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1 **Discovery and characterization of a sulfoquinovose mutarotase using kinetic analysis at**
2 **equilibrium by exchange spectroscopy**

3

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15

16 **Abstract**

17 Bacterial sulfoglycolytic pathways catabolise sulfoquinovose (SQ), or glycosides thereof, to
18 generate a three-carbon metabolite for primary cellular metabolism and a three-carbon
19 sulfonate that is expelled from the cell. Sulfoglycolytic operons encoding an Embden-
20 Meyerhof-Parnas (EMP)-like or Entner-Doudoroff (ED)-like pathway harbour an
21 uncharacterized gene (yihR in *Escherichia coli*; PpSQ1_00415 in *Pseudomonas putida*) that is
22 upregulated in the presence of SQ and has been annotated as an aldose-1-epimerase and which
23 may encode an SQ mutarotase. Our sequence analyses and structural modelling confirmed that
24 these proteins possess mutarotase-like active sites with conserved catalytic residues. We over-
25 expressed the homologue from the sulfo-ED operon of *Herbaspirillum seropedicaea* (HsSQM)
26 and used it to demonstrate SQ mutarotase activity for the first time. This was accomplished
27 using NMR exchange spectroscopy (EXSY), a method that allows chemical exchange of
28 magnetization between the two SQ anomers at equilibrium. HsSQM also catalyzed the
29 mutarotation of various aldohexoses with an equatorial 2-hydroxy group, including D-
30 galactose, D-glucose, D-glucose-6-phosphate, and D-glucuronic acid, but not D-mannose.
31 HsSQM displayed only 5-fold selectivity in terms of efficiency (k_{cat}/K_M) for SQ versus the
32 glycolysis intermediate glucose-6-phosphate (Glc-6-P), however its proficiency [$k_{uncat} /$
33 (k_{cat}/K_M)] for SQ was 17,000-fold better than for Glc-6-P, revealing that HsSQM preferentially
34 stabilises the SQ transition state.

35 **Introduction**

36 Various prokaryotes metabolise the sugar sulfoquinovose (SQ) to sulfolactaldehyde (SLA) and
37 dihydroxyacetone phosphate (DHAP), via an Embden-Meyerhof-Parnas (EMP)-like pathway
38 [1], or pyruvate, via an Entner-Doudoroff (ED)-like pathway [2, 3] (Figure 1). While the DHAP
39 or pyruvate feed into primary metabolic pathways, SLA is converted to 2,3-
40 dihydroxypropanesulfonate (DHPS) or sulfolactate (SL) by the sulfo-EMP and sulfo-ED
41 pathways, respectively, and excreted from the cell. Yet sulfoquinovose is rarely encountered
42 as a free sugar in nature; rather it is liberated from the plant sulfolipid α -sulfoquinovosyl
43 diacylglycerol (SQDG), or its delipidated form α -sulfoquinovosyl glycerol (SQGro), by the
44 action of glycoside hydrolases termed sulfoquinovosidases (SQases) [4]. SQases are retaining
45 glycosidases, and result in the initial formation of α -SQ, which can undergo mutarotation to β -
46 SQ at an unknown rate. The anomeric preferences of the immediate downstream enzymes that
47 utilize SQ (SQ isomerase for the sulfo-EMP pathway; SQ dehydrogenase for the sulfo-ED
48 pathway) are unknown.

49 All proteins comprising a sulfo-ED or sulfo-EMP pathway are typically encoded within
50 a single gene cluster. These clusters usually include an SQase, which highlights that SQ
51 glycosides are important natural feedstocks for sulfoglycolytic pathways [1, 2]. The gene
52 clusters also encode a conserved uncharacterized protein, annotated as an aldose-1-epimerase,
53 which likely catalyses SQ mutarotation: an enzyme activity yet to be reported. Mutarotases are
54 widely distributed enzymes that facilitate the rapid mutarotation of aldoses to enhance flux
55 through metabolic pathways when enzymes acting on reducing sugars are specific for a single
56 anomer [5, 6].

57 A classical approach to studying mutarotases involves measuring rates of mutarotation
58 by polarimetry [7-10]. These assays are limited by an inability to prepare pure samples of a
59 single anomer of many reducing sugars. Indeed, to date it has not been possible to obtain SQ
60 as a single anomer, which makes this approach of limited use for studying putative SQ
61 mutarotases (SQMs). Alternative approaches to studying mutarotases have employ coupled
62 assays in which only one anomer acts as substrate for a secondary enzyme [11, 12] or a
63 chemical reaction (e.g. bromine oxidation [13]), or use a glycosidase to prepare a single anomer
64 in solution prior to addition of the mutarotase [12]. The high rate of uncatalyzed mutarotation
65 under standard conditions often renders these assays technically demanding, and a requirement
66 for different coupling enzymes for each substrate undermines their generality. An alternative
67 approach is to study reaction rates at equilibrium. NMR spectroscopy is ideally suited to this

68 approach using the technique of exchange spectroscopy (EXSY) [14, 15]. EXSY involves
69 chemical shift labelling of the spin population of nuclei at one site within a substrate, followed
70 by a chemical reaction that changes the chemical environment of individual nuclei resulting in
71 magnetization transfer to the new site, and finally sampling of the magnetization states of the
72 nuclei in the substrate and product. Because of the spectral resolution of NMR spectroscopy
73 and the potential to conduct two-dimensional variants, EXSY can be used to study
74 unidirectional reactions at equilibrium. Several reports have described the development of
75 saturation transfer and inversion transfer NMR methods for analysis of equilibrium exchange
76 rates for mutarotases [16-19].

77 Here, we disclose the first measurement of SQ mutarotase activity, using an enzyme
78 from *Herbaspirillum seropedicaea* (HsSQM) and 2D EXSY, and analyze its selectivity for
79 various reducing sugars with and without an anionic substituent at C6. HsSQM exhibited a
80 broad spectrum of activity for sugars with an equatorial hydroxyl group at C2 and with, or
81 without, charge at C6. 1D EXSY was used to measure reaction rates to obtain Michaelis-
82 Menten kinetics for HsSQM with SQ and glucose-6-phosphate, a common cytoplasmic
83 metabolite, revealing an approximate 5-fold preference for SQ as a substrate. Sequence and
84 structural analyses revealed HsSQM belongs to the galactose mutarotase-like Structural
85 Classification Of Proteins (SCOP) with strictly conserved active site residues that are proposed
86 to be involved in substrate binding and catalysis.

87

88 **Materials and Methods**

89 **Reagents**

90 D-Glucose (Glc), D-mannose (Man), D-galactose (Gal), D-glucuronic acid (GlcA) and D-
91 glucose-6-phosphate (Glc-6-P) were purchased from Sigma Aldrich. D-Sulfoquinovose was
92 purchased from MCAT GmbH (Germany, <http://www.MCAT.de>). 4-Nitrophenyl α -D-
93 sulfoquinovoside and sulfoquinovosidase have been described previously [4].

