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1 **A sulfoglycolytic Entner-Doudoroff pathway in *Rhizobium leguminosarum* bv. *trifolii***
2 **SRDI565**

3

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20 **Keywords**

21 Sulfoglycolysis; metabolomics; sulfur cycle; rhizobia; carbohydrates; metabolism

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25

26 **Abstract**

27 Rhizobia are nitrogen fixing bacteria that engage in symbiotic relationships with plant hosts
28 but can also persist as free-living bacteria with the soil and rhizosphere. Here we show that
29 free living *Rhizobium leguminosarum* SRDI565 can grow on the sulfosugar sulfoquinovose
30 (SQ), or the related glycoside SQ-glycerol, using a sulfoglycolytic Entner-Doudoroff (sulfo-
31 ED) pathway resulting in production of sulfolactate (SL) as the major metabolic end-product.
32 Comparative proteomics supports the involvement of a sulfo-ED operon encoding an ABC
33 transporter cassette, sulfo-ED enzymes and an SL exporter. Consistent with an oligotrophic
34 lifestyle, proteomics data revealed little change in expression of the sulfo-ED proteins during
35 growth on SQ versus mannitol, a result confirmed through biochemical assay of
36 sulfoquinovosidase activity in cell lysates. Metabolomics analysis showed that growth on SQ
37 involves gluconeogenesis to satisfy metabolic requirements for glucose-6-phosphate and
38 fructose-6-phosphate. Metabolomics analysis also revealed the unexpected production of
39 small amounts of sulfofructose and 2,3-dihydroxypropanesulfonate, which are proposed to
40 arise from promiscuous activities of the glycolytic enzyme phosphoglucose isomerase and a
41 non-specific aldehyde reductase, respectively. The discovery of a rhizobium isolate with the
42 ability to degrade SQ builds our knowledge of how these important symbiotic bacteria persist
43 within soil.

44 **Importance**

45 Sulfonate sulfur is a major form of organic sulfur in soils but requires biomineralization
46 before it can be utilized by plants. Very little is known about the biochemical processes used
47 to mobilize sulfonate sulfur. We show that a rhizobial isolate from soil, *Rhizobium*
48 *leguminosarum* SRDI565, possesses the capability to degrade the abundant phototroph-
49 derived carbohydrate sulfonate SQ through a sulfoglycolytic Entner-Doudoroff pathway.
50 Proteomics/metabolomics demonstrated the utilization of this pathway during growth on SQ

51 and provided evidence for gluconeogenesis. Unexpectedly, off-cycle sulfoglycolytic species
52 were also detected pointing to the complexity of metabolic processes within cells under
53 conditions of sulfoglycolysis. Thus rhizobial metabolism of the abundant sulfosugar SQ may
54 contribute to persistence of the bacteria in the soil and to mobilization of sulfur in the
55 pedosphere.

56 **Introduction**

57 Sulfur is essential for plant growth and is the fourth most important macronutrient after
58 nitrogen, phosphorus, and potassium. Up to 10 kg/ha/y of sulfur is deposited in rain,
59 especially near industrialized areas (1). However, sulfur dioxide emissions from industrial
60 sources have decreased in recent decades as a result of pollution mitigation and the move to
61 low sulfur fuels and renewable energy sources, and quantities received from atmospheric
62 sources is now at levels below that required by most crops (2). Sulfur deficiency in soils is
63 primarily combated by application of sulfur-containing fertilizers such as superphosphate,
64 ammonium sulfate and gypsum (3), which are applied across all major cropping and pasture
65 areas worldwide (4). Soils contain significant amount of sulfur, yet plants can only use sulfur
66 in the form of sulfate and it has been shown that 95-98% of sulfur in soils is in the form of
67 unavailable biological sulfur (4). Thus, effective microbial cycling of sulfur from biological
68 to inorganic forms within the soil is important (5) and has the potential to enhance crop yields
69 and reduce reliance on fertilizers.

70 X-ray absorption near-edge spectroscopy measurements have led to estimates that
71 approx. 40% of sulfur within various sediments and humic substances exist as sulfonate (6).
72 Chemical methods of analysis applied to a range of forest soils revealed sulfonate sulfur was
73 at 40% of total organic sulfur pool in the majority of cases (7). Little detail is known on the
74 speciation of organic sulfonates in soils but one important input is phototroph-derived litter.
75 It is estimated that around 10 billion tonnes per annum of the sulfosugar sulfoquinovose (SQ)
76 is produced annually by photosynthetic organisms, including plants, cyanobacteria and algae
77 (8). SQ is primarily found as the glycerolipid sulfoquinovosyl diacylglycerol (SQDG), and
78 land plants can contain as much as 10% SQDG in their thylakoid membrane glycerolipids
79 (9). Very little is known about how SQ is metabolized within soils, although it has been
80 shown to undergo very rapid mineralization to inorganic sulfate (10).

