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Glucosylglycerate Phosphorylase, an Enzyme with Novel Specificity Involved in Compatible Solute Metabolism

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ABSTRACT Family GH13_18 of the carbohydrate-active enzyme database consists of retaining glycoside phosphorylases that have attracted interest with their potential for synthesizing valuable α-sugars and glucosides. Sucrose phosphorylase was believed to be the only enzyme with specificity in this subfamily for many years, but recent work revealed an enzyme with a different function and hinted at an even broader diversity that is left to discover. In this study, a putative sucrose phosphorylase from *Meiothermus silvanus* that resides in a previously unexplored branch of the family's phylogenetic tree was expressed and characterized. Unexpectedly, no activity on sucrose was observed. Guided by a thorough inspection of the genomic landscape surrounding other genes in the branch, the enzyme was found to be a glucosylglycerate phosphorylase, with a specificity never before reported. Homology modeling, docking, and mutagenesis pinpointed particular acceptor site residues (Asn275 and Glu383) involved in the binding of glycerate. Various organisms known to synthesize and accumulate glucosylglycerate as a compatible solute possess a putative glucosylglycerate phosphorylase gene, indicating that the phosphorylase may be a regulator of its intracellular levels. Moreover, homologs of this novel enzyme appear to be distributed among diverse bacterial phyla, a finding which suggests that many more organisms may be capable of assimilating or synthesizing glucosylglycerate than previously assumed.

IMPORTANCE Glycoside phosphorylases are an intriguing group of carbohydrate-active enzymes that have been used for the synthesis of various economically appealing glycosides and sugars, and they are frequently subjected to enzyme engineering to further expand their application potential. The novel specificity discovered in this work broadens the diversity of these phosphorylases and opens up new possibilities for the efficient production of glucosylglycerate, which is a remarkably potent and versatile stabilizer for protein formulations. Finally, it is a new piece of the puzzle of glucosylglycerate metabolism, being the only known enzyme capable of catalyzing the breakdown of glucosylglycerate in numerous bacterial phyla.

KEYWORDS glucosylglycerate phosphorylase, glycoside phosphorylase, sucrose phosphorylase, glycoside hydrolase family GH13

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Sucrose phosphorylase ([SP] EC 2.4.1.7) catalyzes the reversible phosphorolysis of sucrose into α-D-glucose 1-phosphate (Glc1P) and D-fructose (1). As the physiological concentration of phosphate is known to be higher than that of Glc1P, the enzyme is assumed to serve a catabolic function *in vivo* (2). Moreover, phosphorolysis of glycosidic bonds provides an advantageous shortcut in energy metabolism compared to hydrolysis. Glycosyl phosphates can readily enter the glycolytic pathway after having their phosphate groups transferred from the C-1 to the C-6 positions by a phosphomutase, thereby avoiding the need for activation by a hexokinase and saving one

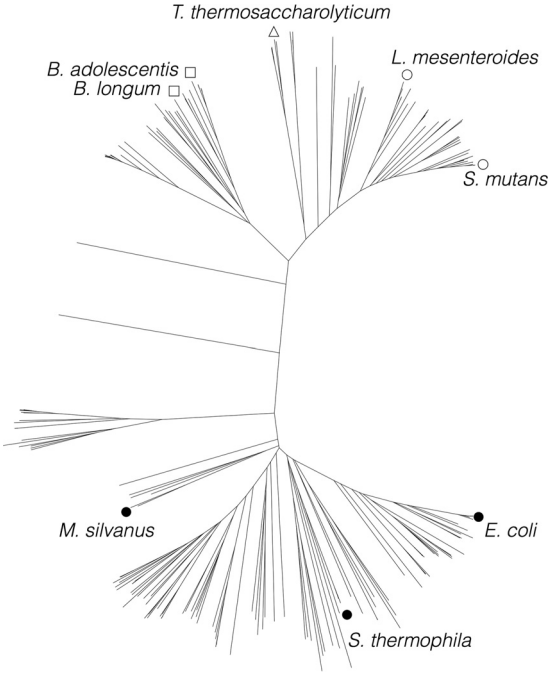

FIG 1 Phylogenetic tree of protein sequences classified in glycoside hydrolase family GH13_18. A selection of representatives described in the literature are indicated. Filled circles, glucosylglycerate phosphorylases (*M. silvanus*, UniProt identifier [ID] D7BAR0; *S. thermophila*, G0GBS4; *E. coli*, P76041); empty squares, *Bifidobacterium*-like sucrose phosphorylases (*B. adolescentis*, UniProt ID Q84HQ2; *B. longum*, Q84BY1); empty circles, lactic acid bacterium-like sucrose phosphorylases (*L. mesenteroides*, UniProt ID Q59495; *S. mutans*, P10249); empty triangle, *T. thermosaccharolyticum* sucrose 6′-phosphate phosphorylase (UniProt ID D9TT09).

molecule of ATP (3). *In vitro*, however, sucrose phosphorylase has attracted the most attention for its synthetic capabilities, producing a wide range of α-glucosides thanks to its broad substrate promiscuity. A noteworthy example of this is its use in the commercial production process of 2-*O*-(α-D-glucopyranosyl)-*sn*-glycerol, a moisturizing agent in cosmetic formulations known under the trade name Glycoin (4).

In the sequence-based carbohydrate-active enzyme (CAZy) database, sucrose phosphorylases are classified in subfamily 18 of glycoside hydrolase family 13 (GH13_18) (5, 6). While it was long thought that this subfamily consisted only of sucrose phosphorylases, recent work has indicated that GH13_18 might actually be more diverse than anticipated. Detailed kinetic characterization of a putative SP from *Thermoanaerobacterium thermosaccharolyticum* affirmed that the enzyme is actually a sucrose 6′-phosphate phosphorylase ([SPP] EC 2.4.1.329) (7). Furthermore, a thorough comparison of the acceptor sites in different branches of the subfamily's phylogenetic tree hinted at the existence of even more specificities. Finding such novel enzymes is not only interesting from a fundamental point of view by unveiling new metabolic pathways, but it can also offer new possibilities for practical applications. This was demonstrated in the case of SPP, for which a mutant could glucosylate a far broader range of bulky acceptors than any other SP known to date (8).

