Glucose-1,6-bisphosphate a key metabolic regulator is synthesized by a distinct family of α-phosphohexomutases widely distributed in prokaryotes

Niels Neumann[1,2], Simon Friz[1,2] and Karl Forchhammer[1,2*]

[1] Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

[2] Cluster of Excellence: EXC 2124: Controlling Microbes to Fight Infection, Tübingen, Germany

* For correspondence: karl.forchhammer@uni-tuebingen.de

Abstract

The reactions of α-D-phosphohexomutases (αPHM) are ubiquitous, key to primary metabolism and essential for several processes in all domains of life**.** The functionality of these enzymes relies on an initial auto-phosphorylation step which requires the presence of α-D-glucose-1,6-bisphosphate (Glc-1,6-BP). While well investigated in vertebrates, the origin of the this activator compound in bacteria is unknown. Here we show that the Slr1334 protein from the unicellular cyanobacterium *Synechocysitis* sp. PCC 6803 is a Glc-1,6-BP-synthase. Biochemical analysis revealed that Slr1334 efficiently converts fructose-1,6-bisphosphate and α-D-glcucose-1-phosphate/α-D-glucose-6-phosphate into Glc-1,6-BP and also catalyzes the reverse reaction. Phylogenetic analysis revealed that the *slr1334* product belongs to a primordial subfamily of αPHMs that is present especially in deeply branching bacteria and also includes human commensals and pathogens. Interestingly, the homologue of Slr1334 in the human gut bacterium *Bacteroides salyersiae* catalyzes the same reaction, suggesting a conserved and essential role for the members of this αPHM subfamily.

Key words:

Phosphoglucomutase, glucose-1,6-bisphosphate, fructose-1,6-bisphosphate, glycolysis, carbon metabolism

31 # Introduction

32 The α-D-phosphohexomutase (αPHM) superfamily is ubiquitous and found in all domains of
33 life . All known members of this superfamily catalyze a reversible intramolecular phosphoryl
34 transfer on their phosphosugar substrates. This reaction requires a bound metal ion (usually
35 Mg^{2+}) together with a conserved phosphorylated seryl residue in the active site and proceeds
36 via a bis-phosphorylated sugar intermediate [1]. While showing similar properties in structure
37 and reaction mechanism, the enzymes differ strongly in substrate specificity . Based on their
38 preferred substrate, the αPHMs are traditionally divided into four main groups: (1)
39 Phosphoglucomutases (PGM), (2) phosphoglucosamine mutases (PNGM), (3)
40 phosphoacetylglucosamine mutases (PAGM) and (4), the group of bifunctional
41 phosphoglucomutases/phosphomannomutases (PGM/PMM) [1]. The most recent expansion to
42 this classification has been the discovery of the mammalian phosphopentomutase (PGM2)
43 and Glucose-1,6-Bisphosphate-synthase (PGM2L1) [2, 3]. This classification, however, lacks a
44 more detailed subdivision in between the main groups and also does not take into account
45 αPHMs preferably catalyzing other reactions (e.g. straight phosphomannomutases) [3]. The
46 NCBI conserved domain database (CDD) classifies the αPHM superfamily in 11 different
47 subfamilies on a phylogenetic basis [4]. This phylogenetic model enables a more detailed
48 classification and includes several groups of bacterial and archaeal enzymes showing typical
49 αPHM properties but are so far only poorly characterized (**Table 1**).
50

CDD group name	CDD identifier	Proposed main function
PGM1	cd03085	Phosphoglucomutase
PGM2	cd05799	Phosphopentomutase (PGM2)/Glc-1,6-BP Synthase (PGM2L1)
PGM3(PAGM)	cd03086	Phosphoacetylglucosamine mutase
PMM/PGM	cd03089	Bi-functional phosphomanno-/phosphoglucomutase
PGM like 1	cd03087	Unknown
PGM like 2	cd05800	Unknown
PGM like 3	cd05801	Phosphoglucomutase
PGM like 4	cd05803	Unknown
ManB	cd03088	Phosphomannomutase
GlmM	cd05802	Phosphoglucosamine mutase
Mpg1 transferase	cd05805	GDP-mannose-pyrophosphorylase

Table 1: Subfamilies in the αPHM superfamily with their identifier and proposed main function according to the NCBI conserved domain database (CDD)

51 The PGM (cd03085) from rabbit and human as well as the bacterial PGM (cd05801) from
52 *Salmonella typhimurium* and the bacterial bifunctional PMM/PGM (cd03089) from
53 *Pseudomonas aeruginosa* are among the best characterized αPHMs with available crystal
54 structures and detailed description of reaction mechanisms [5-9]. Catalyzing the interconversion
55 of α-D-glucose-1P (Glc-1P) and α-D-glucose-6P (Glc-6P) makes PGM a key enzyme in glycogen
56 metabolism, glycolysis and gluconeogenesis. During this reaction, a phosphoserine at the
57 active site of PGM donates the phosphoryl group to the substrate Glc-6P or Glc-1P resulting
58 in the synthesis of the transient intermediate α-D-glucose-1,6-bisphosphate (Glc-1,6-BP).
59 After a reorientation in the catalytic center, the intermediate product re-phosphorylates the
60 active site serine yielding Glc-1P or Glc-6P, respectively. Glc-1,6-BP itself acts as an essential
61 activator of PGM by providing an initial phosphorylation of the active site and was first
62 discovered by Leloir, Trucco [10] in yeast extract [11]. While Glc-1,6-BP is not a free metabolite in
63 any metabolic pathway, it is known to be a potent regulator of several enzymes in central
64 carbon metabolism in eukaryotes: in addition to PGMs and other αPHMs, it was shown to
65 regulate hexokinases, 6-phosphogluconate dehydrogenase, phosphofructokinase and
66 pyruvate kinase [12]. In mammalian tissues the synthesis of Glc-1,6-BP is catalyzed by the
67 PGM2L1 enzyme, a member of the cd05799 subfamily, utilizing 1,3-bisphosphoglyceric acid
68 (1,3-BPG) as phosphoryl donor for Glc-1P [3]. In contrast, despite its essential role in the activity
69 of prokaryotic PGM, the function of Glc-1,6-BP in prokaryotes has been overlooked and its
70 origin remains obscure as no enzyme producing free Glc-1,6-BP has been identified so far [13].
71 The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) has two
72 enzymes that are encoded by the genes *sll0726* and *slr1334* and annotated as PGM-like [4].
73 While Sll0726 exhibits typical properties of PGMs [14-16], little is known about Slr1334. The only
74 study addressing this issue reported that purified *Synechocystis* Sll0726 has about a tenfold
75 higher activity *in vitro* than Slr1334 and that a *sll0726* knockout mutant only shows around
76 3 % PGM activity compared to the wildtype [17]. Despite its low contribution to over-all PGM
77 activity, the Slr1334 product appeared to be essential as it was not possible to acquire fully
78 segregated knockout mutants of the *slr1334* gene, which raised the question of the role of
79 Slr1334 in *Synechocystis*.
80 In this work we show that Slr1334 has a phosphotransferase activity catalyzing the production
81 of free Glc-1,6-BP from Glc-1P/Glc-6P and Fructose-1,6-bisphosphate (Frc-1,6-BP). Thereby,
82 Slr1334 is responsible for the formation of the key activator of PGM, Glc-1,6-BP making it a

83 major regulator in glycolysis and other carbon pathways. Our finding makes Slr1334 the first
84 bacterial enzyme specific for the production of free Glc-1,6-BP. Furthermore, we show that
85 the homologue of Slr1334 in the human gut bacterium *Bacteroides salyersiae* catalyzes the
86 same reaction.
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89 # Results

