccgtcatgt3'[c] 5'cggggtacctttcctcaccatttcttg3'[d]	this study
pLM-YqgP-Km1	pLR-YqgP derivative; *gluP*::*aphA3* Km^r	-	this study
pRK415	Tc^r, low-copy number plasmid		[46]

[a]Size of the cloned PCR fragment after digestion with restriction enzyme as indicated [b]The deletion within the 2112 bp DNA fragment of the pLR-P01, which is digested by *Bst*BI and religated [f]Forward primer; [d]Reverse primer

Determination of glucose content in the cell culture supernatant

Glucose concentration in the growth medium was determined using the Glucose (HK) Assay Reagent (Sigma, Deisenhofen, Germany) according to the manufacturer's suggestion [9]. In brief, 10 µl of cell culture supernatant was mixed with 1.0 ml of the assay reagent and incubated for 5 min at 37°C. Glucose concentration in mM is equal to $[(A_{340}\,\text{Sample}-A_{340}\,\text{Blank})*2.93]$ (mg. ml^{-1}) * 0.05555.

Glucose uptake assay

B. subtilis strains 168, MD153, MD229, and MD232 were grown until an OD_{600} of 1.2 in LB medium supplemented with 50 mM glucose. A 25 ml cell culture was centrifuged. The pellet was then washed three times with ice-cold TM buffer (50 mM Tris-Cl and 20 mM $MgCl_2$, pH 7.2). The cells were adjusted to an OD_{600} equal to 1.0. The uptake of [^{14}C]-glucose by *B. subtilis* cells was assayed at 37°C. [^{14}C]-glucose was added to a final concentration of 25 µM. 70 µl samples were taken at various time points (i.e. 0, 30, 45, 60, 120, and 240 seconds) filtered through cellulose nitrate filters (pore size: 0.45 µM), and washed three times with 5 ml TM buffer. Radioactivity of the wet filter was measured in a scintillation counter (Beckman, Fullerton, USA).

Phase contrast microscopy

Cell culture was prepared either by growing the cells at 37°C in LB or C minimal medium. A 25 µl sample of culture was added to the center of an object glass and then covered by a coverslip. Cells were monitored under a phase contrast microscope (Zeiss, Jena, Germany).

Cytochemistry analysis

Septum formation and chromosomal partitioning of *B. subtilis* wild type and the GluP minus strain MD229 were analyzed using the method described by Pogliano *et al.* [48]. Membrane dye, FM5-95 (Molecular Probe, US) or chromosomal DNA dye, bisbenzimide (Sigma, Deisenhofen, Germany) was added with final concentration of 1 µg ml^{-1} and 0.1 µg ml^{-1} onto fixed *B. subtilis* cell, respectively. The fixed cells were bordered with Dako-pen

(Dako, Glostrup, DK). The cells were then incubated for 15 min and were washed with PBS buffer thoroughly. The cells were then overlaid with mounting medium, elvanol (DuPont, Bad Homburg, Germany). Cells were observed under the Axiophot fluorescence microscope (Zeiss, Jena, Germany). The microscope was equipped with UV filter having an absorption wavelength of 360 nm and an emission wavelength of 461 nm to observe bisbenzimide staining cells. A Cy3 filter with an absorption wavelength of 514 nm and an emission wavelength of 566 nm or a rhodamine filter with an absorption wavelength of 550 nm and an emission wavelength of 573 nm was used for the observation of FM5-95 stained cells. Images were captured using CCD color video camera (Sony, Japan) and processed with AxioVision 2.0 program (Zeiss, Jena, Germany).

Sporulation assay

Sporulation frequency was determined according to Bohin *et al.* [49]. In short, cells were incubated on NB medium at 37°C with strong agitation. The cell growth was monitored with a spectrophotometer at 600 nm. End of the exponential growth was measured as time zero (T_0). At T_0 the viable cells (V_0) were counted as cfu.ml^{-1}. Twenty hours after T_0 (T_{20}), cells were harvested and heated for 10 min at 80°C. The number of spores per milliliter was then counted (S_{20}). The value of S_{20}/V_0*100 was taken as the sporulation frequency.

In silico *analysis of the GluP protein*

GluP sequence (GenBank BG11683), available from the *B. subtilis* genome project [11,12], was used to predict the protein structure. The two-dimensional structure of the transmembrane helices and the entire GluP protein's structure was elucidated using the TMHMM (v2.0) [19] and SOSUI [20]. The three-dimensional structure of the GluP TPR motif was constructed by Geno3D [25] using human PEX5 TPR motifs [26] as template. GluP related protein sequences from other organisms were retrieved from the GenBank database. These sequences were aligned using the MegAlign 4.0 program (DNAStar, Madison, USA). The multiple alignments were based on the Clustal method [50].

Authors' contributions

LRM carried out all aspects of the work and drafted the manuscript. FMM contributed in the cytochemistry and supervised the writing of the manuscript. MKD was supported by DFG and Fonds der Chemischen Industrie and supervised the experimental studies.

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