Curiously, the phylogenetic tree of all sequences classified in CAZy family GH13_18 comprises two major branches (Fig. 1) (7). One of these harbors all experimentally characterized sucrose phosphorylases, as well as all (putative) sucrose 6′-phosphate phosphorylases. However, not a single member from the other branch has been characterized in detail so far. Although these proteins are annotated as sucrose phosphorylases, we hypothesized that they may display a different function. In this work, we determined the properties of a representative enzyme from *Meiothermus silvanus*, a bacterium that is notorious for forming undesirable biofilms in the paper industry (9). Aided by a comprehensive scrutiny of the genomic landscape surrounding

these putative sucrose phosphorylases, the enzyme was discovered to be a glucosylglycerate phosphorylase. Glucosylglycerate belongs to a group of highly soluble molecules, named compatible solutes, which can accumulate in large amounts to protect the cell against fluctuating water activity while still remaining compatible with cellular functions. In addition to the glucosylglycerate phosphorylase reported here, glycoside hydrolases and glycosyltransferases have also been described as being involved in this compound's degradation and synthesis.

RESULTS

Inspection of genomic context and choice of target sequence. To gather clues about the substrate specificity of putative sucrose phosphorylases in the unexplored major branch of GH13_18 (Fig. 1), their genetic organization was examined. It became apparent that a few genes frequently surrounded the putative sucrose phosphorylases in the branch of interest, but not in the branch containing the characterized sucrose or sucrose 6′-phosphate phosphorylases. The genes encoding glycerate kinase, glucosyl 3-phosphoglycerate synthase (gpgS), and glucosyl 3-phosphoglycerate phosphatase (gpgP) are sometimes located adjacent to those of the putative sucrose phosphorylases. The last two are key enzymes in the two-step synthesis of the compatible solute glucosylglycerate ([GGa] R-2-*O*-α-D-glucopyranosyl-glycerate), where gpgS catalyzes the conversion of GDP-glucose and 3-phosphoglycerate into glucosyl 3-phosphoglycerate, which is subsequently dephosphorylated by gpgP (10). Also, putative SPs from organisms where free glucosylglycerate has been detected, such as *Prochlorococcus marinus*, *Chromohalobacter salexigens*, and *Synechococcus* sp. PCC7002, all reside in the clade from which no representatives have been characterized so far.

For this work, the genes originating from thermophilic sources were inspected in more detail, as thermostable proteins are beneficial for reducing enzyme turnover and allowing higher process temperatures (11). In the genome of *Meiothermus silvanus*, the operon adjacent to that of the phosphorylase contains a close homolog to a hydrolase of *Thermus thermophilus* HB27 (53% amino acid identity) that was previously discovered to be active on both glucosylglycerate and mannosylglycerate (12). Considering it is not uncommon for organisms to have concurrent hydrolytic and phosphorolytic pathways for the same substrates (13), this link further strengthened the assumption that the enzymes in the clade are not regular sucrose phosphorylases but may in fact be involved in glucosylglycerate metabolism. Therefore, the gene from *M. silvanus* was selected for expression and characterization.

Expression, purification, and substrate specificity of the putative sucrose phosphorylase from *M. silvanus*. The protein, provided with an N-terminal His_6 tag, was expressed in *Escherichia coli* and purified to apparent homogeneity (>95%) by mild heat treatment and nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography under optimized purification conditions. Although the protein was mainly present in the soluble fraction, expression was rather poor (~400 μg enzyme from a 500-ml culture medium), with comparable results using several different expression vectors and conditions (see Fig. S2 in the supplemental material). An analysis by SDS-PAGE (see Fig. S3) showed a single band with an apparent molecular mass of 63.0 kDa, which is in agreement with the theoretical mass deduced from the amino acid sequence.

Activity measurements showed that, quite unexpectedly, the putative sucrose phosphorylase could not catalyze the phosphorolysis of sucrose or synthesize sucrose from Glc1P and D-fructose. The measurements did show hydrolysis of Glc1P (0.10 ± 0.1 U/mg), proving that the enzyme was catalytically active and could bind the glucosyl moiety in subsite −1. Indeed, the hydrolysis of the donor substrate is a typical side activity inherent to the double-displacement mechanism of retaining glycoside phosphorylases (14). Considering the evident link with glycosylglycerate, D-glycerate was tested as an alternative acceptor substrate in the synthetic direction. The protein indeed showed high activity toward this compound, with a specific activity (110 ± 4 U/mg) that is in line with values of wild-type reactions from similar glycoside phosphorylases. True sucrose phosphorylases catalyze this conversion as well, albeit at

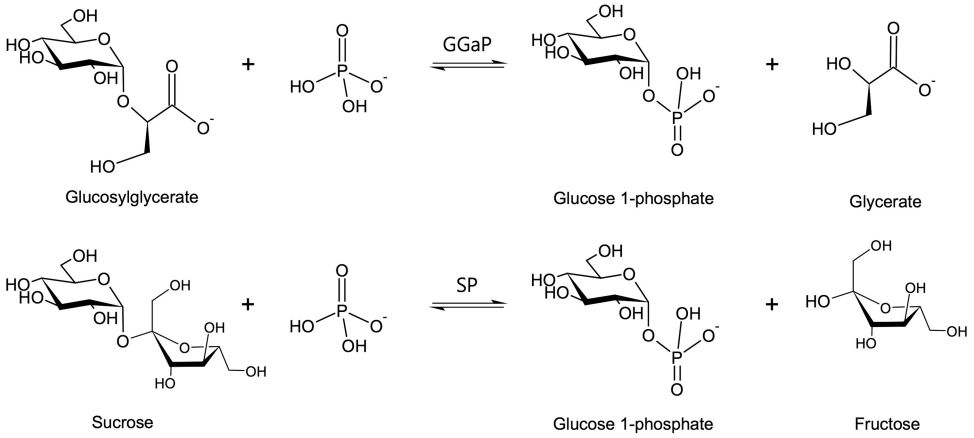

FIG 2 Reactions catalyzed by glucosylglycerate phosphorylase (GGaP) and sucrose phosphorylase (SP).

a much lower rate. For comparison, the same synthetic reaction was monitored with the SP from *Bifidobacterium adolescentis*, resulting in a specific activity of just 0.32 ± 0.05 U/mg. More than 50 additional acceptors were tested to further evaluate substrate flexibility, including various monosaccharides, disaccharides, oligosaccharides, sugar alcohols, substituted monosaccharides and acids (see Table S2). However, the enzyme was found to have a very strict specificity, showing significant activity only toward the enantiomer L-glycerate (7.5 ± 0.4 U/mg). No activity was detected on mannosylglycerate. The putative sucrose phosphorylase from *M. silvanus* can thus be designated as a glucosylglycerate phosphorylase (GGaP) (Fig. 2), an enzyme with a novel specificity that provides intriguing implications for our understanding of the metabolic pathways in which the compound is involved.