90 **Classification of the two PGM isoenzymes from *Synechocystis***

91 *Synechocystis* possesses two genes annotated as PGMs, the genes *sll0726* and *slr1334*. In
92 order to classify them within the αPHM superfamily, we performed a phylogenetic analysis by
93 multi-level alignment of all αPHM subfamilies that are considered PGM or "PGM_like". For
94 this, we collected the entry sequences from the NCBI Conserved Domains Database (CDD) [4]
95 and retrieved further sequences by performing BLAST searches in the NCBI RefSeq_protein
96 database. To focus on glucose-specific αPHMs, the groups of enzymes specific for other sugars
97 like PMMs, PNGM and PAGMs (cd03086 cd03088, cd05802, cd05805) were omitted from the
98 analysis (**Fig. 1**). Slr1334 is part of the cd05800 (PGM like 2) subfamily present in many
99 bacterial groups. In agreement with the CDD our phylogenetic analysis shows that this
100 subfamily forms a distinct cluster of PGMs which clearly separates from other PGM and PGM
101 like enzymes. The Sll0726 PGM, by contrast, is a member of the cd05801 subfamily, to which
102 the well characterized PGM from *S. typhimurium* belongs.
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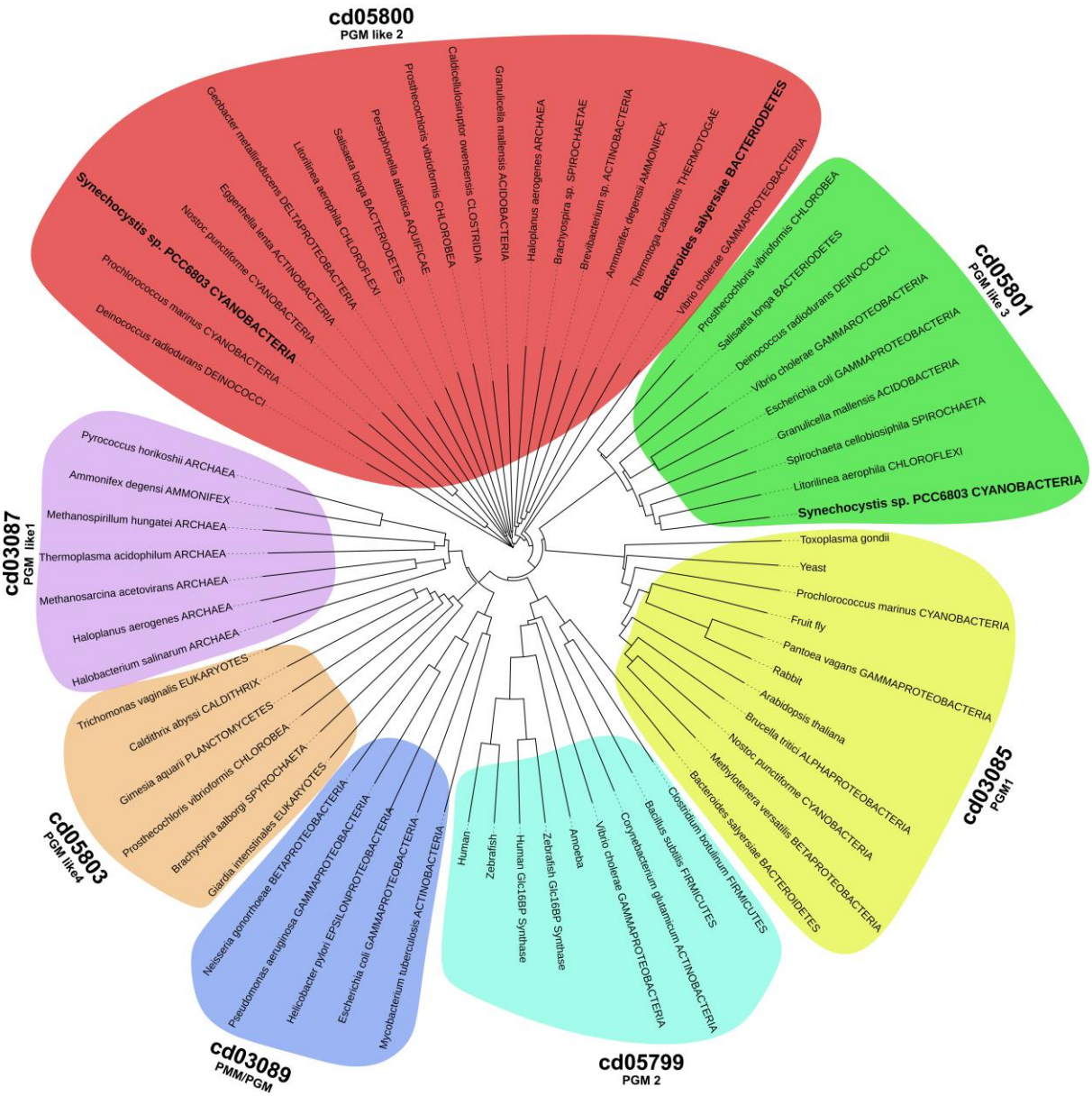

Figure 1: **Phylogenetic tree of the αPHM subfamilies that are considered to be "PGM-like" according to CDD**

The tree was built on the taxonomy based on the protein sequences present in the NCBI RefSeq_protein database. The CDD subfamilies cd03086, cd03088, cd05802 and cd05805, which are known to catalyze distinct reactions, were omitted from the analysis. The organisms investigated in this study are highlighted in bold letters.

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113 **Slr1334 shows low PGM activity compared to Sll0726 but behaves differently in presence of**
114 **Fructose-1,6-BP**
115 To gain deeper insights into the role of the enigmatic Slr1334, we overexpressed Sll0726 along
116 with Slr1334 in *Escherichia coli* and assessed the PGM enzymatic activity of the purified
117 proteins by measuring the interconversion of Glc-1-P to Glc-6-P in an assay that couples Glc-
118 6-P formation to its subsequent oxidation using Glucose-6-phosphate-dehydrogenase
119 (G6PDH) and $NADP^+$. We could show that Slr1334 only displays about 5% of catalytic efficiency
120 compared to Sll0726 when both PGMs were tested with saturating amounts of the activator
121 Glc-1,6-BP (60 µM) (**Fig. 2, Table 2. See also Fig. 3**). Interestingly, the PGM activity of both
122 enzymes was also measurable without addition of Glc-1,6-BP, which is considered to be an
123 essential activator. However, in the absence of Glc-1,6-BP, the catalytic efficiency was about
124 70 times lower due to a significantly increased Michaelis-Menten constant (K_m) for Sll0726 and
125 Slr1334 alike.
126 Fructose-1,6- bisphosphate is an endogenous intermediate of the glycolytic pathway and is
127 known to be an inhibitor of PGM most likely by competing with the reaction intermediate Glc-
128 1,6-BP [18, 19]. In a preliminary experiment we tested the effect of different concentrations of
129 Frc-1,6-BP on Sll0726 and observed major inhibition already at concentrations of 100 µM (data
130 not shown). When testing PGM activity by replacing 60 µM Glc-1,6-BP with 100 µM Frc-1,6-
131 BP, we detected a decrease in catalytic efficiency to 19 % as a consequence of a strongly
132 increased K_m (**Fig. 2, Table 2**). Strikingly, however, for Slr1334 we detected a different effect:
133 while the catalytic rate constant (k_{cat}) decreased by about 2-fold, we measured an
134 approximately 100-fold reduced K_m resulting in a 50 times increased catalytic efficiency. This
135 raised the question of how Frc-1,6-BP affected the PGM-activity of Slr1334. We speculated
136 that Frc-1,6-BP might either function as a direct activator similar to Glc-1,6-BP or be required
137 for the synthesis of the activating compound Glc-1,6-BP during the reaction of Slr1334. As we
138 will show below in fact Glc-1,6-BP turned out to be the activating compound.
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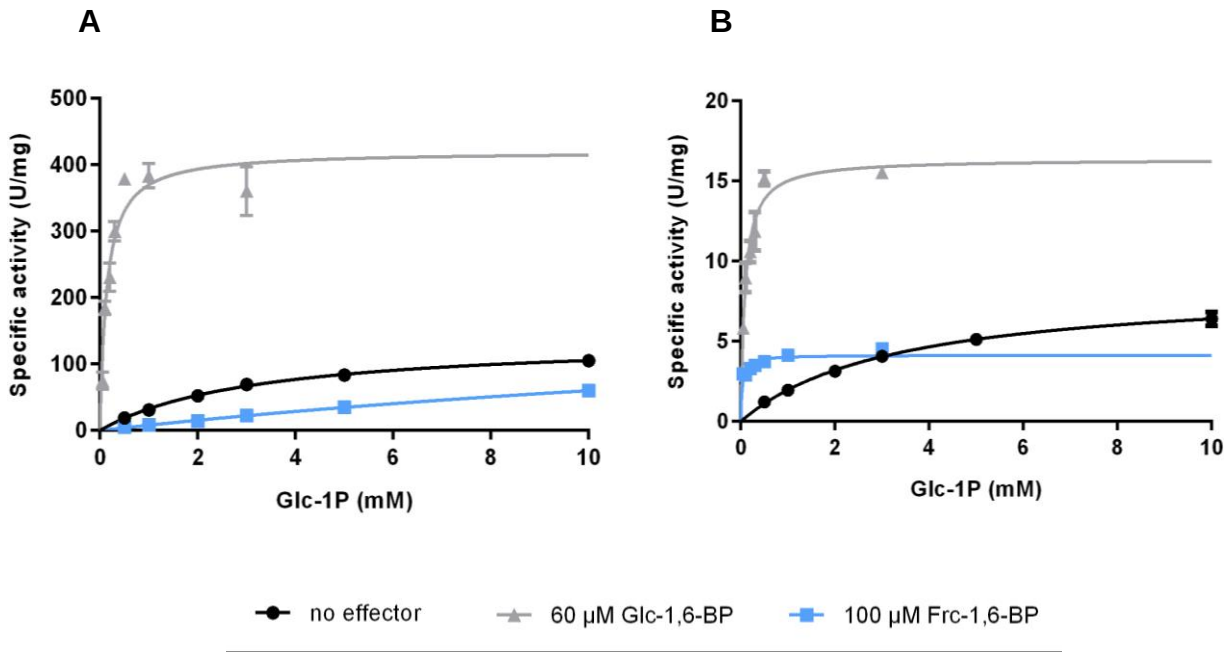