81 Bacteria are likely to be primarily responsible for the biomineralization of SQ,
82 possibly by using SQ as a carbon source and catabolizing it via a modified version of
83 glycolysis, termed sulfoglycolysis (11). Two sulfoglycolytic processes have been described:
84 the sulfoglycolytic Embden-Meyerhof-Parnas (sulfo-EMP) pathway (12), and the
85 sulfoglycolytic Entner-Doudoroff (sulfo-ED) pathway (Fig. 1) (13). The sulfo-ED pathway
86 was first reported in *Pseudomonas putida* strain SQ1, a bacterium isolated from freshwater
87 sediment, catabolised of SQ with excretion of equimolar amounts of sulfolactate (SL) (13).
88 The sulfo-ED operon of *P. putida* SQ1 contains 10 genes including a transcriptional
89 regulator, an SQ importer and SL exporter, a sulfoquinovosidase, SQ mutarotase, SQ
90 dehydrogenase, SL lactonase, SG dehydratase, KDSG aldolase and SLA dehydrogenase
91 enzymes. Based on genome-wide annotation studies, the sulfo-ED pathway is predicted to
92 occur in a range of alpha-, beta- and gamma-proteobacteria (13). However, no direct
93 evidence for this pathway has been reported for any organism other than *P. putida* SQ1.
94 Other members of the microbial community can catabolize SL and 2,3-
95 dihydroxypropanesulfonate (DHPS; the product of the sulfo-EMP pathway) to inorganic
96 sulfur (14), completing the biomineralization of SQ.

97 *Rhizobium leguminosarum* bv. *trifolii* SRDI565 (syn. N8-J), hereafter *Rl*-SRDI565,
98 was isolated from a soil sample collected in western New South Wales but has the capacity to
99 colonize *Trifolium subterraneum* subsp. *subterraneum* (subterranean clover) and other
100 *Trifolium* spp. (15). Colonization of trifolium species with *Rl*-SRDI565 results in suboptimal
101 nodulation and nitrogen fixation in some species and ineffective nitrogen fixation in others,
102 leading to reduced shoot nitrogen content relative to other commercial strains (16).
103 Interestingly, the genome of *Rl*-SRDI565 encodes all the genes needed for a functional sulfo-
104 ED pathway (17), although there is no evidence to show that this is operative and/or that *Rl*-
105 SRDI565 can use SQ as a major carbon source.

106 Rhizobia participate in sophisticated symbiotic relationships with leguminous host
107 plants that allow them to fix atmospheric dinitrogen to provide a growth advantage to the host
108 (18). Symbiosis is triggered by molecular communication between the bacterium and the host
109 resulting in nodule formation on the root and colonization by the bacterium. Within nodule
110 bacteroids the energy intensive fixation of nitrogen is supported by C₄-dicarboxylates
111 (primarily malate, fumarate, and succinate) sourced from glycolysis of sucrose photosynthate
112 within the plant host (18). Owing to the importance of biological nitrogen fixation for input
113 of nitrogen into the biosphere, the symbiosis of rhizobia and leguminous hosts has been well
114 studied. However, rhizobia can also exist as free-living bacteria within the soil and
115 rhizosphere (19). Here, like other soil bacteria, they adopt a saprophytic and oligotrophic
116 lifestyle where they utilize a variety of alternative carbon sources, including a wide range of
117 carbohydrates (20). Most likely, the ability of various rhizobia to persist in the pedosphere
118 depends upon their ability to utilize diverse carbohydrate and non-carbohydrate substrates
119 and establish an appropriate niche. SQ or its glycosides are likely to be a common soil
120 constituent and nutrient given its ubiquitous production by plants. Possibly, the sulfo-ED
121 pathway in *Rl-SRDI565* might provide it with the capacity to survive on plant derived SQ or
122 SQDG in the rhizosphere and in the soil.