Optimal pH and temperature and kinetic properties. The optimal pH of *M. silvanus* GGaP (*Ms*GGaP) was 6 and 6.5 in phosphorolytic and synthetic directions, respectively, comparable to those of the other enzymes in family GH13_18 (Fig. 3). Its optimal temperature for activity was determined to be 42°C, which is at the lower end of the organism's growth range of 40 to 65°C (with an optimal growth rate at 55°C) (15). Furthermore, the thermostability of *Ms*GGaP was assessed by measuring the residual

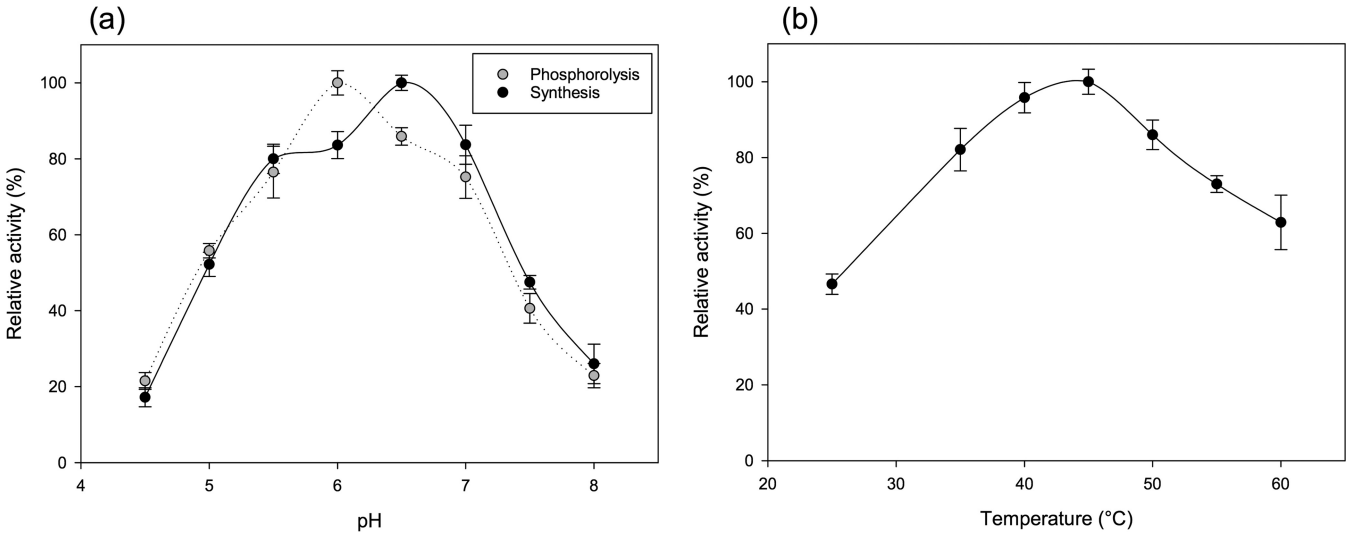

FIG 3 The effect of pH (a) and temperature (b) on *M. silvanus* glucosylglycerate phosphorylase activity. The pH profile was determined in the synthetic (black circles, 100% = 88 U/mg) and phosphorolytic (gray circles, 100% = 0.81 U/mg) directions. The temperature profile was determined in the synthetic direction (100% = 104 U/mg).

TABLE 1 Apparent kinetic parameters of *M. silvanus* glucosylglycerate phosphorylase[a]

Reaction	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phosphorolysis	Glucosylglycerate	3.5 ± 0.5	0.83 ± 0.17	0.24
	Phosphate	2.5 ± 0.4	0.98 ± 0.15	0.40
Synthesis	α-D-Glucose 1-phosphate	8.1 ± 0.6	96 ± 4	11.8
	D-Glycerate	2.6 ± 0.5	128 ± 6	49.4

[a]Parameters at 42°C and 50 mM MES pH 6.0 (for the degradation direction) or pH 6.5 (synthetic direction).

activity after incubating at various temperatures for 10 min. Full activity (105 ± 8 U/mg) was retained at up to 55°C, but only 35% was left after incubating at 60°C.

The apparent kinetic parameters of *Ms*GGaP were determined at an optimal pH and temperature, for the donor as well as for the acceptor, in both directions of the reversible reaction (Table 1; see also Fig. S4). The enzyme exhibited Michaelis-Menten kinetics at the tested substrate concentrations, and the Michaelis constants were in the same range as those of other phosphorylases in GH13_18 (7, 16, 17). The turnover numbers in the synthetic direction were consistent with data from other phosphorylases as well, although it is rather unusual that those in the phosphorolytic direction were considerably lower. Conversion starting from 200 mM Glc1P and D-glycerate was monitored until the substrate and product concentrations remained constant. The equilibrium constant K_{eq} at 42°C, calculated as ([Glc1P] × [D-glycerate])/([P_i] × [glucosylglycerate]) at the end of conversion, equaled 0.013 ± 0.004. The reaction equilibrium thus seems to be far on the side of glucosylglycerate, reflecting the great stability of this compound and explaining the lower rate of its phosphorolysis compared to its synthesis.

Homologous sequences. A BLAST search with the *Ms*GGaP sequence uncovered many homologous sequences in numerous bacterial genomes from different lineages. The closest homologs are found in other strains from the same genus, such as *M. ruber* (75% identity), but closely related genes are also present in species from the phyla *Chloroflexi* (e.g., *Caldilinea aerophila*, 56% identity), *Proteobacteria* (*Shigella sonnei*, 53% identity), *Planctomycetes* (*Rhodopirellula islandica*, 52% identity), *Firmicutes* (*Caloranaerobacter azorensis*, 52% identity), and *Spirochaetes* (*Spirochaeta thermophila*, 51% identity). More distant homologs are also found in the phyla of *Cyanobacteria* (e.g., *Lyngbya aestuarii*, 46% identity), *Nitrospirae*, *Bacteroidetes*, and many others. Interestingly, organisms also known to accumulate glucosylglycerate *in vivo* appear to possess a putative GGaP gene (Fig. 4). Furthermore, a distant homolog was also found in one unclassified archaeon species (UniProt identifier A0A0M0BH93, 40% identity). To verify the ubiquity of the novel glucosylglycerate phosphorylase specificity, the putative GGaPs from *Spirochaeta thermophila* and *Escherichia coli* were expressed and tested with a variety of substrates (Table S2). As expected, both enzymes catalyzed only the phosphorolysis and synthesis of glucosylglycerate and showed no activity on sucrose. The affinity toward D-glycerate was determined for these GGaPs and for three previously characterized representatives from the same family, which again demonstrated a clear difference between glucosylglycerate phosphorylases and sucrose or sucrose 6′-phosphate phosphorylases (Table 2). Because these GGaPs are scattered throughout the clade, this newly identified specificity may be very abundant within the GH13_18 family.