Figure 2: Slr1334 shows lower PGM activity then Sll0726 and behaves differently in presence of Frc-1,6-BP

Shown are the Michaelis-Menten kinetics of Sll0726 (A) and Slr1334 (B): in black, without addition of effectors; in light grey in the presence of 60 µM Glc-1,6-BP; in blue, in the presence of 100 µM Frc-1,6-BP. Three replicates were measured for each data-point. Error bars represent the SD.

Effector	Sll0726			Slr1334			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Catalytic efficiency ratio (%) (Slr1334/Sll0726)
No effector	3.27 ± 0.21	138 ± 3.7	42.2 *10^3	3.31 ± 0.14	7.5 ± 0.1	2.3 * 10^3	**5.5**
Glc-1,6-BP	0.19 ± 0.04	415 ± 30.6	3038.9 * 10^3	0.09 ± 0.02	14.4 ± 0.7	158.1 * 10^3	**5,2**
Frc-1,6-BP	26.86 ± 3.35	218 ± 21.3	8.1 * 10^3	0.03 ± 0.01	3.6 ± 0.2	117.5 * 10^3	**1450**

Table 2: Kinetic parameters of Sll0726 and Slr1334 with different effector molecules
KM and Kcat values are means of triplicates with ± SD. Catalytic efficiency ratio is given in percentage and was calculated by the ratio of K_{cat}/K_m of Slr1334 and Sll0726

169 **Slr1334 forms a product out of Frc-1,6-BP and Glc-1P/Glc-6P that strongly activates Sll0726**

170 To determine the amount of Glc-1,6-BP needed to achieve activation of Sll0726 at 1 mM Glc-
171 1P we first tested enzymatic activity of Sll0726 at different Glc-1,6-BP concentrations.
172 Maximum activation was achieved at Glc-1,6-BP concentrations between 30 and 60 µM
173 resulting in a 5-fold increased activity as compared to the zero Glc-1,6-BP reaction (**Fig. 3**).
174 Next, we incubated Slr1334 with the phosphorylated sugars Frc-1,6-BP, Glc-1P and Glc-6P (1
175 mM each) in different combinations and subsequently tested whether Sll0726 could be
176 activated by the corresponding reaction products (**Fig. 3**). As negative control, Frc-1,6-BP and
177 Glc-1P were allowed to react without any enzyme or with Sll0726 instead of Slr1334. After
178 running the Slr1334 reaction for 1.5 h, the reaction was stopped by heat inactivation and the
179 supernatant was added in a dilution of 1:10 to the reaction mixture of the Sll0726 assay, which
180 was then started by adding 1 mM Glc-1P. Only after adding the supernatant of the Slr1334
181 reaction containing Frc-1,6-BP and Glc-1P (or Glc-6P) as substrates the velocity of the Sll0726
182 reaction was increased by 5-fold in comparison to the control reaction at zero Glc-1,6-BP (**Fig.**
183 **3**). When the Sll0726 test reaction was performed with the supernatant of the Sll0726 control
184 reaction or the reaction products of Slr1334 using Frc-1,6-BP as sole substrate, the enzyme
185 velocity was lower than the zero Glc-1,6-BP reaction. This reduction can be explained by the
186 inhibitory effect of Frc-1,6-BP on Sll0726, as exemplified by the "no enzyme control" reaction
187 (**Fig. 3**).
188 In addition to Frc-1,6-BP, we also tested whether Slr1334 can utilize 1,3-BPG instead of Frc-
189 1,6-BP for Glc-1,6-BP formation as described for the mammalian PGM2L1. Since 1,3-BPG is not
190 commercially available, we established an assay whereby we continuously produced 1,3-BPG
191 by a phosphoglycerate kinase (PGK) reaction using a His-tagged purified PGK in combination
192 with 1 mM 3PG and 1 mM ATP (see methods part). This reaction was directly coupled to the
193 reaction of Slr1334 with Glc-1P or Glc-6P or no substrate. As a control the same reaction was
194 performed with Sll0726 instead of Slr1334. To test the possible formation of Glc-1,6-BP, the
195 supernatants of these reactions were added to the Sll0726 reaction before performing the
196 enzymatic assay as described above.
197 We detected an increase in Sll0726 activity by approx. 2.5-fold compared to the zero Glc-1,6-
198 BP reaction when the supernatant of the Slr1334 reaction contained 1,3-BPG and either Glc-
199 1P or Glc-6P. No increase in activity was detected when the supernatant solely contained 1,3-

BPG. The supernatant of the control reaction with Sll0726 instead of Slr1334 resulted in no marked change in activity.

Comparing the activation of Sll0726 by the Slr1334 reaction products to the activation at different Glc-1,6-BP levels reveals the efficiency of Glc-1,6-BP formation from Frc-1,6-BP or 1,3-BPG. With Frc-1,6-BP as substrate for the Slr1334 reaction, a maximum activation of Sll0726 was achieved, which corresponded to activation of Sll0726 with concentrations of Glc-1,6-BP between 30 and 60 µM (**Fig. 3**). When 1,3-BPG was used as substrate as described above, the increase in Sll0726 activity corresponded to a Glc-1,6-BP concentrations between 1 and 5 µM. From this experiment we conclude that i) Slr1334 forms a product out of Glc-1P/Glc-6P and either Frc-1,6-BP or 1,3-BPG, which is able to activate Sll0726 like defined amounts of Glc-1,6-BP; ii) when normalizing the Sll0726 activation to Glc-1,6-BP concentrations, the reaction product of Slr1334 using Frc-1,6-BP as phosphorylating sugar is 10-fold more effective in activating Sll0726 than using 1,3-BPG.

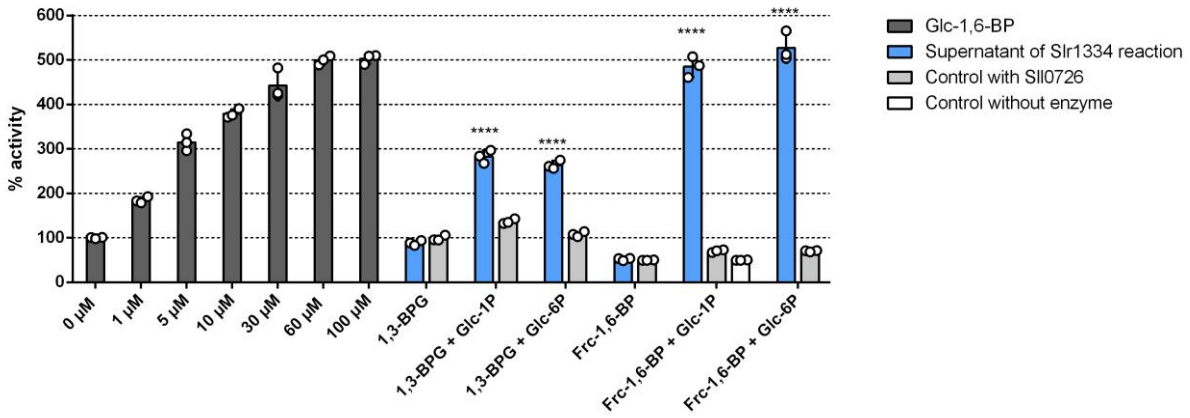

Figure 3: Slr1334 produces a compound out of Frc-1,6-BP that strongly activates Sll0726