94

95 **Cloning, expression and purification of HsSQM**

96 A gene encoding the *Herbaspirillum seropedicae* strain AU14040 protein WP_069374721.1
97 was codon harmonised for *E. coli*, synthesised and cloned into the pET29 vector using the NdeI
98 and XhoI restriction sites to provide pET29-HsSQM (see Supporting Information). This
99 plasmid was transformed into chemically competent ‘NEB Express’ *E. coli*, plated onto LB-
100 agar (50 μ g/ml kanamycin) and incubated at 37 °C for 16 h. A single colony was used to
101 inoculate 10 ml of LB media containing 50 μ g/ml kanamycin followed by incubation at 37 °C
102 for 16 h. This culture was used to inoculate 1000 ml of “S-broth” (35 g tryptone, 20 g yeast
103 extract, 5 g NaCl, pH 7.4) containing 50 μ g/ml kanamycin, which was incubated with shaking
104 (250 rpm) at 37°C until it reached an OD₆₀₀ of 0.7. The culture was cooled to room temperature,
105 IPTG added to a final concentration of 100 μ M, and then incubated with shaking (200 rpm) at
106 18 °C for 19 h. The cells were harvested by centrifugation at 17,000 g for 20 min at 4 °C then
107 resuspended in 40 ml of binding buffer (50 mM NaP_i, 500 mM NaCl, 5 mM imidazole, pH 7.5)
108 containing protease inhibitor (Roche complete EDTA-free protease inhibitor cocktail) and
109 lysozyme (0.1 mg/ml) by nutating at 4 °C for 30 min. Benzonase (1 μ l) was added to the
110 mixture and lysis was effected by sonication. The lysate clarified by centrifugation (17,000 g
111 for 20 min at 4 °C), the supernatant filtered (0.45 μ m) and loaded onto a 1 ml HiTrap TALON
112 column (GE Healthcare). The column was washed with 15 ml binding buffer and the protein
113 was eluted using elution buffer (50 mM NaP_i, 500 mM NaCl, 500 mM imidazole, pH 7.5).
114 Fractions containing the protein of interest (as determined by SDS-PAGE) were further
115 purified by size exclusion chromatography on a HiLoad 16/600 Superdex 75 column using 50
116 mM NaP_i, 150 mM NaCl, pH 7.5. Fractions containing the protein of interest were further
117 purified on a MonoQ 5/50 column with protein bound in buffer A (20 mM Tris, pH 7.5) and
118 eluted with a linear gradient from 100% buffer A to 100% buffer B (20 mM Tris, NaCl 500
119 mM, pH 7.5) over 15 ml at 1 ml/min. Protein yield was \approx 5 mg per litre of culture.

120

121 **NMR magnetization-transfer experiments**

122 The theoretical background of NMR for the study of kinetics of chemical exchange has been
 123 well documented [14, 20] and only a brief overview is presented. The interconversion of
 124 anomers by mutarotation is described by a simple two-state equilibrium:



126
 127
 128 For a species exchanging between isomers, the dependence upon time (t) of the longitudinal
 129 nuclear magnetizations of corresponding nuclei from the α and β anomer spin populations (I_α)
 130 and (I_β), corresponding to the integrals, are given by the Bloch-McConnell equation:

$$131 \quad \frac{d}{dt} \begin{pmatrix} I_\alpha \\ I_\beta \end{pmatrix} = \begin{pmatrix} -R_\alpha - k_1 & k_{-1} \\ k_1 & -R_\beta - k_{-1} \end{pmatrix} \begin{pmatrix} I_\alpha \\ I_\beta \end{pmatrix} + \begin{pmatrix} R_\alpha & 0 \\ 0 & R_\beta \end{pmatrix} \begin{pmatrix} I_\alpha^0(t) \\ I_\beta^0(t) \end{pmatrix} \quad (2)$$

132
 133
 134 Where R_α and R_β are the longitudinal relaxation rates, characterizing the return of the
 135 magnetizations towards their respective equilibrium values, I_α^0 and I_β^0 ; k_1 and k_{-1} are the
 136 extrinsic rate constants for the forward and reverse reactions for each anomer, α and β
 137 respectively. The solution of this equation gives the intensity of the transferred magnetization
 138 at τ_{mix} :

$$139 \quad \begin{pmatrix} I_\alpha(\tau_{\text{mix}}) \\ I_\beta(\tau_{\text{mix}}) \end{pmatrix} = \exp \left[\begin{pmatrix} -R_\alpha - k_1 & k_{-1} \\ k_1 & -R_\beta - k_{-1} \end{pmatrix} \tau_{\text{mix}} \right] \times \begin{pmatrix} I_\alpha(0) - I_\alpha^0 \\ I_\beta(0) - I_\beta^0 \end{pmatrix} + \begin{pmatrix} I_\alpha^0 \\ I_\beta^0 \end{pmatrix} \quad (3)$$

140
 141
 142 Under the condition of chemical equilibrium, the net flux is zero. However, the unidirectional
 143 fluxes remain and are equal in both directions. Thus,

$$144 \quad k_1[\alpha]^{\text{eq}} = k_{-1}[\beta]^{\text{eq}} \quad (4)$$

145
 146
 147 And

$$148 \quad K_{\text{eq}} = \frac{[\beta]^{\text{eq}}}{[\alpha]^{\text{eq}}} = \frac{k_1}{k_{-1}} = \exp\left(-\frac{\Delta G}{RT}\right) \quad (5)$$

150

151 To obtain the rate constants, k_1 and k_{-1} , at a given concentration ratio of the substrate and
152 enzyme, a series of 1D ^1H EXSY spectra with mixing time τ_{mix} , ranging from 5 ms to 1.5 s
153 were acquired using a 1D selective NOESY pulse sequence. For each substrate concentration,
154 the normalized integrals of the substrate (H1 α) and product (H1 β) anomeric signals are plotted
155 against mixing time, providing buildup and decay curves, respectively, as described previously
156 [21]. The data were then fitted to a second-order polynomial, an approximation to Eq 3, which
157 is valid for short mixing times. The tangent at $\tau_{\text{mix}} = 0$, the so-called initial rate approach [22],
158 provides estimates of both exchange-rate constants k_1 and k_{-1} .

159

160 Michaelis–Menten kinetics

161 The equilibrium-exchange kinetics (reaction rate, v^{eq}) of a mutarotase-mediated reaction can
162 be expressed as:

163

$$164 \quad v^{\text{eq}}([\alpha]) = \frac{V_{\text{max}}^{\text{eq}}[\alpha]}{K_{\text{M}}^{\text{eq}} + [\alpha]} \quad (9)$$

165

166 $V_{\text{max}}^{\text{eq}}$ is the maximum rate of reaction, reached under saturating equilibrium exchange
167 conditions when $[\alpha] \gg K_{\text{M}}^{\text{eq}}$. The rate of the mutarotation process can be expressed in terms of
168 the Michaelis-Menten equation:

169

$$170 \quad v^{\text{eq}} = k_1[\alpha]^{\text{eq}} = \frac{V_{\text{max}}^{\text{eq}}[\alpha]}{K_{\text{M}}^{\text{eq}} + [\alpha]} \quad (10)$$

171

172 k_{cat} is calculated by dividing $V_{\text{max}}^{\text{eq}}$ by the enzyme concentration.

173

174 2D ^1H - ^1H EXSY experiments

175 2D ^1H - ^1H EXSY spectra for all substrates (SQ, Glc-6-P, Glc, Gal, Man, GlcA) were acquired
176 at 25 °C on a Bruker Avance II 800 spectrometer equipped with a TXI cryoprobe using the
177 standard 2D NOESY/EXSY pulse sequence (noesyphpr). Spectra were collected with 4 and 8
178 scans in the absence and presence of enzyme, respectively. A mixing time, τ_{mix} of 1.1 s was
179 used for all the experiments. Spectra were processed and analysed using TOPSPIN (version
180 3.2 Bruker) and ^1H chemical shifts were referenced indirectly to DSS at 0 ppm via the H_2O
181 resonance, 4.77 ppm at 25 °C. Samples were prepared in D_2O buffer consisting of 50 mM

182 sodium phosphate, 150 mM NaCl (pD 7.5) with a substrate concentration 5 mM in the absence
183 or presence of 1.51 μ M HsSQM. pD was measured using a pH meter and the relationship pD
184 = pH + 0.4.