Homology modeling and docking. To expose possible interactions between *Ms*GGaP and its substrate glucosylglycerate, a homology model of the enzyme was constructed using the crystal structure of sucrose phosphorylase from *B. adolescentis* as the template. Afterwards, the binding of GGa was simulated by automated docking (Fig. 5). All residues in subsite −1 that interact directly with the glucose moiety are identical between sucrose phosphorylase and glucosylglycerate phosphorylase (see Fig. S5), thereby explaining the lack of activity of *Ms*GGaP on mannosylglycerate. Moreover, two notable interactions in the acceptor site could be inferred. Asn275, located near the general acid/base catalytic residue, appears to be in a position

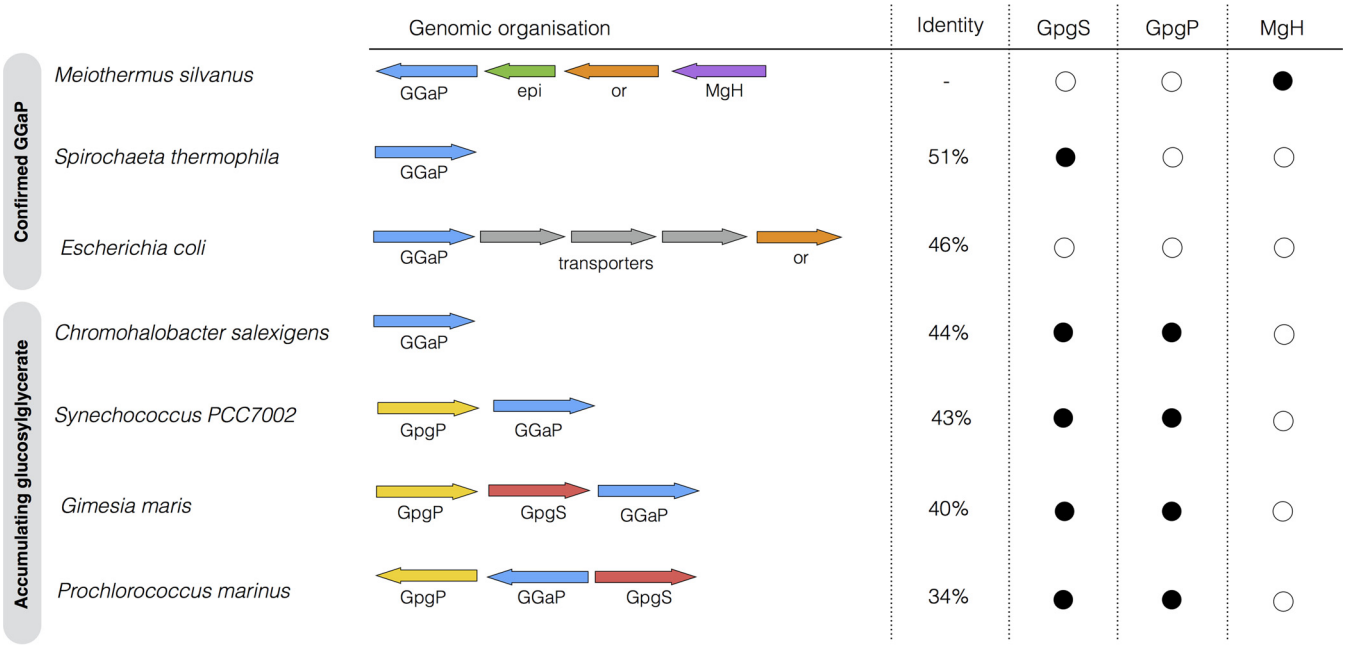

FIG 4 Genomic organization of confirmed glucosylglycerate phosphorylases (GGaP) and several putative GGaPs from organisms known to accumulate glucosylglycerate *in vivo*. epi, carbohydrate epimerase; or, oxidoreductase; MgH, mannosylglycerate hydrolase; GpgS, glucosyl 3-phosphoglycerate synthase; GpgP, glucosyl 3-phosphoglycerate phosphatase. Also shown is the amino acid identity compared to *M. silvanus* GGaP and the presence (filled circle) or absence (open circle) of relevant genes in the genome.

favorable for hydrogen bonding with the glycerate moiety. It is completely conserved in putative GGaPs, whereas a histidine is present in all sucrose phosphorylases at the corresponding positions. A second interaction may be established by the conserved Glu383 in loop A, a flexible region that is known to be important for recognition of the acceptor moiety in sucrose phosphorylases (18). There, this position is occupied by Gln and forms a hydrogen bond with O-3 and O-6 of fructose (19). Mutagenesis was performed to confirm the importance of these residues. Both positions were mutated to alanines to remove their side chains without affecting the main chain conformation and also to correspond to the amino acid in the sequence of *B. adolescentis* SP. Mutants N275A and N275H retained specific activities of 1.5 and 2.9 U/mg, respectively, compared with 110 U/mg for the wild-type enzyme. An even more drastic decrease was observed with E383A and E383Q, both showing an activity of less than 1 U/mg.

DISCUSSION

Glucosylglycerate (GGa) was long considered to be a rare glycoside and had only been detected in a select few organisms. However, new developments have shown that it is in fact a widespread compatible solute, primarily protecting organisms facing salt stress and very specific nutritional constraints. Furthermore, it is a precursor for the synthesis of several macromolecules that sometimes provide a vital function in their

TABLE 2 Affinity of phosphorylases from family GH13_18 for D-glycerate[a]

UniProt ID	Organism	Specificity	$K_{m\ \text{D-glycerate}}$ (mM)
D7BAR0	*M. silvanus*	GGaP	2.6 ± 0.5[b]
G0GBS4	*S. thermophila*	GGaP	0.8 ± 0.3[b]
P76041	*E. coli*	GGaP	3.4 ± 0.9[c]
Q84HQ2	*B. adolescentis*	SP	353 ± 78[b]
P10249	*S. mutans*	SP	175 ± 58[c]
D9TT09	*T. thermosaccharolyticum*	SPP	162 ± 71[b]

[a]Measurements were carried out in 50 mM MES pH 6.5 with 50 mM Glc1P as the cosubstrate.
[b]Determined at 42°C.
[c]Determined at 30°C.