Shown is the Sll0726 activity: in dark grey, at different Glc-1,6-BP concentrations; in blue, after addition of the supernatant of the Slr1334 reaction; in light grey, after addition of the supernatant of the Sll0726 reaction as a control; in white, after addition of the sole phosphosugars without enzyme as further control. The different combinations of substrates used for the Slr1334 reaction and controls are indicated on the X-axis. Values represent relative activity compared to the Sll0726 reaction with 0 µM Glc-1,6-BP, which was considered 100%. Three biological replicates were measured for each datapoint. Error bars represent the SD; asterisks represent the statistical significance

232 **The product of the Slr1334 reaction is not degradable by the Frc-1,6-BP-aldolase**

233 To confirm that the activating compound formed from Glc-1P/Glc-6P and Frc-1,6-BP through
234 the Slr1334 reaction is in fact Glc-1,6-BP, we analyzed the reaction product via liquid
235 chromatography-mass spectrometry (LC/MS). Therefore, we incubated Slr1334 together with
236 500 µM Frc-1,6-BP and 1 mM Glc-1P for 1.5 h. As a control, the same substrates were
237 incubated without enzyme. Since LC/MS analysis does not allow discriminating Glc-1,6-BP
238 from Frc-1,6-BP, we performed an additional reaction step to specifically degrade Frc-1,6-BP.
239 Therefore, after heat inactivation, the reaction mixture was incubated with Frc-1,6-BP-
240 aldolase (Fba) and glycerol-3-phosphate-dehydrogenase (GPDH) as well as 1 mM NADH: Fba
241 specifically cleaves Frc-1,6-BP into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-
242 3P while Glc-1,6-BP is unaffected. GPDH subsequently forms glycerol-3-phosphate (glycerol-
243 3P) and NAD^+ out of DHAP and NADH. This is necessary to enable full degradation of Frc-1,6-
244 BP.
245 After this treatment, LC/MS analysis revealed that a compound with a mass corresponding to
246 either Glc-1,6-BP or Frc-1,6-BP was present in the Slr1334 reaction while this mass was not
247 detectable in the control reaction where no Slr1334 was present (**Fig. 4**). This indicates that
248 Frc-1,6-BP in the control reaction was completely degraded in the subsequent reaction by Fba
249 as expected, while Frc-1,6-BP in the Slr1334 reaction was metabolized into a molecule of the
250 same mass that was not degraded by Fba. This is precisely what is expected when Glc-1,6-BP
251 is produced form Frc-1,6-BP turn-over. Furthermore, the reaction products from the coupled
252 GPDH reaction, NAD+ and glycerol-3P were more abundant in the control reaction without
253 Slr1334 as compared to the reaction with Slr1334. This indicated that more Frc-1,6-BP was
254 degraded out of the control reaction than out the reaction containing Slr1334 due to partial
255 conversion of Frc-1,6-BP into Glc-1,6-BP by Slr1334 (**Fig. 4**). Altogether, this experiment
256 strongly indicated that in the presence of Slr1334, Frc-1,6-BP was partially utilized to form Glc-
257 1,6-BP. The fact that only a part of Frc-1,6-BP was consumed could be either due to a too short
258 reaction time or because the reaction reached an equilibrium state.
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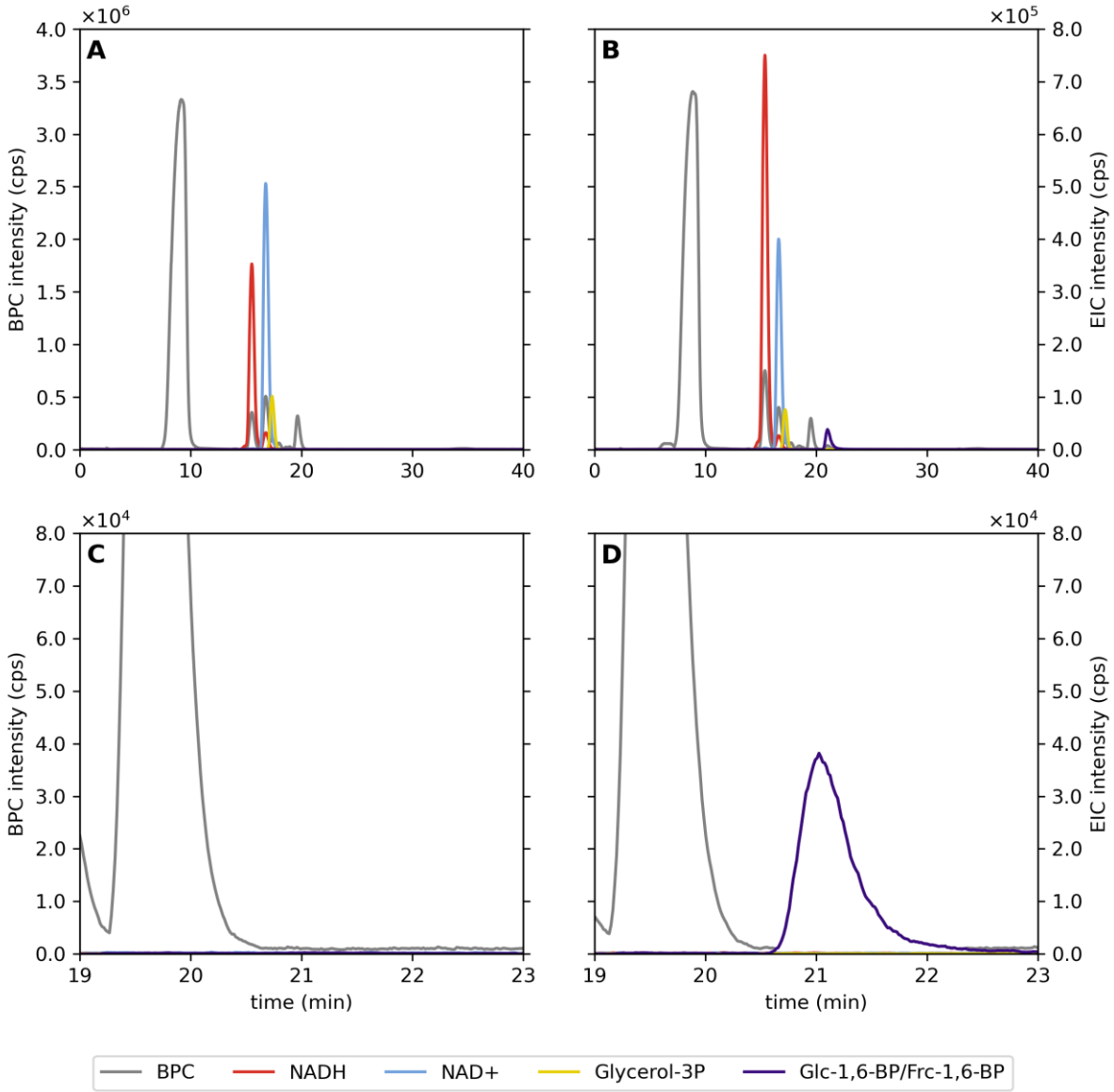

Figure 4: LC-MS analysis reveals that Slr1334 metabolizes Frc-1,6-BP into a molecule of identical mass that is not degraded by Frc-1,6-BP-aldolase

Analysis of the products of the reaction without (A) and with Slr1334 (B) via LC/MS. Shown are the base peak chromatograms (BPC) in the mass range $(M\text{-}H)^-$ m/z = 79–1,601 (grey) and the extracted ion chromatograms (EIC) based on the exact masses of NAD+ $(M\text{-}H)^-$= 662.102 m/z (light blue), NADH $(M\text{-}H)^-$= 664.118 m/z (red), Glycerol 3-P $(M\text{-}H)^-$= 171.006 m/z (yellow), Glc-1,6-BP/Frc-1,6-BP $(M\text{-}H)^-$= 338.989 m/z (purple). The presence of the NAD+ (blue) and Glycerol-3P (yellow) peaks in (B) shows that Frc-1,6-BP was not fully metabolized into Glc-1,6-BP. Magnification and adjustment of the graphs in (A) and (B) is clear evidence of the absence (**C**) or formation (**D**) of Glc-1,6-BP.