185

186 **1D 1 H EXSY experiments**

187 Michaelis-Menten parameters

188 1D 1 H EXSY spectroscopic studies for SQ and Glc-6-P were performed at 25 °C on a Bruker
189 Avance III 600 spectrometer equipped with a TCI cryoprobe using a 1D selective NOESY
190 pulse sequence (selnogp, Bruker). Samples were prepared in D₂O buffer consisting of 50 mM
191 sodium phosphate, 150 mM NaCl (pD 7.5) using 1.50 μ M HsSQM at substrate concentrations
192 ranging from 0.5-15.0 mM (for SQ) or 0.5-30.0 mM (for Glc-6-P). pD values were calculated
193 using the following relationship: pD = pH + 0.4. For each sample, 1D 1 H EXSY spectra with
194 mixing time, τ_{mix} , ranging from 5 ms to 1.5 s were acquired with number of scans varying
195 between 32 and 256 depending on the concentration of substrate. A recycle delay of 13.4 s (c.a.
196 3-5 times of measured 1 H T₁ relaxation times) between scans was used for the acquisition of
197 1D 1 H EXSY spectra. Spectra were subsequently processed and analysed using TOPSPIN
198 (version 3.2 Bruker). Kinetic data for conversion of the β -anomer of SQ to the α -anomer were
199 obtained by selectively inverting the resonance of H5 β at 3.72 ppm using a Gaussian-shaped
200 pulse of 20 ms, and vice versa, for conversion of the α -anomer to the β -anomer, the same
201 selective pulse was applied to the resonance of H5 α at 4.15 ppm. Similarly, for the conversion
202 of the β -anomer of Glc-6-P to the α -anomer, kinetic data were obtained by selectively inverting
203 the signal for H1 β at 4.57 ppm. A build-up curve for the β -anomer could not be obtained since
204 the signal for H1 β at 4.57 ppm was affected by the nearby HOD peak at 4.77 ppm (see SI data
205 for representative 1 H NMR spectra). Instead, the rate constant for the conversion of the α -
206 anomer to the β -anomer were calculated using Eq 4. Rates for each concentration were
207 calculated using the Prism 6 software package (GraphPad Scientific Software). Data were fitted
208 to a second-order polynomial function as described previously by Aski et al. [21].

209

210 pD dependence of activity for HsSQM

211 The Michaelis-Menten parameter $k_{\text{cat}}/K_{\text{M}}$ was measured for Glc-6-P mutarotation in D₂O buffer
212 consisting of 50 mM citrate/phosphate, 150 mM NaCl at a range of pD values (5.6, 6.1, 6.5,
213 7.0, 7.5, 8.1, 9.0, 9.4, 9.8, 10.4 and 10.9) at 25 °C. Reactions were initiated by adding 1.48-
214 2.96 μ M HsSQM to Glc-6-P (5.0 mM) in buffer and the rate measured by 1D 1 H EXSY as

215 described above with mixing time, τ_{mix} , ranging from 5 ms to 1.0 s and number of scans from
 216 64 and 128. Kinetic data were obtained for the conversion of the β -anomer of Glc-6-P to the α -
 217 anomer, by selectively inverting the signal for H1 β at 4.57 ppm. k_{cat}/K_M and $\text{p}K_a$ values were
 218 calculated using the Prism 6 software package (Graphpad Scientific Software). pH dependence
 219 was fit to the following function:

$$220 \quad y = \frac{k_{\text{cat}}}{K_M} \left[\left(\frac{1}{1 + \left(\frac{10^{-\text{pH}}}{10^{-\text{p}K_{a1}} + 10^{-\text{pH}}} \right)} \right) \right] + c \quad (11)$$

221

222 **Uncatalyzed mutarotation rate measurement**

223 The uncatalyzed rate constant for SQ mutarotation was measured using polarimetry on a Jasco
 224 DIP-1000 digital polarimeter equipped with Na 589 nm lamp and 100.00 mm cell, using a
 225 sulfoquinovosidase to generate α -SQ from 4-nitrophenyl α -D-sulfoquinovoside (PNPSQ).
 226 Analysis by ^1H NMR spectroscopy and thin layer chromatography revealed that hydrolysis of
 227 PNPSQ was complete within 5 min. SQase (final concentration 2.13 μM) was added to a
 228 solution of PNPSQ (final concentration 12.4 mM) in buffer in a final volume of 2 ml. Buffers
 229 consisted of: 50 mM sodium phosphate, 150 mM NaCl in H_2O (pH 7.1); 50 mM sodium
 230 phosphate, 150 mM NaCl in D_2O (pD 7.5); or 10-50 mM sodium phosphate, 2 M NaCl in D_2O
 231 (pD 7.5). The reaction mixture was transferred to the polarimetry cell and after 5 min the
 232 mutarotation rate was monitored continuously at 26 ± 1 $^\circ\text{C}$ by reading specific rotation at various
 233 times. The rate constant was calculated using the Prism 6 software package (Graphpad
 234 Scientific Software). Data were fitted to a one phase decay function, $t_{1/2} = \ln(2)/k$.

235 For mutarotation as described in equation 1, the rate of change in the concentration of
 236 the α -anomer has two contributions: it is depleted by the forward reaction at rate $k_1[\alpha]$ and is
 237 replenished by the reverse reaction at rate $k_{-1}[\beta]$. The net rate of change is therefore:

238

$$239 \quad \frac{d[\alpha]}{dt} = -k_1[\alpha] + k_{-1}[\beta] \quad (12)$$

240

241 The solution of this first-order differential equation is:

242

$$243 \quad [\alpha] = \left\{ \frac{k_{-1} + k_1 e^{-(k_1 + k_{-1})t}}{k_1 + k_{-1}} \right\} [\alpha]_0 \quad (13)$$

244

245 And thus the first order decay constant (k) is related to the forward and reverse rates as:

246

$$247 \quad k = k_1 + k_{-1} \quad (14)$$

248

249 Using equation (5), which relates the equilibrium constant to the forward and reverse rates, this
250 shows that:

251

$$252 \quad K^{eq} = \frac{[\beta]^{eq}}{[\alpha]^{eq}} = \frac{k_1}{k-k_1} \quad (15)$$

253

254

255 **Results**

256 Our initial efforts to characterize SQ mutarotases focused on expressing YihR from *E. coli*
257 (which utilizes the sulfo-EMP pathway) [1], and PpSQ1_00415 from *P. putida* SQ1 (which
258 utilizes the sulfo-ED pathway) [2] in an *E. coli* expression system. However, despite screening
259 several expression constructs and conditions, only a poor yield of low quality protein was ever
260 obtained. Thus, we turned our attention to other putative SQ mutarotases from various bacteria
261 that possess sulfoglycolytic gene clusters and succeeded in obtaining WP_069374721.1
262 (hereafter HsSQM) from *Herbaspirillum seropedicaea* in high yield and purity. *H.*
263 *seropedicaea* is a nitrogen-fixing endophytic bacterium capable of colonizing the intercellular
264 spaces of grasses such as rice and sugar cane [23], and contains a predicted sulfo-ED operon
265 analogous to that of *P. putida* SQ1 but lacking in synteny (Figure 2A) [2]. A sequence
266 alignment of these three putative sulfoquinovose mutarotases, as well as two structurally
267 characterized hexose mutarotases, is provided in Figure 2B: HsSQM shares 37% similarity
268 (19% identity) with *E. coli* YihR, and 50% similarity (35% identity) with *P. putida* SQ1
269 PpSQ1_00415 (SI Table 1). All three proteins retain the highly conserved residues of other
270 hexose mutarotases (His92, His162 and Glu254 in HsSQM). The equivalent residue to HsSQM
271 His162 in galactose mutarotase from *E. coli* has previously been proposed to be involved in
272 substrate binding [24], whereas the equivalent residues to His92 and Glu254 are proposed to
273 act in roles of general acid and general base, respectively, in the first half of the reaction leading
274 to the acyclic aldehyde, in galactose mutarotases from both *L. lactis* and *E. coli* [24, 25].

275 The iTASSER server [26-28] provided a homology model of HsSQM with a C-score
276 of 0.87, suggesting it to be a good approximation for the protein's native fold. This was
277 compared to existing structures by using TM-align [29, 30] and returned only other (predicted)
278 mutarotase structures. Greatest structural similarity was found between the HsSQM model and

279 a galactose mutarotase-like protein from *Clostridium acetobutylicum* (PDB 3OS7, Figure 3A),
280 with a RMSD of 1.55 Å between structural models, despite both proteins sharing <15%
281 identity. Structural alignment of the HsSQM homology model and the galactose mutarotase
282 domain of gal10 from *S. cerevisiae* with D-galactose bound (PDB 1Z45) [31] reveals that the
283 conserved active site residues His92, His162 and Glu254 in the homology model are positioned
284 appropriately for catalysis (Figure 3B).