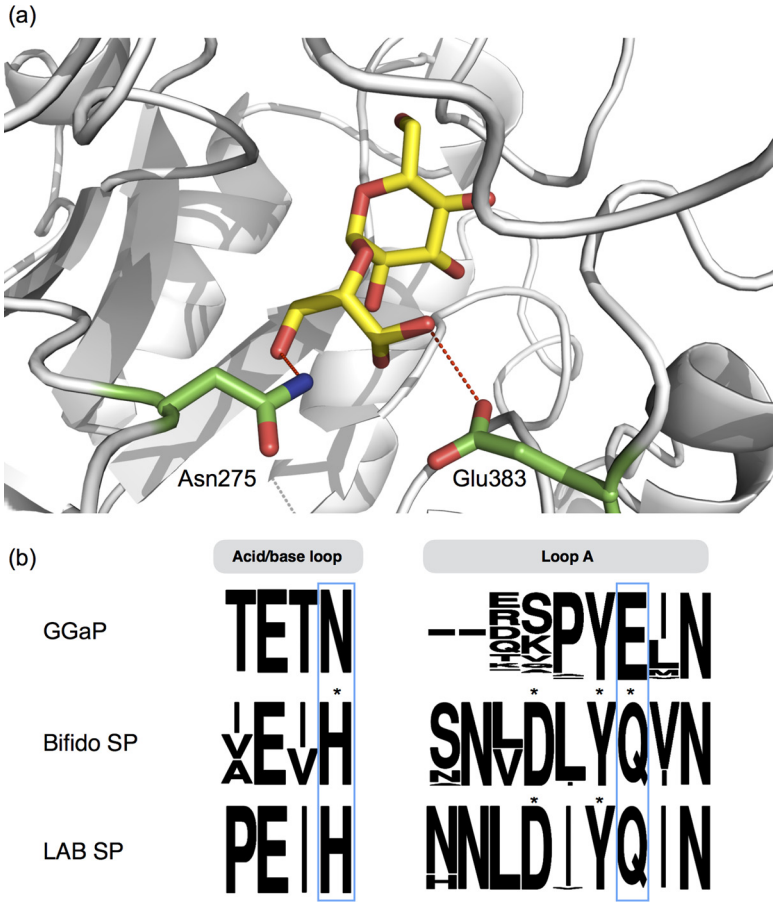

FIG 5 (a) Docking of glucosylglycerate in the homology model of *M. silvanus* glucosylglycerate phosphorylase. (b) Sequence logos of the loop containing the general acid/base catalyst and loop A for all putative glucosylglycerate phosphorylases (GGaP), *Bifidobacteriaceae* sucrose phosphorylases (Bifido SP), and lactic acid bacteria sucrose phosphorylases (LAB SP). Residues previously confirmed to be involved with specificity are indicated by asterisks.

host (20, 21). The metabolic routes in which GGa is involved remained unknown for decades after its discovery, until two widely distributed biocatalysts were found to synthesize it from nucleoside diphosphate (NDP)-glucose and 3-phosphoglycerate through a glucose 3-phosphoglycerate intermediate (Fig. 6). A second single-step process wherein glucosylglycerate synthase catalyzes the direct condensation of NDP-glucose and D-glycerate was described not long after, although this enzyme appears to be rather rare. Finally, the fact that putative sucrose phosphorylases are sometimes associated with GGa-synthesizing proteins while also being capable of transferring the glucosyl moiety of sucrose to D-glycerate had raised speculation that they may constitute a third synthetic pathway (20). This hypothesis has never been verified *in vivo*, however.

In this study, the putative sucrose phosphorylase from the moderately thermophilic bacteria *Meiothermus silvanus* was cloned and characterized. The enzyme did not recognize sucrose at all, and extensive substrate screening revealed that it strictly catalyzes the reversible phosphorolysis of glucosylglycerate. The enzyme thus presents a novel specificity, i.e., glucosylglycerate phosphorylase, for which no EC number is available at this time. The confirmed glucosylglycerate phosphorylases from *M. silvanus*, *E. coli*, and *S. thermophila*, as well as the homologs from organisms known to accumulate GGa, all reside in a distinct large branch in the phylogenetic tree of CAZy family GH13_18. All actual sucrose phosphorylases are found in a different branch. The GH13_18 enzymes that were presumed to have some sort of connection with GGa

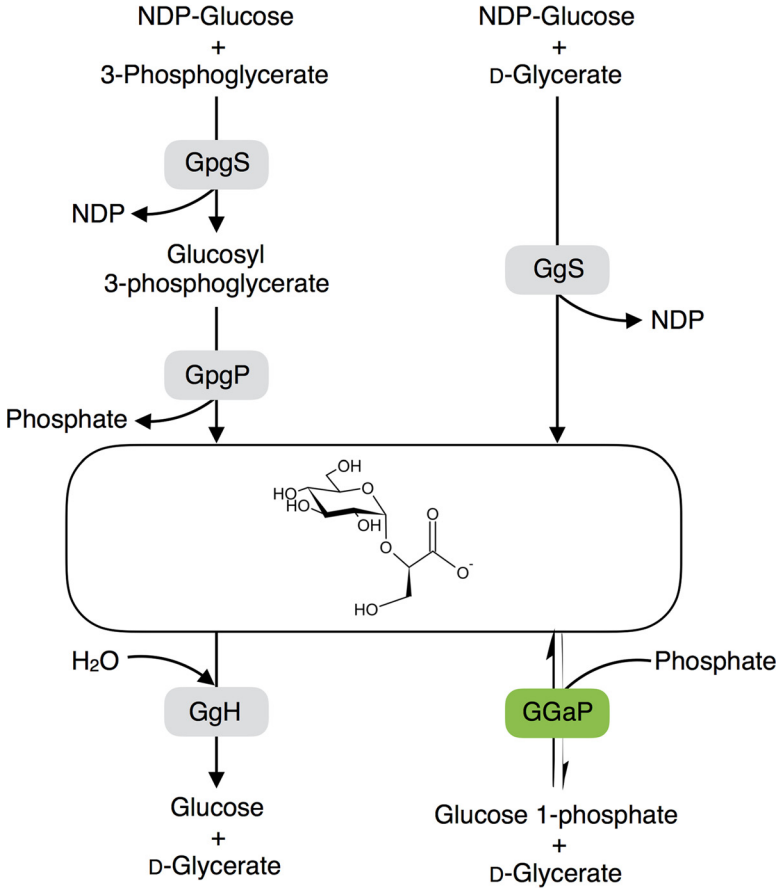

FIG 6 Summary of enzymes involved in glucosylglycerate metabolism that have been discovered so far. GpgS, glucosyl 3-phosphoglycerate synthase; GpgP, glucosyl 3-phosphoglycerate phosphatase; GgS, glucosylglycerate synthase; GGaP, glucosylglycerate phosphorylase; GgH, glucosylglycerate hydrolase.

metabolism due to their genomic context are thus likely glucosylglycerate phosphorylases rather than sucrose phosphorylases. The fact that sucrose phosphorylase is also capable of using D-glycerate as an acceptor in the synthetic direction (22) is not at all surprising in light of its renowned acceptor promiscuity, and assuming that this activity has any metabolic relevance would be premature. However, it is definitely tempting to speculate that the GGaP specificity may have emerged from specialization of this side activity of sucrose phosphorylase throughout evolution (23).