296 **Slr1334 keeps Frc-1,6-BP and Glc-1,6-BP in an equilibrium**

297 For investigating whether the formation of Glc-1,6-BP out of Glc-1P/Glc-6P and Frc-1,6-BP is
298 an equilibrium reaction, we performed an enzymatic assay to determine the amount of the
299 remaining Frc-1,6-BP. Therefore, Slr1334 was incubated with 500 µM of Frc-1,6-BP and 1 mM
300 Glc-1P for 1.5 h. As controls, the same assays were performed with Sll0726 or without any
301 enzyme. After the reaction was stopped by heat inactivation, the supernatant was used to run
302 the coupled Fba/GPDH assay in a dilution of 1:10, which would correspond to a final
303 concentration of 50 µM Frc-1,6-BP if no Frc-1,6-BP was consumed. As further controls,
304 standards of 50 µM and 25 µM Frc-1,6-BP were used in the Fba/GPDH assay. This experiment
305 showed that the concentration of Frc-1,6-BP decreased by about 50 % when in the first
306 reaction Slr1334 was present, while apparently no Frc-1,6-BP was utilized in the control
307 reactions (**Fig. 5**). This result agreed with the assumption that the reaction reaches an
308 equilibrium at 50 % product formation, either because of the reversibility of the reaction or
309 product inhibition.
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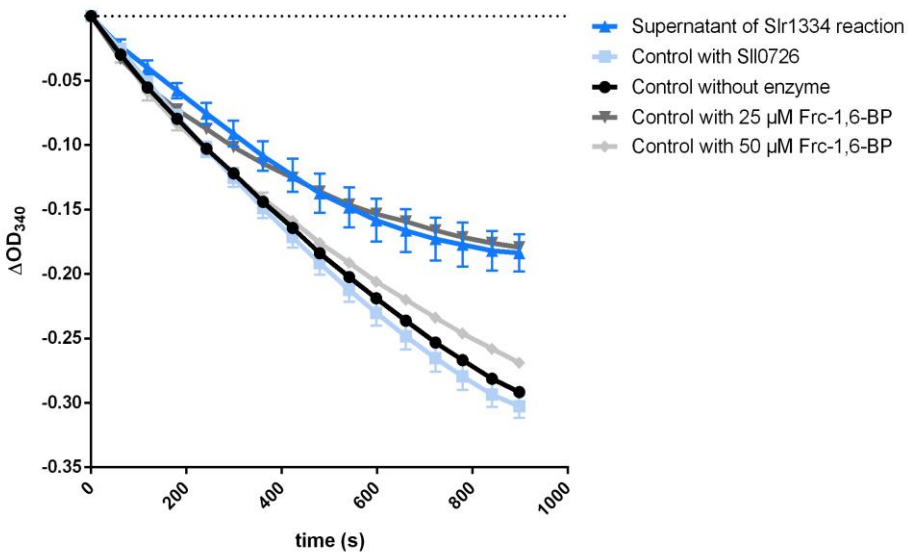

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322 **Figure 5: Slr1334 produces Glc-1,6-BP out of Frc-1,6-BP in an equilibrium reaction**

323 Shown is the Frc-1,6-BP-aldolase activity: in dark blue, after addition of the supernatant of
324 the Slr1334 reaction; in light blue, after addition of the supernatant of the Sll0726 reaction
325 as a control; in black, after addition of the supernatant without any enzyme. In dark grey
and light grey, the aldolase activity measured at Frc-1,6-BP concentrations of 25 µM and
50 µM, respectively, as further control.
326 Frc-1,6-BP was used at concentrations of 500 µM in the Slr1334 reaction and respective
327 control reactions. Therefore, its final concentration in the Sll0726 assay after 1:10 dilution

328 **The Slr1334 reaction is reversible**

329 To reveal whether Slr1334 can catalyze the proposed reverse reaction and synthesize Frc-1,6-
330 BP from Glc-1,6-BP and Fructose-1P (Frc-1P)/Fructose-6P (Frc-6P), formation of Frc-1,6-BP was
331 coupled to the Fba and GPDH reaction. For the assay, 50 µM Glc-1,6-BP was used and the
332 reaction was started by the addition of 5 mM Frc-6-P. As control reactions, *Synechocystis*
333 Sll0726 as well as PGM1 from rabbit were used. The result clearly showed that Slr1334 could
334 produce Frc-1,6-BP, resulting in measurable aldolase activity while the controls showed no
335 activity (**Fig. 6a**). Using this assay, we tried to determine the kinetic constants for the two
336 substrates Frc-6-P and Glc-1,6-BP. For Frc-6P, a K_m of 1 mM and a K_{cat} of 1.2 s^{-1} could be
337 determined (**Fig. 6b**). By contrast, the affinity of Slr1334 for Glc-1,6-BP was so high that even
338 at the lowest measurable substrate concentrations (2 µM) the reaction proceeded with
339 maximal velocity (**Fig. 6c**). Altogether these experiments unequivocally demonstrate that
340 Slr1334 is the first characterized bacterial Glc-1,6-BP synthase. In contrast to the eukaryotic
341 Glc-1,6-BP synthase, the prokaryotic enzyme uses Frc-1,6-BP as major phosphoryl donor for
342 Glc-1,6-BP production.
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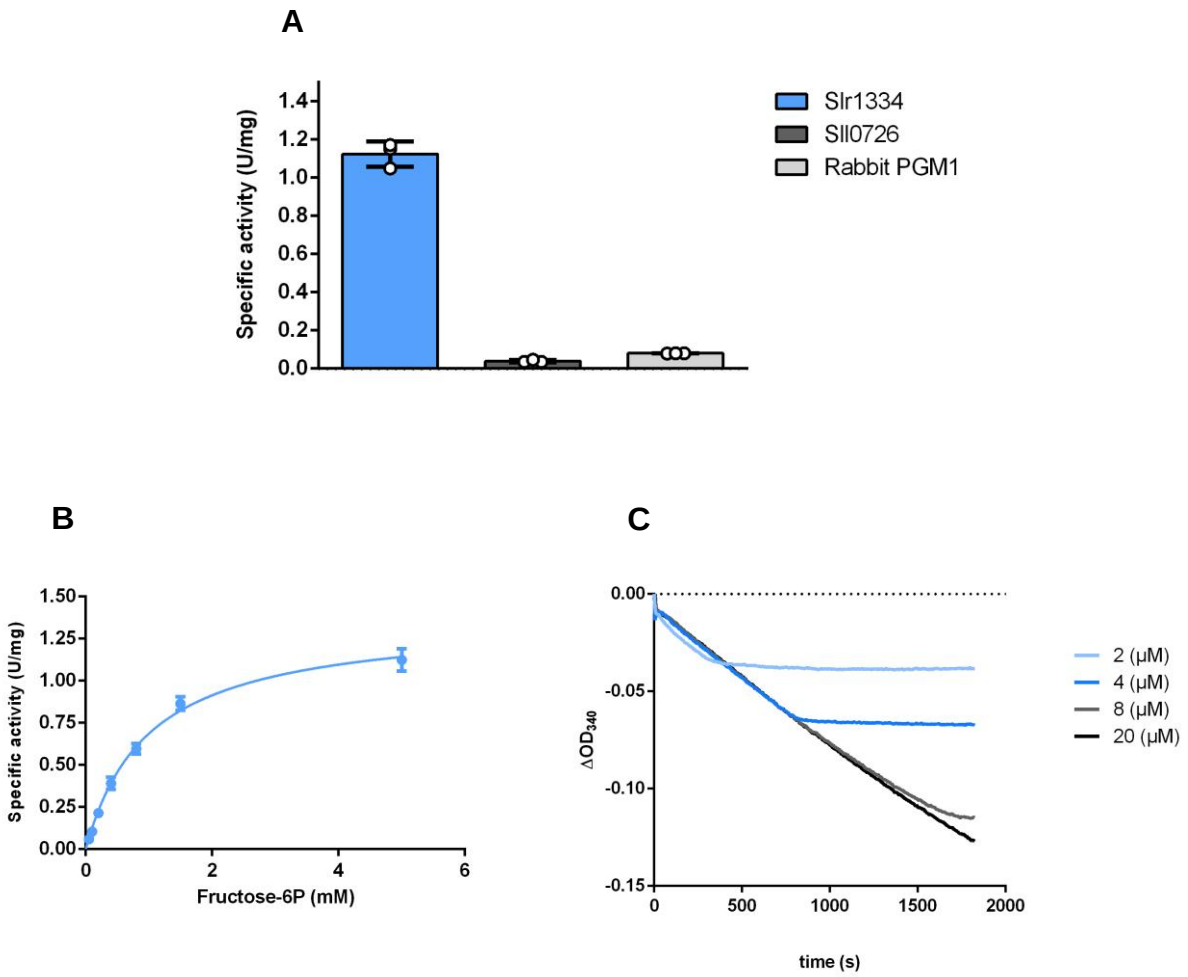

Figure 6: Slr1334 catalyzes the reverse reaction to form Frc-1,6-BP out of Glc-1,6-BP and Frc-6P