285 2D ¹H-¹H EXSY was used to assess whether HsSQM can catalyze mutarotation. A 2D
286 pulse sequence equivalent to that used for a 2D ¹H-¹H NOESY experiment was employed to
287 provide a visual representation of the chemical exchange network [15, 20]. A similar approach
288 was utilized by Graille and coworkers to study a hexose-6-phosphate mutarotase [32], building
289 on work by Balaban and Ferretti for the study of anomerization/isomerization of Glc-6-P by
290 phosphoglucose isomerase using 2D ³¹P-³¹P EXSY [17], and earlier work by Kuchel and
291 coworkers, who studied mutarotation catalyzed by porcine mutarotase using ¹³C-¹³C EXSY
292 [16]. In this approach, all nuclei are excited using a 90° excitation pulse, which is allowed to
293 evolve over a t_1 period. A second 90° pulse is then applied to rotate the y-component of
294 magnetization onto the z-axis, and a mixing interval τ_{mix} , allows magnetization transfer through
295 chemical exchange. A final 90° pulse creates transverse magnetization in the xy plane, which
296 is detected. Molecules that undergo chemical interconversion display cross-peak signals
297 between a signal from the substrate along one axis of the 2D spectrum, and the corresponding
298 nucleus in the product on the other axis. Careful choice of mixing time to be shorter than that
299 required for chemical exchange by spontaneous mutarotation can allow qualitative detection
300 of enzyme-catalyzed mutarotation in a single NMR experiment. Figure 4A shows that a
301 solution of SQ displays H1 of the α - and β -anomers as independent sets of signals, and upon
302 addition of HsSQM off-diagonal cross-peaks appear between the anomeric protons. This data
303 provides evidence that chemical exchange is occurring and that HsSQM is catalyzing the
304 mutarotation of SQ.

305 To qualitatively explore the substrate specificity of HsSQM, we assessed the ability of
306 the enzyme to catalyze mutarotation of other simple hexoses (Figures 4B-F). HsSQM could
307 catalyze the mutarotation of D-glucose-6-phosphate and D-glucuronic acid, showing that the
308 sulfonate group is not required and that HsSQM can tolerate other anionic groups. A set of
309 simple aldohexoses was also studied. HsSQM catalyzed mutarotation of D-glucose and D-
310 galactose, showing that the enzyme is tolerant of either stereochemistry at C4. However, using
311 this pulse sequence we could not detect HsSQM-mediated mutarotation of D-mannose on the

312 NMR time-scale, demonstrating the preference of HsSQM for substrates with an equatorial
313 C2-hydroxyl group.

314 While these experiments provide insights into the substrate specificity of HsSQM,
315 simple hexoses are not present at any appreciable concentration within bacterial cells, as shown
316 for *E. coli* grown on various carbon sources [33]. Other than SQ, the only other physiologically
317 plausible substrate for HsSQM is glucose-6-phosphate (Glc-6-P): an abundant primary
318 metabolite produced by bacteria grown on glucose, glycerol or acetate [33]. To explore the
319 selectivity of HsSQM for SQ and Glc-6-P, we used EXSY to determine kinetic parameters
320 under conditions of equilibrium exchange. While it is possible to use 2D methods to determine
321 rates, accurate determination requires a priori knowledge of optimum mixing times and a
322 lengthy experiment, and so we elected to use the alternative 1D EXSY approach. Measurement
323 of the equilibrium-exchange rate constants was achieved by selective inversion of a distinctive
324 signal from one anomer and then monitoring the return to equilibrium intensity of signals from
325 both anomers. For quantitative determination of rates, the selective irradiation must be of
326 sufficient power and duration to invert the targeted magnetization, yet should not directly affect
327 the equilibrium magnetization of the other spin population. Due to the chemical exchange of
328 the anomers, the inverted magnetization redistributes into the un-irradiated site. The
329 magnetization can then be sampled by normal methods. Figures 5A,B show build-up curves of
330 H1 α arising from the irradiation of H1 β of SQ and Glc-6-P, respectively; and Figures 5C,D
331 show the respective decay curves from the same experiment for H1 β . Also shown is the tangent
332 at $\tau_{\text{mix}} = 0$, which provides the exchange-rate constant. Figures 5E,F show Michaelis-Menten
333 plots for conversion of the β -anomers of SQ and Glc-6-P, respectively. While SQ exhibited
334 saturation kinetics, allowing calculation of k_{cat} , K_{M} and $k_{\text{cat}}/K_{\text{M}}$ values, Glc-6-P did not and thus
335 only $k_{\text{cat}}/K_{\text{M}}$ could be calculated. As the equilibrium concentrations of the two anomers of each
336 substrate are known, this data allows calculation of kinetic parameters for the reverse reaction.
337 The complete set of kinetic parameters for the forward and reverse mutarotation reactions are
338 shown in Table 1, and reveals that HsSQM displays an approximate 5-fold selectivity for SQ
339 over Glc-6-P in terms of the $k_{\text{cat}}/K_{\text{M}}$ values.

340

341 **Table 1.** Michaelis Menten kinetic parameters for HsSQM catalyzed mutarotation of SQ and
342 Glc-6-P under conditions of equilibrium exchange.

substrate	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1} \text{s}^{-1}$)
α -SQ	$(3.08 \pm 0.19) \times 10^2$	1.00 ± 0.20	$(3.07 \pm 0.44) \times 10^5$

β -SQ	$(3.37 \pm 0.20) \times 10^2$	2.08 ± 0.34	$(1.62 \pm 0.18) \times 10^5$
α -Glc-6-P	—	—	$(5.87 \pm 0.57) \times 10^4$
β -Glc-6-P	—	—	$(3.26 \pm 0.31) \times 10^4$

343

344 To gain insight into the rate enhancement achieved by HsSQM we measured the rate
345 of mutarotation of SQ by polarimetry using a coupled assay. Incubation of 4-nitrophenyl α -D-
346 sulfoquinovoside with a retaining sulfoquinovosidase occurs with retention of configuration to
347 afford α -SQ. Monitoring the rate of mutarotation of α -SQ in 50 mM phosphate buffer revealed
348 a half-life of 50 min (SI Figure 1). By comparison, mutarotation of α -Glc-6-P has been reported
349 to occur at 25 °C with a half-life of 6.0 s [34, 35]. However, phosphate buffer is known to
350 catalyze mutarotation.[36] In order to measure the rate of mutarotation in phosphate-free
351 conditions, and to subsequently allow comparison with enzyme-catalyzed rates measured in
352 D₂O we measured mutarotation rates of SQ in D₂O at various concentrations of phosphate at
353 pseudo-constant ionic strength achieved with 2 M NaCl (Figure 6A). Extrapolation of this data
354 to [phosphate] = 0 mM, gave a predicted mutarotation rate (k) of $3.87 \times 10^{-5} \text{ s}^{-1}$ (0.00232 min^{-1})
355 corresponding to a half-life of 299 min. Using the equilibrium constant $K^{\text{eq}} = 1.5$ allows
356 calculation of forward and reverse rates of $k_1 = 2.3 \times 10^{-5} \text{ s}^{-1}$ and $k_{-1} = 1.5 \times 10^{-5} \text{ s}^{-1}$. A similar
357 calculation for Glc-6-P mutarotation ($K^{\text{eq}} = 1.8$) using the published data gave forward and
358 reverse rates of $k_1 = 7.7 \times 10^{-2} \text{ s}^{-1}$ and $k_{-1} = 4.3 \times 10^{-2} \text{ s}^{-1}$; strictly speaking these 'uncatalyzed'
359 rates are for $k_{(\text{H}_2\text{O})} + k_{(\text{H}^+)}[\text{H}_3\text{O}^+] + k_{(\text{HO}^-)}[\text{HO}^-]$.