The question remains as to what the physiological function of GGaP could be. At this time, not a single glycoside phosphorylase is known to fulfill a synthetic role inside the cell. The intracellular phosphate concentration is expected to be much higher than that of Glc1P, because the latter can enter the glycolytic pathway easily. However, since GGa is typically accumulated under very specific stressful conditions, it cannot be excluded that the cellular environment favors the synthetic reaction under certain circumstances regardless. The poor k_{cat} in the phosphorolytic direction would be beneficial in this regard. Peculiarly, the operon adjacent to *Ms*GGaP already encodes a hydrolase dedicated to the breakdown of GGa. On the one hand, having both hydrolytic and phosphorolytic degradation routes may seem redundant, but nevertheless, some species do possess alternative pathways that accomplish similar goals (13, 24).

Proteins involved in glucosylglycerate assimilation have only been discovered very recently. A family GH63 glycoside hydrolase from *Thermus thermophilus* HB27 and its ortholog from *Rubrobacter radiotolerans* specifically hydrolyze glucosylglycerate as well as mannosylglycerate, a related osmolyte that is frequently observed in hyperthermophilic archaea and thermophilic bacteria (20). However, *T. thermophilus* and *Rubrobacter*

spp. accumulate only mannosylglycerate; therefore, this hydrolase was named mannosylglycerate hydrolase (MgH) (12). Conversely, a mycobacterial MgH homolog turned out to be highly specific toward GGa exclusively and was therefore designated glucosylglycerate hydrolase (GgH) (25). Many GgH homologs exist in the genomes of rapidly growing mycobacteria, but from all other organisms known to accumulate GGa *in vivo*, only *Persephonella marina* has one.

The distribution of these hydrolases degrading GGa is thus not in line with expectations. It would make sense that all organisms producing and accumulating the solute have a way of getting rid of it when conditions in the environment shift. Mycobacteria, for example, accumulate GGa during growth under nitrogen deprivation but rapidly deplete the compound by upregulating GgH transcription as soon as assimilable nitrogen is replenished (25). A similar behavior can be anticipated in other GGa-producing bacteria, which typically accumulate GGa to cope with salt stress when nitrogen is scarce. Since the genomes of such organisms often contain a glucosylglycerate phosphorylase homolog, it is possible that this enzyme is a regulator of intracellular glycoside levels. The transcript of a putative GGaP in *Gimesia maris* does get pronouncedly more abundant under nitrogen-limiting conditions, hinting at some role within this metabolic context (26). In addition, extensive turnover of GGa was observed very recently in *Synechococcus sp.* PCC7002 by isotope probing (27). This finding was very surprising considering the lack of any known metabolic sinks for GGa in this organism. It turns out that *Synechococcus sp.* PCC7002 has a GGaP homolog that harbors the same sequence motifs in the acceptor site that are characteristic of *Ms*GGaP and might thus possibly be responsible for the observed turnover.

Another way of reducing the intracellular stocks of compatible solutes is to release them to the environment. When osmoprotectants are accumulated by microorganisms to compensate for high extracellular osmolality, sudden hypoosmotic shock caused by events such as rain or washout into freshwater sources can quickly trigger efflux mechanisms to prevent osmolysis (28). They can then serve as a carbon source for other members of the microbial community (29). Indeed, putative GGaPs are found in many organisms that have never been observed to accumulate GGa and do not possess any known routes for GGa synthesis. It would be interesting to determine the true function of these homologs to verify whether the organisms are actually capable of scavenging GGa from the environment or if the homologs are instead indicative of a synthetic pathway. A specific uptake transporter for glucosylglycerate has not yet been reported to our knowledge, but the GGaP homolog of *E. coli* does share its operon with several ABC transporters that could be possible candidates in this respect. For the related mannosylglycerate, an *E. coli* phosphotransferase system has already been identified (30).

In addition to the interesting biological implications, the newly identified glucosylglycerate phosphorylase specificity might open up new options for the commercial production of GGa. This compound has been reported to be an exceptionally potent and versatile stabilizer of proteins at elevated temperatures, usually outperforming glucosylglycerol and trehalose. For this reason, it has captivated interest along with glucosylglycerate and similar negatively charged glycosidic solutes for the development of more stable protein formulations with extended shelf lives (31, 32). Sucrose phosphorylase could be applied to produce Glc1P, which can immediately be converted into GGa by glucosylglycerate phosphorylase in a one-pot system. Similar coupled processes have already been designed for the conversion of sucrose into cellobiose or trehalose with yields of ~60 to 70%, but higher yields can be expected for glucosylglycerate due to the highly beneficial thermodynamic equilibrium of the reaction (33, 34). Because of the excellent specific activity of *Ms*GGaP toward glycerate, such a process may lead to increased space-time yields compared with that of the one-step transglycosylation process based on the side activity of sucrose phosphorylase.

TABLE 3 Genotypes and sources of the strains used in this study

Strain	Genotype	Source
E. coli BL21(DE3)	*fhuA2 lon ompT gal* λ(*s*BamH*lo* ΔEcoRI-B *int*::[*lacI*::*PlacUV5*::T7 gene1] *i21* Δ*nin5*) *dcm* Δ*hsdS*	New England Biolabs
E. coli CGSC 8974	Δ(*araD-araB*)*567* Δ*lacZ4787*::*rrnB-3* λ$^-$ Δ*agp-746*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Coli Genetic Stock Center

MATERIALS AND METHODS

Materials. Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich, Merck, or Carbosynth and were of the highest purity. α-D-Glucose 1-phosphate was produced in-house (35). Glucosylglycerate and mannosylglycerate were kindly provided by Bernd Nidetzky (Graz University of Technology) and Bitop (Witten, Germany), respectively. Expression vectors used in this study and their properties are listed in Table S1 in the supplemental material, while the genotypes of all used strains are listed in Table 3.