(A) Activity of Slr1334 (blue), Sll0726 (grey), and rabbit PGM1 (black) using 50 µM Glc-1,6-BP and 5 mM Frc-6P. (B) Michaelis-Menten kinetics of Slr1334 using different concentrations of Frc-6P and 50 µM Glc-1,6-BP. (C) Slr1334 reaction at different concentrations of Glc-1,6-BP and 5 mM Frc-1P. Three replicates were measured for each

392 **The Glc-1,6-BP synthase reaction is also performed by other members of the cd05800 group**

393 To determine whether the reaction catalyzed by Slr1334 is representative for αPHM enzymes
394 of the cd05800 family, we decided to analyze the corresponding homologue from the human
395 microbiome associated bacterium *Bacteroides salyersiae*. Therefore, we tested formation of
396 Glc-1,6-BP under the same conditions as described above for Slr1334 by incubating the
397 recombinant cd05800 enzyme from *B. salyersiae* with Frc-1,6-BP and Glc-1P or Glc-6P and
398 subsequently adding the supernatant of this reaction to the Sll0726 assay. As a positive control
399 we used the reaction of Slr1334.
400 The addition of the supernatant from the *B. salyersiae* cd05800 PGM increased the velocity of
401 Sll0726 in a comparable manner as the supernatant of the Slr1334 reaction (**Fig. 7**). This
402 indicates that similar amounts of Glc-1,6-BP have been formed in both reactions, hence
403 revealing that *B. salyersiae* cd05800 PGM catalyzes the same reaction as Slr1334.
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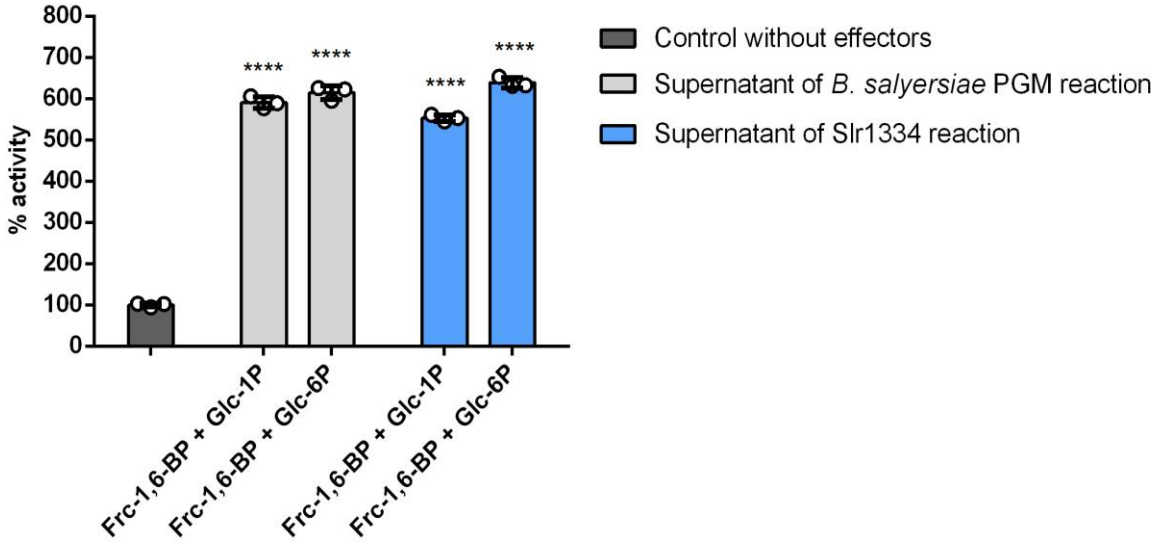

415
416 **Figure 7: Slr1334 homologue from *B.salyersiae* also forms Glc-1,6-BP out of Frc-1,6-BP**

417 Shown is the activity of Sll0726: blue, after addition of the supernatant of the *B. salyersiae*
418 PGM reaction; in light grey, after addition of the supernatant of the Slr1334 reaction as a
419 positive control; in dark grey, without any effectors as a negative control. The different
420 combinations of substrates used for the *B. salyersiae* PGM and Slr1334 control reaction are
indicated on the X-axis. Values represent relative activity compared to the Sll0726 reaction
without any effectors, which was considered 100%. Error bars represent the SD; asterisks
represent the statistical significance.

421 # Discussion

422 Among the key reactions in carbon metabolism are the phosphoryl transfer reactions on
423 different phosphosugars, which are catalyzed by the members of the αPHM superfamily.
424 These reactions are essential for glycogen metabolism, amino sugar metabolism as well as cell
425 wall synthesis and are conserved throughout all domains of life. The interconversion of Glc-1P
426 to Glc-6P and vice versa by PGM is by far the best studied of these reactions and investigation
427 of PGM goes back to the 1940s. It has long been known that PGM requires Glc-1,6-BP as a
428 activator [10]. While in mammals free Glc-1,6-BP has been shown to derive from the reaction of
429 the PGM2L1 enzyme utilizing Glc-1P and 1,3-BPG as substrates, its origin in prokaryotes has
430 remained unknown [3].
431 In this study we used the unicellular cyanobacterium *Synechocystis* sp. PCC6803 as a model
432 organism to identify the first bacterial glucose-1,6-BP synthase. This reversible reaction is a
433 novel function of a member of a distinct αPHM subfamily producing free α-D-Glc-1,6-BP and
434 Frc-6P from Frc-1,6-BP and Glc-1P/Glc-6P.
435 Glc-1,6-BP is essential for PGM activity due to the requirement for phosphorylation of the
436 serine residue at the active site for enzyme activation. The activating reaction converts Glc-
437 1,6-BP into either Glc-1P or Glc-6P. After this initial activation, the interconversion of Glc-1P
438 to Glc-6P is mediated by a transfer of the phosphate group from the serine residue at the
439 active site to the 1' or 6' position of the substrate respectively, forming a Glc-1,6-BP
440 intermediate. This intermediate has to reconfigure inside the enzyme and transfer the
441 phosphate of its 6' or 1' position back to the active site serine leading to the release of Glc-6P
442 or Glc-1P, respectively. As a result of this mechanism, the active site remains phosphorylated
443 and the enzyme is ready for a new cycle. However, it has been shown that the active site
444 phosphorylation of PGM is lost after an average of 15-20 reaction cycles by a spontaneous
445 dissociation of the intermediate Glc-1,6-BP leaving behind an inactive dephosphoenzyme.
446 Therefore, a constant level of Glc-1,6-BP is necessary to ensure that PGM can be kept in its
447 active state [20, 21]. While the loss of the intermediate might be considered an unfavorable event,
448 it can also be considered as a regulatory mechanism that enables regulation of PGM activity
449 based on Glc-1,6-BP levels. For other αPHMs like PMM and PNGM it is known that the
450 corresponding intermediates (e.g. α-D-mannose-1,6-BP and α-D-glucosamine-1,6-BP) are
451 formed during the reaction of the respective enzymes in the same way as for PGM but no

452 source of those intermediates is known that would enable an initial activation of these αPHMs
453 or would keep them in an active state overtime when phosphorylation is lost. However, since
454 many members of the αPHM family show at least some residual PGM activity, Glc-1,6-BP was
455 suggested to be the activating compound for these enzymes as well. This has in fact been
456 shown for PNGM from *E. coli* which is considered to be an essential enzyme [22]. This makes the
457 role of Glc-1,6-BP in carbon metabolism even more relevant and would explain the failure in
458 deleting the slr1334 gene shown by [17].
459 In mammals, it was shown that Glc-1,6-BP can also regulate central enzymes of carbon
460 pathways by inhibiting hexokinases, 6-phosphogluconate dehydrogenase and fructose-1,6-
461 bisphosphatase and by activating phosphofructokinase and pyruvate kinase [12]. Further studies
462 in mammals showed that Glc-1,6-BP is especially elevated in brain tissue to a level that highly
463 exceeds the regulatory concentrations needed for PGM activity, and that reduced levels of
464 Glc-1,6-BP cause severe neurological problems [23]. This indicates that at least in mammals
465 there are likely more functions of Glc-1,6-BP to be discovered. Given the important role of Glc-
466 1,6-BP in eukaryotic metabolism, it is surprising that very little is known on the role of Glc-1,6-
467 BP in bacterial physiology. The only study about the origin of α-D-Glc-1,6-BP production in
468 bacteria proposed a phosphodismutase reaction with Glc-1P as the sole substrate in *E. coli*
469 [24]. Moreover, the levels of Glc-1,6-BP in eukaryotes are affected by a specific Glc-1,6-BP
470 phosphatase, PMM1, which is a phosphomannomutase that is not part of the αPHM
471 superfamily. Whether a homologue exists in bacteria is currently unknown [24].
472 In absence of Glc-1,6-BP, both Slr1334 and Sll0726 showed low PGM activity. This residual
473 activity can be explained by the fact that a phosphorylation of the active site serine can also
474 be achieved at high concentrations of Glc-1P but at a much lower efficiency, leading to high
475 K_m values and overall strongly reduced activity [11]. In the presence of Frc-1,6-BP, Sll0726 was
476 strongly inhibited, which agrees with the inhibitory effect of Frc-1,6-BP on other PGMs [19, 25].
477 By contrast, Frc-1,6-BP enhanced the overall catalytic activity of Slr1334, primarily because of
478 a strongly decreased K_m. This effect can now be attributed to the formation of the activator
479 Glc-1,6-BP.
480 Like mammalian PGM2L1, Slr1334 can utilize 1,3-BPG as a phosphorylating compound for Glc-
481 1,6-BP formation, however with a much lower efficiency than with Frc-1,6-BP. For the
482 mammalian PGM2L1 a reverse reaction (formation of 1,3-BPG and Glc-1P from Glc-1,6-BP and
483 3-PG) has not been shown. For Slr1334, we could clearly show reversibility of the reaction,