123 Here we investigated whether the sulfo-ED pathway is active in *Rl-SRDI565* and its
124 potential role in utilizing plant-derived SQ or SQDG in the rhizosphere and in the soil. We
125 show that *Rl-SRDI565* can grow on SQ and sulfoquinovosyl glycerol (SQGro) as sole carbon
126 source. Growth on SQ leads to excretion of SL into the growth media indicating active
127 sulfoglycolysis. This was supported by proteomic analyses, which showed that several
128 proteins in the sulfo-ED operon show increased expression when bacteria are grown on SQ,
129 while metabolomic analyses confirm the presence of characteristic intermediates of the sulfo-
130 ED pathway, as well as the unexpected production of intracellular DHPS. Overall, we show

131 that *RI-SRDI565* has an active pathway for SQ utilization which may support growth of this
132 bacterium in the environment, and in turn provides a new model organism for the study of the
133 sulfo-ED pathway.

134

135

136 **Results**

137 Analysis of the genome of *Rl-SRDI565* revealed a sulfo-ED operon that had the same genes,
138 but no synteny with the *P. putida* SQ1 operon (Fig. 1). Genes with high sequence identity to
139 the *P. putida* proteins included a putative SQase, SQ dehydrogenase, SL lactonase, SG
140 dehydratase, KDSG aldolase and SLA dehydrogenase, and an SL exporter (see Fig. S1-S6).
141 The *Rl-SRDI565* operon contains some important differences compared to that of *P. putida*
142 SQ1. In particular, it lacks a putative SQ mutarotase (21), and appears to use an ABC
143 transporter cassette to import SQ/SQGro in place of an SQ/SQGro importer/permease. The
144 putative sulfo-ED pathway in *Rl-SRDI565* is consistent with the proposed protein functions
145 outlined in Fig. 1b, with a comparison to the classical ED pathway in Fig. 1c.

146 Initial attempts were made to grow *Rl-SRDI565* in completely defined medium, such
147 as M9 minimal media containing $125 \mu\text{g mL}^{-1}$ biotin (22), to allow assessment of different
148 carbon sources on bacterial growth. However, optimal growth could only be achieved using a
149 yeast extract-based medium (16). In particular robust growth was achieved using a 5%
150 dilution of 1 g L^{-1} yeast extract ($Y_{5\%}$ media) containing 5 mmol mannitol ($Y_{5\%}M$), while no
151 detectable bacterial growth was observed on $Y_{5\%}$ media alone. Significantly, *Rl-SRDI565*
152 also grew robustly on $Y_{5\%}$ media containing 5 mM SQ ($Y_{5\%}SQ$) and reached the same final
153 OD_{600} value as in $Y_{5\%}M$ (Fig. 2a). *Rl-SRDI565* also grew on $Y_{5\%}$ media containing glucose,
154 although to a lower final OD_{600} than in $Y_{5\%}M$ or $Y_{5\%}SQ$. ^{13}C NMR spectroscopic analysis of
155 the culture media of stationary phase *Rl-SRDI565* grown in $Y_{5\%}SQ$ revealed the presence of
156 three major signals corresponding to SL (Fig. 2b). A fourth signal was also observed but not
157 assigned and was also present in stationary phase media of cells grown on $Y_{5\%}M$, suggesting
158 it is derived from other carbon sources in the yeast extract. *Rl-SRDI565* also grew on $Y_{5\%}$
159 containing SQGro, but less robustly than on SQ.

160 We next examined changes in the proteome of *Rl*-SRDI565 cultivated on mannitol
161 versus SQ. Label-free based quantitative proteomic analysis of five experimental replicates
162 of *Rl*-SRDI565 cultivated on each carbon source, identified 2954 proteins, with 1943 proteins
163 quantified in at least 3 experimental replicates under each growth condition (Supplementary
164 Table 1). Expression levels of 17 proteins potentially associated with SQ metabolism were
165 significantly elevated ($-\log_{10}(p) > 2$ and a fold change greater than $2 \log_2$ units) in bacteria
166 cultivated in $Y_{5\%}$ SQ (Fig. 2e and 2f). In particular, a suspected KDSG aldolase (annotated as
167 alpha-dehydro-beta-deoxy-D-glucarate aldolase, WP_017967308.1), a member of the
168 proposed sulfo-ED pathway, was significantly increased ($-\log_{10}(p) = 4.74429$ and a fold
169 change of $2.38 \log_2$). Consistent with the involvement of this pathway we also observed a
170 significant yet less dramatic increase in the proposed SQase (annotated alpha-glucosidase,
171 WP_017967311.1) ($-\log_{10}(p) = 1.43643$ and a fold change of $1.02 \log_2$). Additional members
172 of the predicted pathway expressed at higher levels in SQ-fed bacteria included the suspected
173 SQ dehydrogenase (annotated as SDR family oxidoreductase, WP_017967310.1) identified
174 by MS/MS events in 4 out of 6 SQ experiments compared to 1 mannitol experiment and the
175 suspected SG dehydratase (annotated as dihydroxy-acid dehydratase, WP_017967307.1)
176 identified by MS/MS events in 3 out of 6 SQ experiments compared to 0 mannitol
177 experiments; however, owing to their low abundance they could not be accurately quantified
178 (Fig. S7).