360 The above data allow us to calculate the catalytic proficiency of HsSQM for catalyzing
361 the mutarotation of SQ and Glc-6-P. The proficiency constants [$k_{\text{uncat}} / (k_{\text{cat}}/K_{\text{M}})$] for HsSQM
362 are $7.6 \times 10^{-11} \text{ M}$ for α -SQ and $1.3 \times 10^{-6} \text{ M}$ for α -Glc-6-P, revealing that HsSQM binds the
363 SQ transition state some 17,000-fold tighter than for Glc-6-P and is thus best considered a
364 sulfoquinovose mutarotase. We note that the mutarotation rate for Glc-6-P was measured in
365 H₂O, whereas all other measurements were performed in D₂O, thus a solvent isotope effect
366 may somewhat confound this analysis; nonetheless the broad conclusions still apply.

367 The pD dependence of activity for HsSQM was determined using 1D ¹H EXSY for
368 Glc-6-P. At a concentration of 5 mM, Glc-6-P does not show saturation (unlike for SQ), and
369 so rates measured at this concentration provide an estimate of $k_{\text{cat}}/K_{\text{M}}$. Figure 6 shows the pD
370 dependence of activity is bell-shaped, with a broad maximum of activity of pD 7-9. The
371 simplest interpretation of these results is that it arises from ionization of two catalytically
372 important residues with pK_a values of 6.3 ± 0.2 and 10.3 ± 0.2 . The ionization of the acidic limb

373 presumably reflections ionization of the general acid Glu254, and the basic limb ionization of
374 the general base His92. By comparison, the intrinsic pK_a values for the ionizable side-chains
375 of Glu and His are 4.5 and 6.4, respectively [37]. In both cases, and particularly the latter, these
376 intrinsic pK_a values are perturbed, presumably because of the active-site environment including
377 charge-charge and charge-dipole interactions, as well as desolvation effects [37]. The pD
378 dependence of this enzyme is similar to the pH dependence of green pepper mutarotase [34],
379 but broader than that of *E. coli* galactose mutarotase [38].

380

381 Discussion

382 Many enzymes involved in the metabolism of free sugars are stereospecific for one anomer of
383 their substrate. For example, yeast galactokinase specifically phosphorylates α -galactose to
384 produce α -D-galactose-1-phosphate [39], glucose dehydrogenase from *Bacillus megaterium*
385 uses only β -D-glucose as a substrate [40]; yeast phosphoglucose isomerase only uses α -D-
386 glucose-6-phosphate as substrate [41]; and yeast phosphomannose isomerase is specific for α -
387 D-mannose-6-phosphate [42]. For these enzymes, access to the appropriate substrate anomer
388 would be rate-limiting in vivo if the process relied on spontaneous mutarotation. Mutarotases
389 ensure that there is rapid equilibration between anomers to eliminate this metabolic bottleneck.
390 While some mutarotases have been linked to specific metabolic pathways [6], it has been
391 difficult to assign many others to a single substrate or metabolic process [32]. It is unclear if
392 the inherent promiscuity of many mutarotases confers an advantage to their hosts or simply
393 impose no cost to fitness.

394 Sulfo-EMP and sulfo-ED gene clusters both encode putative mutarotases, homologous
395 to HsSQM, that are upregulated in response to growth on SQ [1, 2], suggesting that their
396 primary function is as an SQ mutarotase. We show here that HsSQM from the sulfo-ED cluster
397 of *H. seropedicaea* can catalyze SQ mutarotation with a greater k_{cat}/K_M value than for the
398 possible alternative substrate Glc-6-P, which is itself an important metabolite that feeds into
399 the Embden-Meyerhof-Parnas and Entner-Doudoroff glycolytic pathways, as well as the
400 pentose phosphate pathway. It is possible that the activity of the enzyme on Glc-6-P is
401 advantageous to a bacterium transitioning between growth on SQ and growth on Glc.
402 Furthermore, due to the broad substrate tolerance of many mutarotases, it is possible that other
403 enzymes may play a role in catalysing SQ mutarotation within the cell, though any enzyme
404 that does so is unlikely to be transcriptionally regulated by SQ concentration as HsSQM and
405 its homologs are.

406 The substrate specificity of HsSQM bears similarities to the aldose-1-epimerase from
407 *E. coli* (*galM*), which is tolerant to functional group changes at C6, and stereochemistry
408 changes at C4 (Gal) but not at C2 (Man) [9]. On the other hand it is distinguished from yeast
409 *ymr099c*, which encodes a hexose-6-phosphate mutarotase with activity on Glc-6-P, Gal-6-P
410 and Man-6-P and thus tolerates stereochemical inversion at C2 [32]. Sequence alignments and
411 structural modelling reveal that HsSQM likely acts through a mechanism conserved with all
412 other mutarotases. The substrate tolerance of HsSQM for substituent variation at C6 lies in
413 contrast to *E. coli* SQase, which exhibited negligible activity on α -glucosides due to its
414 specialized set of conserved residues that recognize the sulfonate group of SQ glycosides [4].

415 The effectiveness of an enzyme as a catalyst can be quantified by measuring the rate
416 enhancement it provides relative to the rate of the uncatalyzed reaction [43]. This work reveals
417 that uncatalyzed SQ mutarotation is a relatively slow reaction, with the rate for α -SQ some
418 3400-fold lower than that of α -Glc-6-P. On the other hand, HsSQM catalyzes mutarotation
419 with a k_{cat}/K_M value around 5-fold higher than for Glc-6-P. Combining these values reveals that
420 HsSQM is approximately 17,000-fold more proficient as a catalyst in catalyzing the
421 mutarotation of SQ and Glc-6-P. As enzymes achieve their rate enhancement through selective
422 stabilization of the transition state relative to the ground state, these data suggest that the
423 affinity for the transition state of SQ mutarotation is approximately 17,000-fold greater than
424 that for Glc-6-P mutarotation. As has been noted by others, the unusually high rate of
425 mutarotation of Glc-6-P is much greater than that of other hexoses [44], and appears to be a
426 result of neighboring group participation by the pendant phosphate group [36]; our data
427 suggests that the sulfonate group of SQ does not provide neighbouring group participation.

428 Understanding the precise role of SQMs in the sulfo-EMP and sulfo-ED pathways will
429 require knowledge of the substrate specificities for upstream and downstream processes.
430 Upstream processes include SQ importers and SQases. For organisms grown on a mixture of
431 SQ anomers, the SQ importers may exhibit a preference for only one SQ anomer; an SQM may
432 be required for re-establishing an equilibrium mixture of SQ anomers. A pertinent example is
433 that of red blood cells in which it is known that glucose importers exhibit a preference for α -
434 glucose [45], and a potential role for glucomutarotase in a permease system involved in re-
435 establishing this equilibrium has been advanced [34]. For cells grown on SQ glycosides, SQ is
436 released by action of SQases; the immediate product is α -SQ, and SQMs may be required to
437 enhance conversion of α -SQ to β -SQ, to match the preference of a downstream enzyme, or to
438 act in the reverse direction to ‘rescue’ β -SQ that accumulates as a result of the spontaneous

439 mutarotation of α -SQ. Downstream enzymes include SQ isomerases and SQ dehydrogenases.
440 As yet the anomeric preference, if any, of these enzymes is unknown. The NMR EXSY
441 experiments used herein to probe SQM activity, which build on pioneering work reaching back
442 many decades, could be of use for studying the substrate preferences of the downstream
443 enzymes, and in so doing could help provide the biological context for the SQMs.

444

445 **Abbreviations**

446 DHAP, dihydroxyacetone phosphate; DHPS, 2,3-dihydroxypropanesulfonate; Gal, galactose;
447 Glc, glucose; GlcA, glucuronic acid; Man, mannose; ED, Entner-Doudoroff; EMP, Embden-
448 Meyerof-Parnas; EXSY, exchange spectroscopy; NMR, nuclear magnetic resonance; PNPSQ,
449 4-nitrophenyl α -D-sulfoquinovoside; SL, sulfolactate; SQ, sulfoquinovose; SQase,
450 sulfoquinovosidase; SQM, sulfoquinovose mutarotase.