484 producing Frc-1,6-BP from Glc-1,6-BP and Frc-6P. Provided that both directions of the reaction
485 have similar kinetic properties, Slr1334 appears to be a bona-fide Glc-1,6-BP synthase, which
486 is prone to activate the main PGM Sll0726.
487 In contrast to Glc-1,6-BP, Frc-1,6-BP is an obligatory intermediate in glycolysis,
488 gluconeogenesis and Calvin-Benson-Bassham cycle. In contrast to mammalian PGM2L1,
489 *Synechocystis* Slr1334 uses this sugar as the major substrate for Glc-1,6-BP synthesis. This links
490 the synthesis of Glc-1,6-BP to the levels of Frc-1,6-BP. Its steady state level depends on the
491 activity of the opposing reactions of phosphofructokinase (Pfk) and Fructose-1,6-
492 bisphosphatase (FBPase), both of which are regulated in an opposite manner: While PFK is
493 usually activated by the low energy state metabolites ADP and AMP and downregulated by
494 the high energy state metabolites ATP and citrate, FBPase is downregulated by AMP while
495 unaffected by high energy compounds. The level of Frc-1,6-BP is further determined by the
496 Frc-1,6-BP-aldolase activity. Frc-1,6-BP is also known to be an allosteric activator of pyruvate
497 kinase (PK), the final step in glycolysis [26].
498 Altogether, in the cyanobacterium *Synechocystis* PCC6803, Frc-1,6-BP appears to have two
499 counteracting effects on PGM activity. On the one hand, it acts as a direct inhibitor of PGM;
500 on the other hand, it gives rise to the synthesis of the PGM activator Glc-1,6-BP through the
501 activity of Slr1334. Furthermore, since these double phosphorylated sugars may affect further
502 enzyme reactions, Slr1334, which connects these two metabolites, might represent a crucial
503 regulatory point in glycolysis (**Fig. 8**). This conclusion would further underline the essential
504 role of *slr1334* [17].
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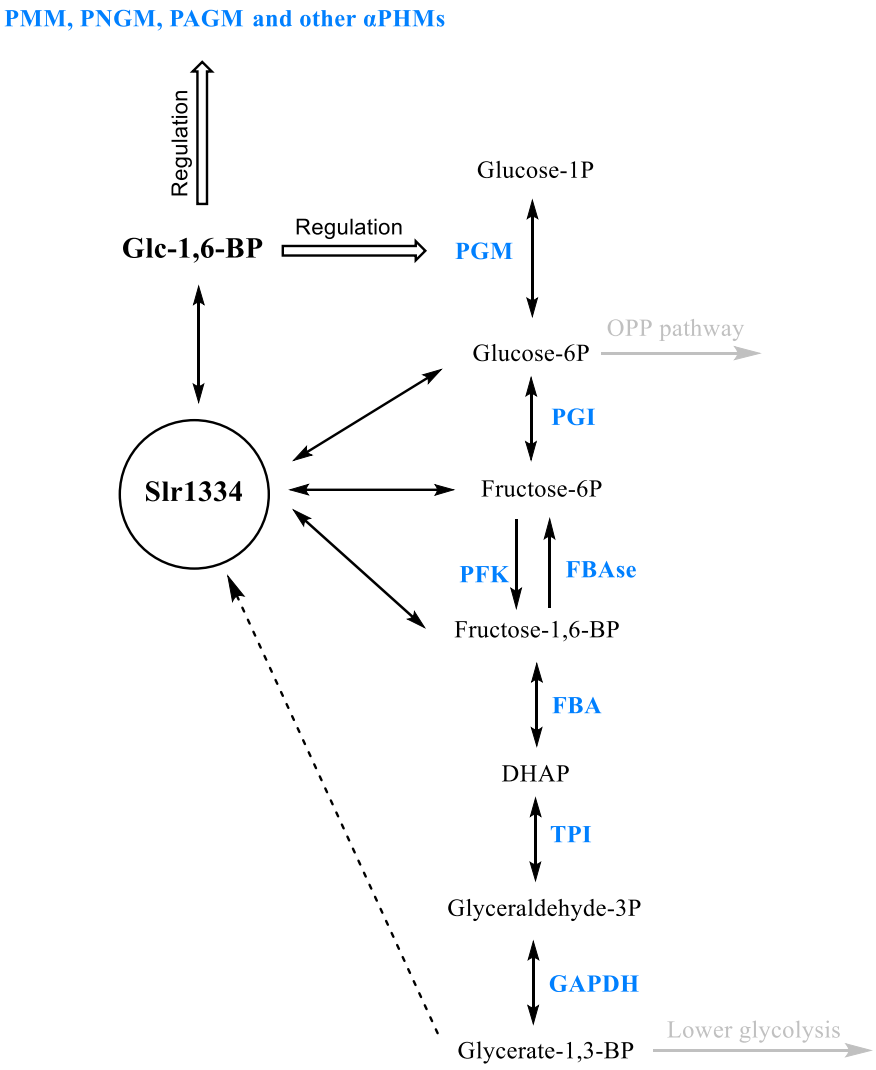

Figure 8: Potential role of Slr1334 in glycolysis

Shown is the potential role of Slr1334 in regulating activity of αPHMs and the concentration of key metabolites in glycolysis. PGM: Phosphoglucomutase; PGI: Glc-6P-Isomerase; PFK: Phosphofruktokinase; FBAse: Fructose-6P phosphatase; FBA: Frc-1,6-BP-aldolase; TPI: Triosephosphate isomerase; GAPDH: Glyceraldehyde-3P dehydrogenase; DHAP: Dihydroxyacetone phosphate

529 Interestingly, our phylogenetic analysis revealed the cd05800 subfamily as the most
530 primordial group of phosphoglucomutases. This group appears to be especially present in
531 bacterial classes that are considered deeply branching bacteria meaning they are relatively
532 close to the last common universal ancestor (LUCA). Examples for this are the classes of
533 Deinococci, Aquificae, Thermotogae, Bacteroidetes and Cyanobacteria, where this PGM
534 subfamily is widely distributed. This finding is supported by the fact that cd05800 members
535 can also be found in archaea. In agreement with this, the first characterized representative of
536 this group, Slr1334 shows both PGM and Glc-1,6-BP activity. This suggests that in primordial
537 systems these PGMs with broad functionality could have performed PGM reactions without
538 the requirement for specific Glc-1,6-BP synthase enzymes. During subsequent functional
539 diversification, the PGMs may have adopted unique functions, with specialization towards
540 efficient Glc-6-P to Glc-1P conversion on the one hand, and more specialized formation of the
541 activating compound Glc-1,6-BP on the other hand. This assumption agrees with the fact that
542 many bacteria, which have a cd05800 family member also have a specialized PGM of the
543 cd05801 or cd03085 subfamily for efficient Glc-6P – Glc-1P conversion. This includes the
544 ensemble of all cyanobacteria. Conversely, many pathogenic bacteria that have a PGM of the
545 cd03089 type lack a cd5800 representative (**Table S4**) which raises the question how those
546 strains produce Glc-1,6-BP. To clarify these issues further investigations on αPHM members
547 from various bacterial groups are required.
548 We were able to show that the cd05800 PGM from the human gut commensal bacterium *B.*
549 *salyersiae* also catalyzes the synthesis of Glc-1,6-BP out of Frc-1,6-BP like Slr1334 (**Fig. 7**). In
550 case of the PGM from *B. salyersiae* the enzyme appears to have an even higher catalytic
551 efficiency for this reaction than for the standard PGM reaction (**Fig. S1**). Therefore, we
552 conclude that the formation of Glc-1,6-BP is a general feature of this group of PGMs.
553 Interestingly, members of the cd05800 group are also present in eminent and emerging
554 human pathogenic species like *Vibrio, Eggerthella* and *Brachyospyra* (**Fig. 1**) and may play a
555 key role in the metabolism of these pathogens. In fact, due to their crucial role in capsule and
556 cell wall synthesis PGMs have been suggested as novel targets for the development of
557 antiinfectives in prophylactic and therapeutic interventions [5, 27]. It is tempting to speculate
558 that Glc-1,6-BP synthases of the cd05800 family, in virtue of their absence in eukaryotes, may
559 also be interesting candidates for the development of novel drugs modulating the microbiota
560 with little collateral damage to the host.