Sequence analysis. All full-length protein sequences classified in subfamily GH13_18 were extracted from the CAZy database (http://www.cazy.org) (36) and aligned with ClustalO (37) using default parameters. Then, a phylogenetic tree was generated with PhyML 3.1 with default parameters (38). BLAST analyses with the amino acid sequences of *M. silvanus* glucosylglycerate phosphorylase and other relevant genes were performed using the NCBI server (http://blast.ncbi.nlm.nih.gov/). The organization of operons containing a (putative) SP or GGaP gene was analyzed in different organisms from different branches of the phylogenetic tree using the DOOR 2.0 web interface (http://csbl.bmb.uga.edu/DOOR/) (39).

Gene cloning and transformation. The genes for *M. silvanus* glucosylglycerate phosphorylase ([*Ms*GGaP] UniProt identifier D7BAR0, codon optimized for *E. coli*), *S. thermophila* GGaP (UniProt identifier G0GBS4, codon optimized for *E. coli*), and *E. coli* GGaP (UniProt identifier P76041) were synthesized by GenScript (Piscataway, NJ, USA; sequences are reported in Fig. S1) and subsequently subcloned into a constitutive pCXP34h vector (40) at NheI and SpeI restriction sites. The *Ms*GGaP gene was also cloned into a pET21a vector at NdeI and XhoI restriction sites and into the pTrc99a expression vector by means of circular polymerase extension cloning (CPEC) (41). First, the gene and vector backbone were amplified in a separate high-fidelity PCR using primers Gene_Fw and Gene_Rv and Vector_Fw and Vector_Rv, respectively (Q5 high-fidelity DNA polymerase [NEB], standard protocol; primer sequences are shown in Table 4). PCR products were treated with DpnI (Westburg) to remove template DNA and were subsequently purified using the innuPREP PCR purification kit (Analytik Jena) and verified on a 1% agarose gel. The DNA concentration was measured with a Nanodrop ND-1000 (Thermo Scientific). The fragments were fused by adding 100 ng of the linearized vector backbone and an equimolar amount of the gene fragment to the CPEC reaction mixture. The following program was used: initial denaturation for 30 s at 98°C, 5 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 55°C, and elongation for 15 s/kb at 72°C, followed by a final elongation of 2 min at 72°C. Constructs were subjected to nucleotide sequencing (Macrogen, Amsterdam, The Netherlands).

Plasmids with a pCXP34h or pTrc99a expression vector were transformed into *E. coli* CGSC 8974, an acid glucose-1-phosphatase-negative strain (Coli Genetic Stock Center, New Haven, CT, USA), while plasmids with a pET21a vector were transformed into *E. coli* BL21(DE3).

Site-directed mutagenesis. Site-directed mutations were introduced with a modified two-stage megaprimer-based whole-plasmid PCR method (42), using primers described in Table 4 to amplify the megaprimers. The PCR mixtures contained 0.05 U/μl *Pfu*Ultra high-fidelity DNA polymerase (Stratagene), 0.2 mM deoxynucleoside triphosphate (dNTP) mix, 2 ng/μl template, and 0.1 pmol/μl of each primer in a total volume of 50 μl. The program started with an initial denaturation (3 min at 94°C) followed by 5

TABLE 4 Primers used in this study

Fragment	Primer	Sequence (5′→3′)[a]
*Ms*GGaP gene	Gene_Fw	CACAGGAAACAGACCATGGGCGGTAG
	Gene_Rv	TGCCTGCAGGTCGACTTAGTCGATAATCC
pTrc99a vector	Vector_Fw	GGATTATCGACTAAGTCGACCTGCAGGCATG
	Vector_Rv	TACCGCCCATGGTCTGTTTCCTGTG
Megaprimer N275A	N275A_Fw	GTTAGCGAAACGGCCGCACCGCACCG
	N275A_Rv	TACTGCCGCCAGGCAAATTC
Megaprimer N275H	N275H_Fw	GTTAGCGAAACGCACGCACCGCACCG
	N275H_Rv	TACTGCCGCCAGGCAAATTC
Megaprimer E383A	E383A_Fw	GGTTGCCAACGATCAGATG
	E383A_Rv	AGCGTCAGGCACAGTGCATACGGGACC
Megaprimer E383Q	E383Q_Fw	GGTTGCCAACGATCAGATG
	E383Q_Rv	AGCGTCAGGCACAGTTGATACGGGACC

[a]Mutations are underlined.

cycles of denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 1 min/kb (size of the megaprimer) at 72°C. The second stage consisted of 30 cycles of 10 s at 94°C and extension for 1 min/kb (size of the entire plasmid) at 72°C followed by one final extension of 2 min at 72°C. After digestion by DpnI (Westburg), the plasmids were transformed and subjected to nucleotide sequencing as described above.

Protein expression and purification. Expression of *Ms*GGaP was optimized by inoculating 2% of an overnight culture of *E. coli* transformed with the pCXP34h, pTrc99a, or pET21a expression plasmid in 250 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin in a 1-liter shake flask at 37°C. When the cultures reached an optical density at 600 nm (OD_{600}) of 0.6, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of either 0.1 mM or 1.0 mM in cultures transformed with the pTrc99a or pET21a plasmid, respectively. Cultures transformed with the pCXP34h plasmid expressed the protein constitutively. For each of these conditions, two expression temperatures (37°C for 6 h and 20°C for 16 h) were tried. All cultures were centrifuged and the cell pellets were frozen at −20°C for at least 4 h. For enzyme extraction and purification, cell pellets were thawed and dissolved in 8 ml lysis buffer consisting of 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml lysozyme, and 50 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.5). These suspensions were incubated on ice for 15 min and sonicated 3 times for 3 min (Branson sonifier 250, level 3, 50% duty cycle). Finally, cell debris was removed by centrifugation at 9,000 rpm for 1 h.

For enzyme production, 2% of an overnight culture was inoculated in 500 ml LB medium containing 100 μg/ml ampicillin in a 2-liter shake flask and incubated at 37°C with continuous shaking at 200 rpm. The culture was grown to an OD_{600} of 0.6 and the expression of *M. silvanus* GGaP (in pTrc99a) was induced by adding IPTG to a final concentration of 0.1 mM, while the expression of *E. coli* and *S. thermophila* GGaPs (in pCXP34h) was constitutive. After 6 h at 37°C, the cultures were centrifuged and the cell pellets were frozen at −20°C for at least 4 h. SPP from *T. thermosaccharolyticum* and SP from *B. adolescentis* and *Streptococcus mutans* were produced as reported earlier (7, 16).