451

452 **Author Contribution**

453 S.J.W., S.Y. and E.D.G-B. conceived and coordinated the study. J.P.L. and J.Y. performed the
454 cloning and protein expression. P.A. and S.Y. conducted the NMR measurements. P.A. and
455 C.B. conducted the polarimetry measurements. All authors contributed to writing of the
456 manuscript and approved the final version of the manuscript.

457

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464

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467

468 **Competing Interests**

469 The Authors declare that there are no competing interests associated with the manuscript.

470

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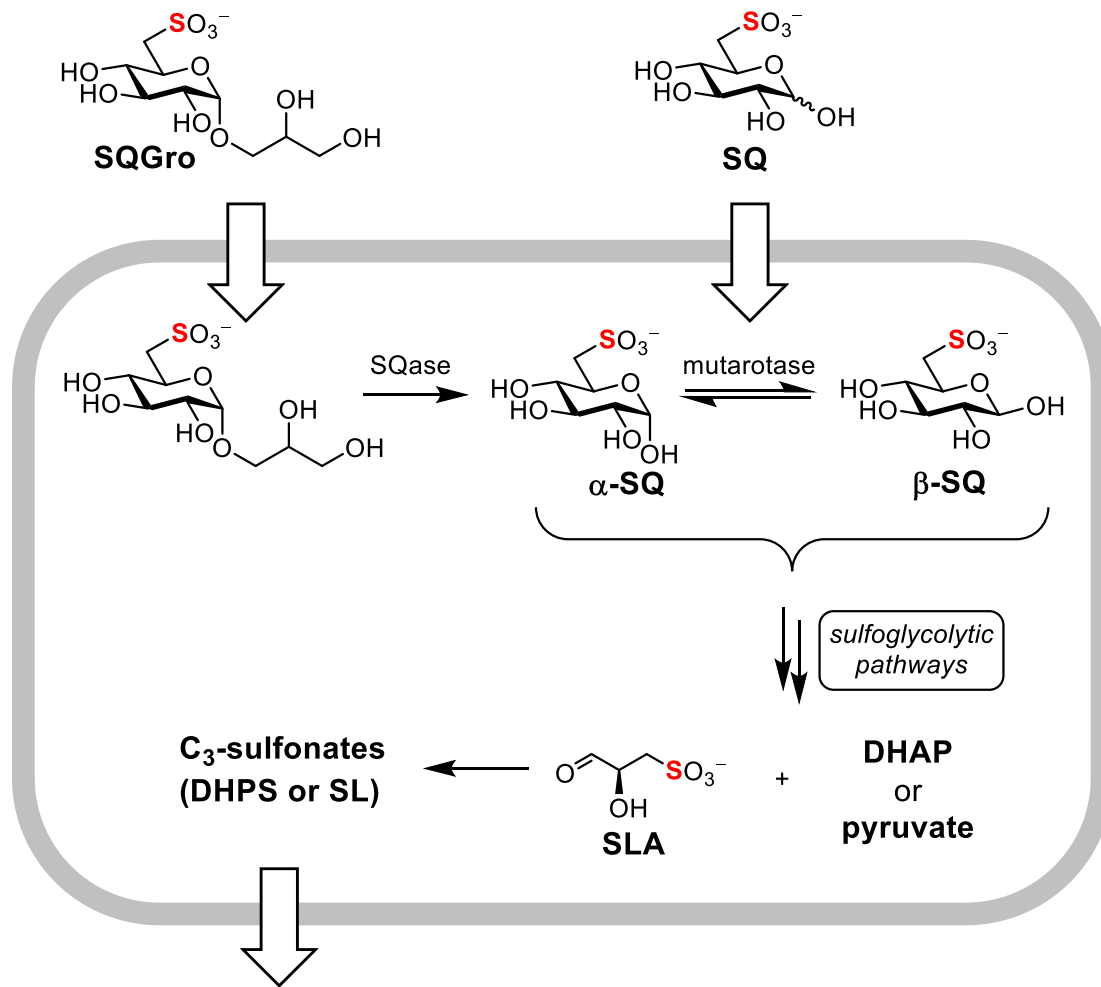
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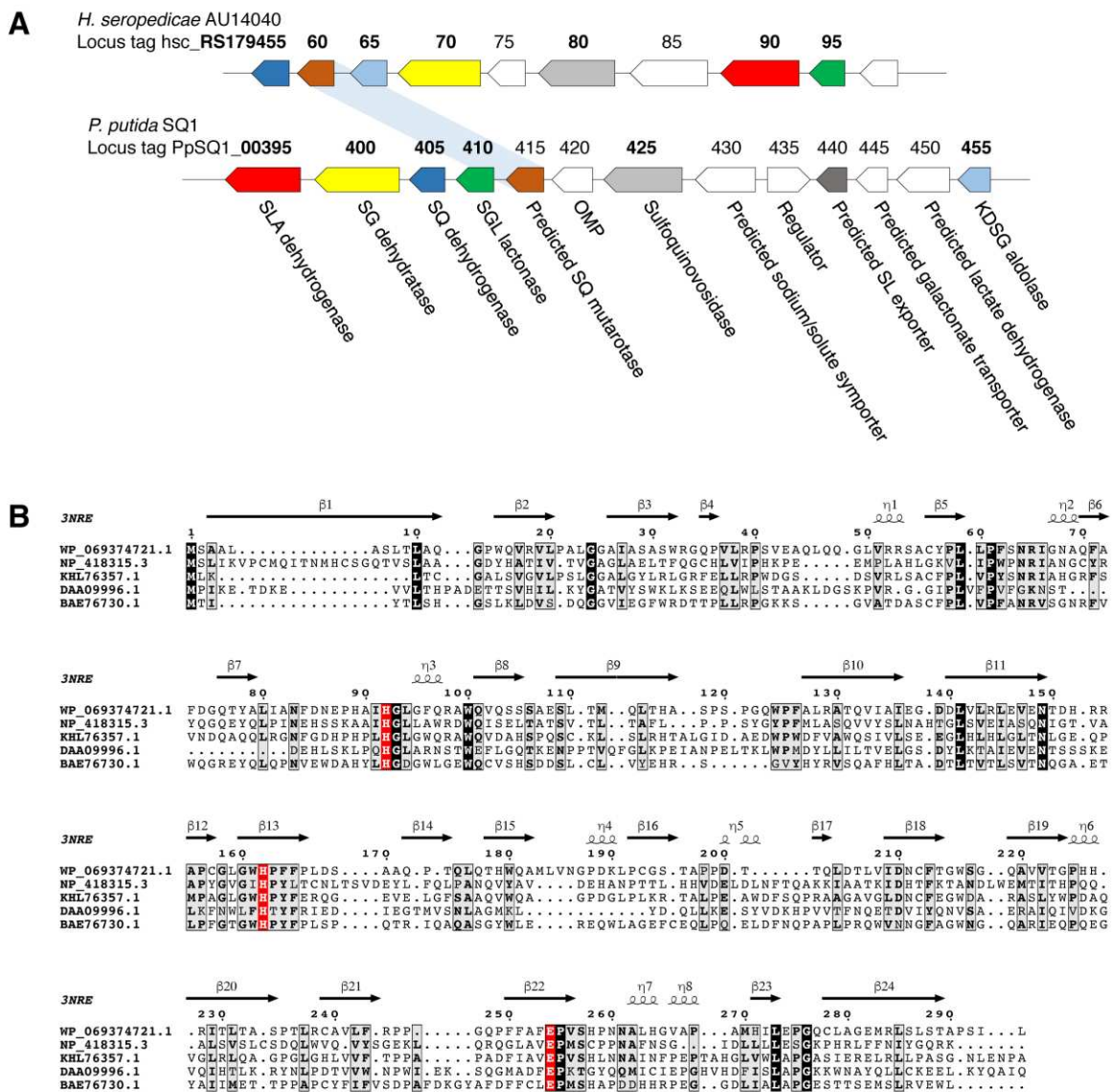


587

588 **Figure 1. Summary of sulfoglycolysis.**

589 Importation of SQGro and cleavage by SQase, or direct importation of SQ, provides an
 590 intracellular pool of SQ anomers that can be interconverted by SQ mutarotase. SQ is
 591 metabolized by sulfo-ED or sulfo-EMP pathways to sulfolactaldehyde (SLA), and then to the
 592 C₃-sulfonates dihydroxypropanesulfonate (DHPS) or sulfolactate (SL), prior to export.