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Author contributions

N.N. performed cloning, protein purification, enzymatic assays phylogenetic analysis and wrote manuscript. S.F. performed LC/MS analysis. K.F. conceived study, interpreted data and edited manuscript.

Competing interests

The authors declare no competing interest.

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670

671 # Methods

672 **Isothermal, Single-Reaction DNA Assembly (Gibson Cloning)**

673 Cloning was performed as described by Gibson, Young [1] using *E. coli* NEB10β cells (details in
674 **Table S5**). All primers and plasmids used are shown in **Table S3** and **Table S4**, respectively.
675

676 **Cultivation of *Escherichia coli***

677 If not otherwise stated *E. coli* was grown in Luria-Bertani medium at 37°C [2]. For growth on
678 plates, 1.5% (w/v) agar-agar was added. For cells containing plasmids, the appropriate
679 concentration of antibiotics was used. All *E. coli* strains used in this study are listed in **Table**
680 **S5.**

681

682 **Protein overexpression and purification**

683 The plasmids used for protein overexpression are shown in **Table S4.** *Escherichia coli* Rosetta-
684 gami (DE3) (details **Table S5**) was used for the overexpression of all proteins. For this, cells
685 were cultivated in 2xYT (3.5% tryptone, 2% yeast extract, 0.5% NaCl; 1L of culture in 5L flasks)
686 at 37 °C until reaching exponential growth (OD_{600} 0.6-0.8). Protein overexpression was
687 induced by adding 75 µg/L anhydrotetracycline, followed by incubation at 20°C for 16 h. Cells
688 were harvested by centrifugation at 4000 g for 10 min at 4 °C. Cell disruption was performed
689 by sonication in 40 mL of lysis buffer (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$,
690 20 mM Imidazole for His-tagged proteins and 100 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM
691 $MgCl_2$ for Strep-tagged proteins) Lysis buffers were supplemented with DNAse I, and
692 cOmplete™ protease inhibitor cocktail (Roche, Basel)). The cell lysate was centrifuged at
693 40,000 g for 45 min at 4°C and the supernatant was filtered with a 0.22 µM filter.
694 For the purification of His-tagged proteins, 5 mL Ni-NTA HisTrap columns (GE Healthcare,
695 Illinois, USA) were used. The cell extracts were loaded onto the columns, washed with wash
696 buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, and 50 mM Imidazole) and eluted with elution
697 buffer (50 mM Tris-HCl pH 7.4, 500 mm NaCl, and 500 mM Imidazole).
698

699 **Measurement of PGM activity of Sll0726 and Slr1334 in vitro**

700 Buffer for enzymatic reactions was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10
701 mM $MgCl_2$, 1 mM $NADP^+$, 1 mM DTT and 1 U/mL G6PDH from *Saccharomyces cerevisiae*
702 (G6378, Sigma Aldrich). For Sll0726 activity, 60 ng of Strep-tagged purified protein was added
703 to each reaction. For slr1334 activity, 650 ng of purified protein was added. For tests with
704 saturating Glc-1,6-BP concentrations, 60 µM Glc-1,6-BP have been used. Reaction was started
705 by the addition of glucose-1P. Reactions were carried out in a total of 300 µl in a 96-well
706 microplate. Absorption change at 340 nm was continuously measured for 15 min at 30 °C in a
707 TECAN Spark® Multiplate reader (Tecan Group AG, Männedorf, Switzerland). At least three
708 replicates were measured.
709
710 **Measurement of Glc-1,6-BP formation**
711 The reaction buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl and 10 mM
712 $MgCl_2$. To test the formation of Glc-1,6-BP out of Frc-1,6-BP 1 mM Frc-1,6-BP, 1 mM Glc1P or
713 1 mM Glc-6P were added in different combinations. To test the formation of Glc-1,6-BP out of
714 1,3-BPG instead of Frc-1,6-BP, 1 mM of ATP, 1 mM 3-PG and 60 µg PGK have been used.
715 Sll0726 and Slr1334 concentration were 30 µg/ml. The reactions were performed for 1.5 h at
716 30 °C followed by heat inactivation at 90°C for 10 minutes with subsequent centrifugation at
717 25000 g for 10 minutes at 4° C. The supernatants of the various reactions were used at 1:10
718 dilution in the Sll0726 activity assay.
719
720 **Frc-1,6-BP aldolase assay**
721 To measure the formation of Frc-1,6-BP by Slr1334, the reaction buffer was composed of 50
722 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.2 mM NAD, 7 µg Slr1334, 1U/ml aldolase
723 from rabbit muscle and 1U/ml GDH from rabbit muscle. Concentrations of Glc-1,6-BP and Frc-
724 6P were varying depending on the experiment. Reaction was started by the addition of Glc-
725 1,6-BP or Frc-6P depending on the experiment.
726 To measure residual Frc-1,6-BP after running the Slr1334 reaction, the buffer was composed
727 of 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.2 mM NAD, 0.02U/ml aldolase
728 from rabbit muscle and 1U/ml GDH from rabbit muscle.
729 All reactions were carried out in a total of 300 µl in a 96-well microplate Absorption change at
730 340 nm was continuously measured for 15 min at 30 °C in a TECAN Spark® Multiplate reader

731 (Tecan Group AG, Männedorf, Switzerland). The enzymatic activity was then calculated. At
732 least three replicates were measured.
733
734 **LC-MS measurement**

735 Sample preparation:
736 Reaction buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl and 10 mM $MgCl_2$.
737 For the reaction without enzyme 500 µM Frc-1,6-BP and 1 mM Glc1-P were added. For the
738 Slr1334 reaction 30 µg of Slr1334 have been used additionally. Reaction was carried out in a
739 total volume of 1 ml at 30 °C for 1h. Afterwards reaction was stopped by heat inactivation at
740 90° C followed by centrifugation at 25000 g for 10 minutes at 4°C.
741 For degradation of Frc-1,6-BP 1 mM NADH, 2U/ml of aldolase from rabbit muscle and 2U/ml
742 of GDH from rabbit muscle were added. Reaction was carried out followed by inactivation at
743 the same conditions as before.
744 LC/MS analysis:
745 LC/MS analysis was performed using an electrospray ionization time of flight (ESI-TOF) mass
746 spectrometer (MicrOTOF II; Bruker Daltonics), operated in negative ion-mode connected to
747 an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). The
748 separation in the HPLC was carried out using a SeQuant ZIC-pHILIC column (PEEK 150 × 2.1
749 mm, 5 µm, 110 Å, Merck) at 30°C with an CH_3CN (buffer A) and 100 mM $(NH_4)_2CO_3$, pH 9
750 (buffer B) buffer system. A single run (injection volume of 5 µl) was performed with a flow rate
751 of 0.2 ml/min and a linear gradient of 25 min, reducing the concentration of buffer A from 82
752 to 42 %. Before (5 min) and after (10 min) the gradient, the column was equilibrated with 82 %
753 buffer A.
754
755 **Phylogenetic analysis of αPHM subfamilies**

756 The occurrence of members of the αPHM subfamilies was investigated in organisms included
757 in the NCBI RefSeq_protein database. Phylogenetic tree data were created using the ClustalW
758 implementation from the European Bioinformatics Institute (EMBL-EBI) and the phylogenetic
759 tree was created using the Interactive Tree Of Life (iTOL) v5 online tool [3, 4].
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Statistical analysis

Statistical details for each experiment can be found in the figure legends. GraphPad PRISM was used to perform one-sided ANOVA to determine the statistical significance. Asterisks (*) in the figures symbolize the p-value: One asterisk represents $p \leq 0.05$, two asterisks $p \leq 0.01$, three asterisks $p \leq 0.001$, and four asterisks $p \leq 0.0001$.

Methods References

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