Cell lysis and removal of cell debris were performed as described above, but the lysis buffer consisted of 10 mM imidazole, 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM phosphate buffer (pH 7.4), followed by His_6-tag purification. For *Ms*GGaP, the resulting supernatant was subjected to mild heat treatment (50°C for 30 min) followed by another centrifugation step. Extracts were further purified by Ni-NTA chromatography as described by the supplier (MCLab). Finally, the buffer was exchanged to 50 mM MES (pH 6.0 or pH 6.5) in a 30-kDa Amicon Ultra centrifugal filter. Ni-NTA chromatography was not performed for the GGaP from *E. coli*, as it showed no retention on different Ni-NTA columns (MCLab or Thermo Scientific) with either a C-terminal or N-terminal His_6 tag.

Protein concentrations were measured in triplicate with a Nanodrop ND-1000 (Thermo Scientific) using extinction coefficients calculated with the ProtParam tool on the ExPASy server (http://web.expasy.org/protparam/). Molecular weights and purity were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis ([SDS-PAGE] 10% gels).

Colorimetric assays. Phosphorylase activity could be monitored in both directions by measuring the release of inorganic phosphate with the phosphomolybdate assay (43), and that of Glc1P was measured with an enzymatic coupled assay that measures the reduction of NAD^+ in the presence of phosphoglucomutase and glucose 6-phosphate dehydrogenase (44). Glucose released due to hydrolysis could be quantified with an enzymatic coupled assay using glucose oxidase and peroxidase (GOD-POD) (45). Transglucosylation activity was calculated by subtracting this hydrolytic activity from the total activity measured by the phosphomolybdate assay. Samples were inactivated by the acidic conditions of the assay solution (phosphomolybdate assay) or by heating for 5 min at 95°C (other assays).

Characterization of glucosylglycerate phosphorylase. To compare the enzymatic activities in cell extracts obtained under different expression conditions, 80 μl of each extract was incubated with 100 mM Glc1P and 25 mM D-glycerate in 50 mM MES (pH 6.5) at 37°C (1-ml reaction volume). Samples of 50 μl were taken every min for 6 min and analyzed with the phosphomolybdate assay to quantify the released phosphates.

The substrate scope in the synthetic direction was evaluated by incubating 20 μg/ml purified enzyme, 100 mM Glc1P as the donor substrate, and 100 mM acceptor substrate in 50 mM MES (pH 6.5) at 37°C (1-ml reaction volume, no agitation). For *E. coli* GGaP, 150 μl cell extract was added instead of purified enzyme and reactions with cell extracts from *E. coli* CGSC 8974 transformed with an empty pCXP34h vector were performed as negative controls. Samples of 50 μl were taken every min for 8 min and analyzed with the phosphomolybdate assay to quantify the released phosphate and with the GOD-POD assay to quantify glucose released by the hydrolysis of Glc1P. For substrates D-glycerate and L-glycerate, these reactions were repeated with 3 μg/ml purified enzyme (or 30 μl cell extract) to ensure measurements of the initial velocity. In the phosphorolytic direction, 100 mM phosphate and 100 mM sucrose or glucosylglycerate were incubated with 20 μg/ml enzyme in 50 mM MES (pH 6) at 37°C. Samples of 50 μl were analyzed with the Glc1P assay every min for 8 min.

The influence of pH on enzyme activity was checked in the synthetic direction in 50 mM acetate (pH 4.5), MES (pH 5.0 to 6.5), or 3-(*N*-morpholino)propanesulfonic acid (pH 7.0 to 8.0) at 37°C, and the optimal temperature was determined in 50 mM MES (pH 6.5). For each reaction, 2 μg/ml enzyme was incubated with 100 mM Glc1P and 20 mM D-glycerate. Samples of 50 μl were taken every 30 s for 4 min and analyzed with the phosphomolybdate and GOD-POD assays.

The apparent kinetic parameters of *Ms*GGaP for glucosylglycerate, inorganic phosphate, Glc1P, and D-glycerate were determined at the optimal temperature and pH in 50 mM MES buffer. Michaelis-Menten curves were obtained using either 20 mM glucosylglycerate or 100 mM phosphate as the fixed cosubstrate in the phosphorolytic direction and either 20 mM D-glycerate or 100 mM Glc1P in the

synthetic direction. The concentration range examined was 0 to 100 mM for Glc1P and inorganic phosphate and 0 to 20 mM for glucosylglycerate and D-glycerate. The enzyme concentrations in the phosphorolytic and synthetic directions were 20 μg/ml and 3.2 μg/ml, respectively. The affinity of other enzymes for D-glycerate was determined at concentrations between 0 to 15 mM (GGaP) or 0 to 500 mM (SP and SPP), with 50 mM Glc1P as a cosubstrate at the same pH and temperature. For GGaP from *E. coli* and SP from *S. mutans*, the temperature was 30°C. The enzyme concentration was 0.5 mg/ml (SP and SPP) or 5 μg/ml (*S. thermophila* GGaP). Because it could not be purified (see above), 5% (vol/vol) cell extract was added for *E. coli* GGaP, and reactions with cell extracts from *E. coli* CGSC 8974 transformed with an empty pCXP34h vector were performed in parallel as negative controls. Parameters were calculated by nonlinear regression of the Michaelis-Menten equation using SigmaPlot 11.0. The molecular mass of 63.0 kDa was used to calculate turnover number, k_{cat}, for *Ms*GGaP.

The enzyme's stability was examined by incubating purified protein (4 μg/ml) for 10 min at various temperatures in 50 mM MES (pH 6.5), after which the residual activity was measured in the synthetic direction (42°C with 100 mM Glc1P and 20 mM D-glycerate) and compared to the activity of untreated enzyme.

Homology modeling and automated docking. A homology model of glucosylglycerate phosphorylase from *M. silvanus* was generated with YASARA (46) using default parameters. The crystal structure of the sucrose phosphorylase from *B. adolescentis* from the same family GH13_18 served as the template (PDB entries 1R7A, 2GDU, and 2GDV). The binding of glucosylglycerate was simulated by ligand docking using the implemented AutoDock VINA module (47) with default parameters, except the number of runs was increased to 100. The most accurate model was selected on the basis of known interactions in the −1 subsite of sucrose phosphorylase. Figures were made with PyMOL v1.3 (48).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01434-17.

SUPPLEMENTAL FILE 1, PDF file, 5.2 MB.

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