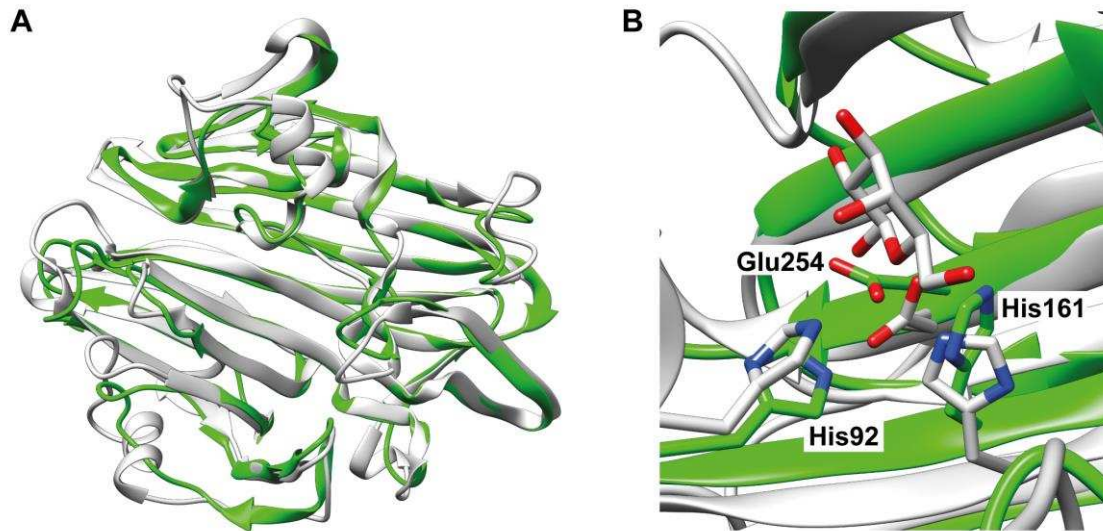
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596 **Figure 2. *Herbaspirillum seropedicae* contains a sulfo-ED operon and a putative SQ**
 597 **mutarotase.**

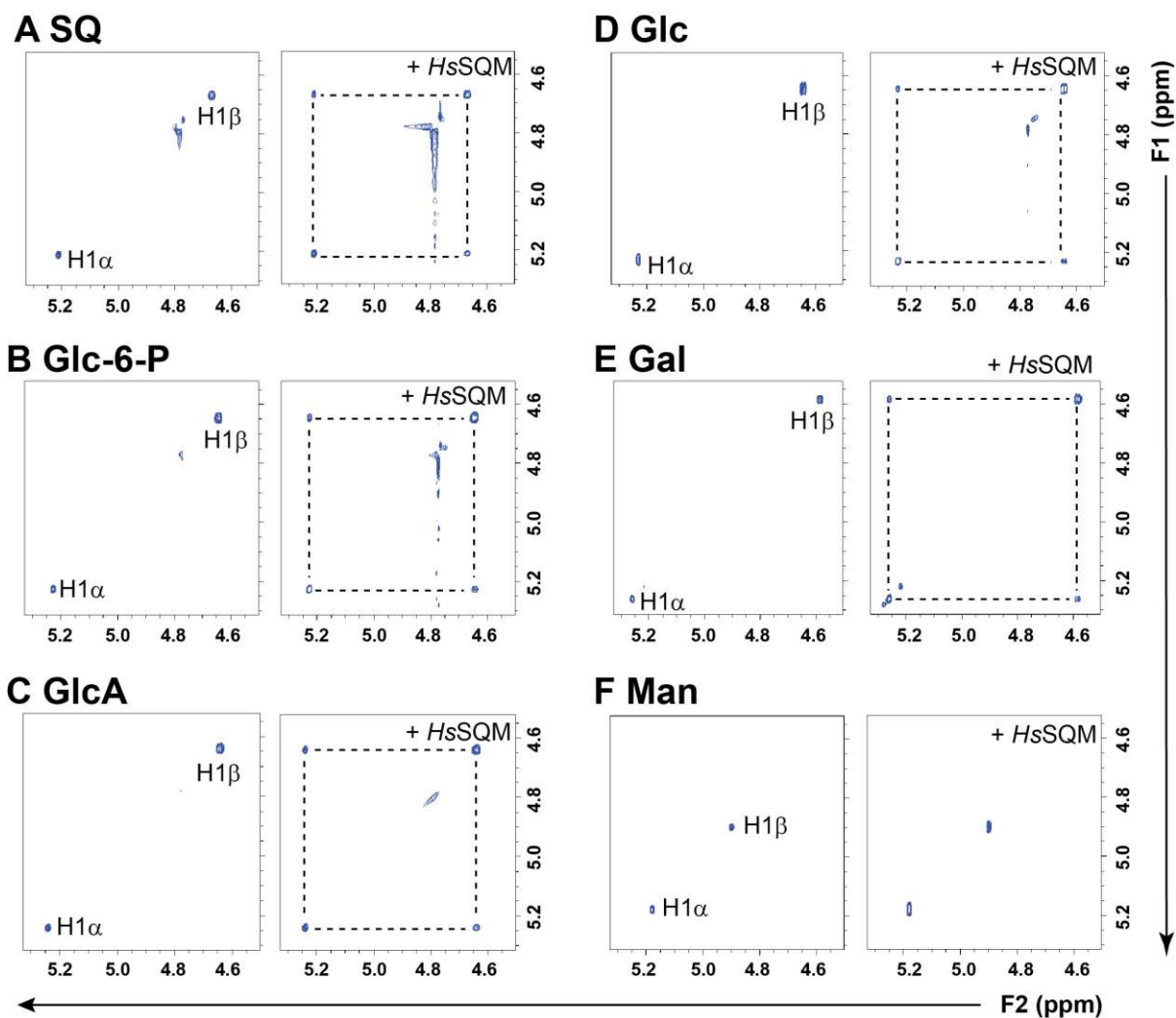
598 A) Operon structure of *P. putida* SQ1 and *H. seropedicae* strain AU14040. Bold indicates
 599 genes for which enzymatic activity has been biochemically determined in at least one organism.
 600 B) Alignment of various putative mutarotases with secondary structural elements.
 601 WP_069374721.1, HsQM from *H. seropedicae*; NP_418315.3, YihQ from *E. coli*;
 602 KHL76357.1, PpSQ1_00415 from *Pseudomonas putida* SQ1; DAA09996.1, YMR099C
 603 hexose-1-phosphate mutarotase from *S. cerevisiae*; BAE76730.1, YphB aldose-1-epimerase
 604 from *E. coli* (BAE76730.1). The secondary structural elements are annotated from the structure
 605 of *E. coli* YphB (PDB 3nre).



606

607 **Figure 3. Homology model of HsSQM.**

608 A) Overlay of HsSQM homology model (green) with a putative mutarotase from *Clostridium*
 609 *acetobutylicum* (PDB 3OS7; grey). B) The active sites of the HsSQM homology model (green)
 610 overlaid with the galactose mutarotase domain of gal10 from *S. cerevisiae* with D-galactose
 611 bound (grey, PDB 1Z45). Residue numbers are for HsSQM.

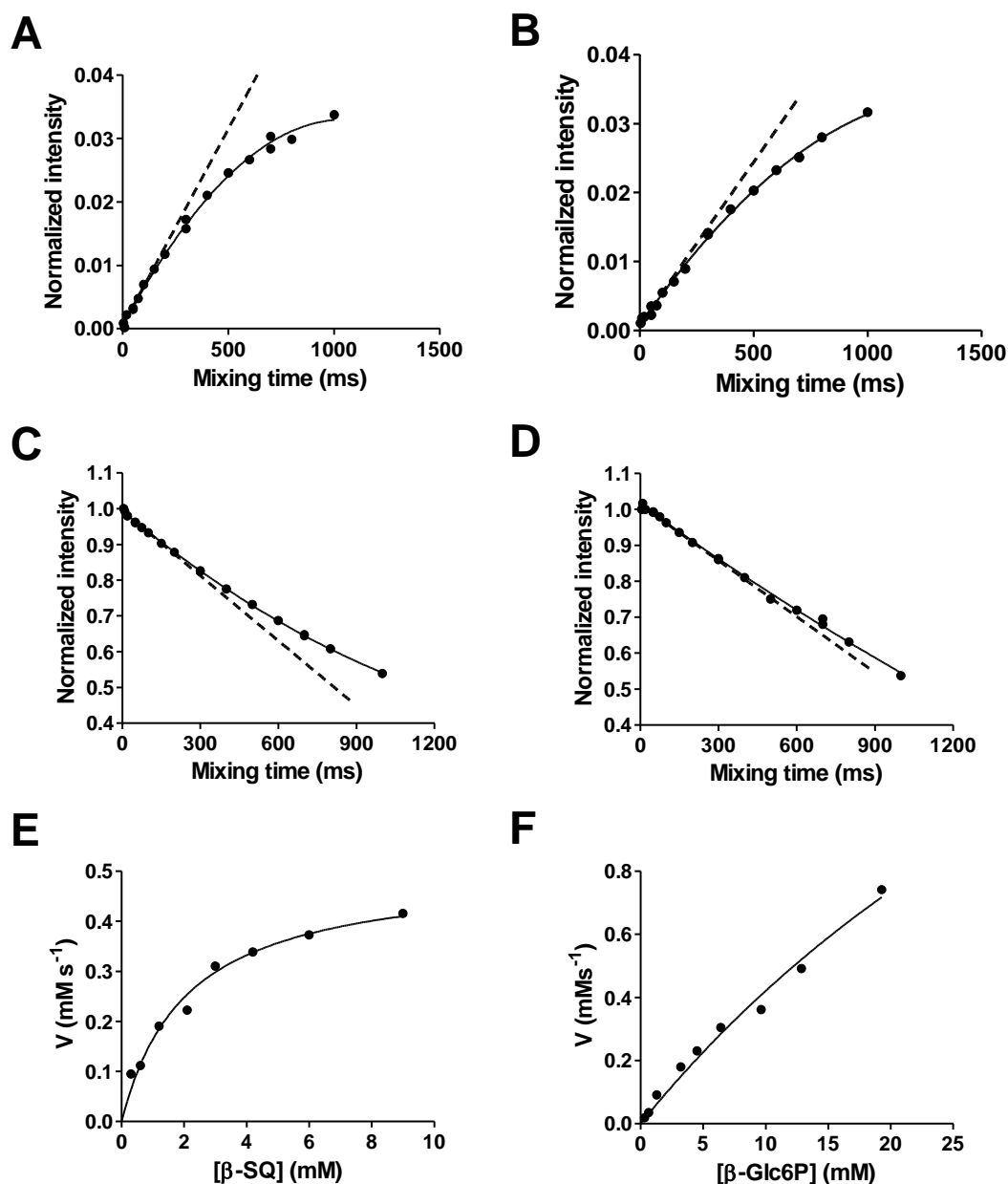


612

613 **Figure 4. Excerpt showing anomeric regions of 2D ^1H - ^1H EXSY plots of various hexoses**
 614 **alone and with *H. seropediacae* mutarotase.**

615 A) sulfoquinovose (SQ), B) D-glucose-6-phosphate (Glc-6-P), C) D-glucuronic acid (GlcA),
 616 D) D-glucose (Glc), E) D-galactose (Gal), F) D-mannose (Man). Hexoses are at 5 mM, 1.51
 617 μM HsSQM in 50 mM sodium phosphate, 150 mM NaCl (pD 7.5).

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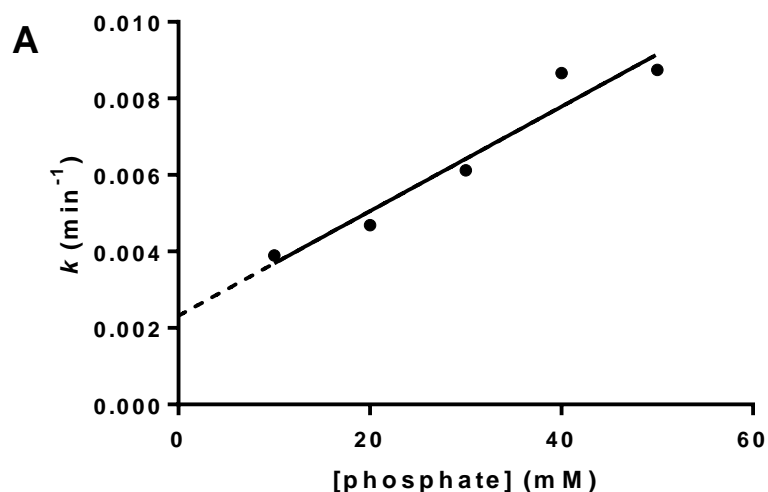


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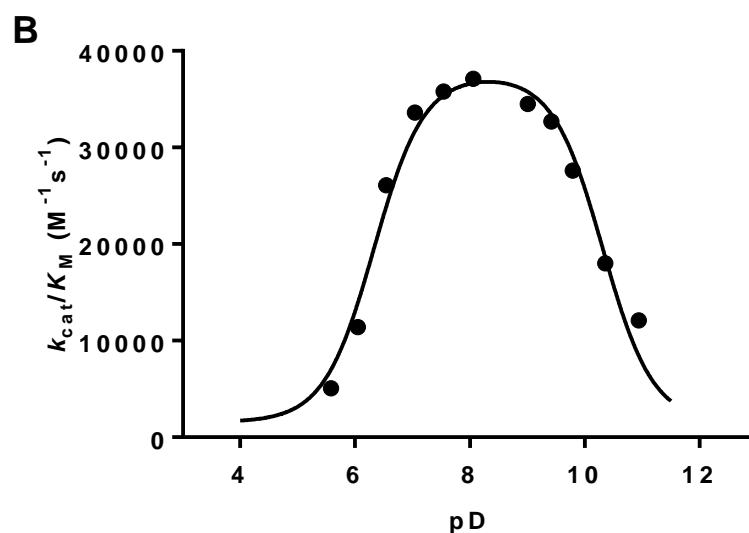
621 **Figure 5. Kinetic analysis of HsSQM by inversion-recovery 1D ^1H EXSY.**

622 Inversion recovery curves for 10 mM SQ or Glc-6-P corresponding to (A) α -SQ at 4.00 mM
623 and (B) α -Glc-6-P at 3.57 mM. Inversion decay curves corresponding to (C) β -SQ at 6.00 mM
624 and (D) β -Glc-6-P at 6.43 mM. Michaelis-Menten plots for (E) β -SQ and (F) β -Glc-6-P.
625 Dashed lines indicate tangents to fitted curve at $t = 0$.

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630

631 **Figure 6. Spontaneous mutarotation of α -SQ and pD dependence of HsSQM.**

632 A) Plot of rates of spontaneous mutarotation of α -SQ versus phosphate buffer concentration
633 at pseudo-constant ionic strength. Buffers consisted of 10-50 mM sodium phosphate, 2 M
634 NaCl in D₂O (pD 7.5). For comparison, $k = 0.0073 \text{ min}^{-1}$ in 50 mM sodium phosphate, 150
635 mM NaCl in D₂O (pD 7.5). B) pD dependence of HsSQM activity for mutarotation of
636 glucose-6-phosphate. Data was fit to a bell-shaped curve leading to estimated pK_a values of
637 5.9 ± 0.1 and 9.9 ± 0.1 .