179 Other proteins that were significantly increased in SQ-fed bacteria included a
180 NAD(P)-dependent oxidoreductase (WP_017965793.1), NADH-quinone oxidoreductase
181 subunit NuoH (WP_017963854.1), a NAD-dependent succinate-semialdehyde
182 dehydrogenase (WP_017967313.1) and a citrate synthase/methylcitrate synthase
183 (WP_017964386.1) supporting an alteration with the TCA cycle and oxidative
184 phosphorylation under conditions of growth on SQ (Fig. 2f).

185 To demonstrate activity for a representative sulfo-ED enzyme from *Rl*-SRDI565, we
186 cloned and expressed the gene encoding the putative SQase. To support future structural
187 studies, we expressed the N-terminal hexahistidine tagged K375A/K376A variant, termed
188 *Rl*SQase*, a mutant enzyme whose design was guided by the Surface Entropy Reduction
189 prediction (SERp) server (Fig. S8) (23). Size exclusion chromatography-multiple angle light
190 scattering (SEC-MALS) analysis of *Rl*SQase* revealed that the protein exists as a dimer in
191 solution (Fig. S8). Enzyme kinetics were performed using the chromogenic SQase substrate
192 4-nitrophenyl α -sulfoquinovoside (PNPSQ). *Rl*SQase* exhibited a bell-shaped pH profile
193 with optimum at pH 7-8 and consistent with titration of catalytically important residues of
194 $\text{pKa}_1 = 6.5 \pm 0.4$ and $\text{pKa}_2 = 8.6 \pm 0.3$. The enzyme displayed saturation kinetics with
195 Michaelis-Menten parameters $k_{\text{cat}} = 1.08 \pm 0.17 \text{ s}^{-1}$, $K_{\text{M}} = 0.68 \pm 0.25 \text{ mM}$, and $k_{\text{cat}}/K_{\text{M}} =$
196 $(1.59 \pm 0.83) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 3a and 3b). For comparison, the kinetic parameters for *A.*
197 *tumefaciens* SQase is $k_{\text{cat}} = 22.3 \pm 0.6 \text{ s}^{-1}$, $K_{\text{M}} = 0.21 \pm 0.03 \text{ mM}$, $k_{\text{cat}}/K_{\text{M}} = (1.1 \pm 0.1) \times 10^5$
198 $\text{M}^{-1} \text{ s}^{-1}$ and *E. coli* SQase YihQ is $k_{\text{cat}} = 32.7 \pm 0.6 \text{ s}^{-1}$, $K_{\text{M}} = 0.15 \pm 0.01 \text{ mM}$, $k_{\text{cat}}/K_{\text{M}} = (2.2$
199 $\pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (24).

200 Direct evidence for enzymatic activity associated with the sulfo-ED operon in *Rl*-
201 SRDI565 was obtained by measuring SQase enzyme activity in cell lysates. The chromogenic
202 substrate 4-nitrophenyl α -sulfoquinovoside (PNPSQ), which was designed as an analogue of
203 the natural substrate SQGro, results in release of the chromophore 4-nitrophenolate, which
204 can be detected using UV-visible spectrophotometry with high sensitivity at 400 nm or at the
205 isosbestic point, 348 nm (24, 25). *Rl*-SRDI565 was grown to mid-logarithmic phase in $\text{Y}_{5\%}\text{M}$
206 and $\text{Y}_{5\%}\text{SQ}$ media, and the harvested cells used to prepare a cell-free lysate containing
207 soluble proteins. Incubation of $\text{Y}_{5\%}\text{M}$ and $\text{Y}_{5\%}\text{SQ}$ -derived lysates with PNPSQ both resulted
208 in production of 4-nitrophenolate at similar rates. The activity in the YSQ -derived lysate was
209 inhibited by the addition of IFG-SQ, an azasugar inhibitor of SQases that makes key

210 interactions in the active site that mimic those required for substrate recognition (Fig. 3c)
211 (24). The similar levels of activity of SQase in both mannitol and SQ grown *Rl-SRDI565* is
212 consistent with the abundance of the putative SQase WP_017967311.1 detected by proteomic
213 analysis.

214 To further confirm that a sulfo-ED pathway was operative in cells, a targeted
215 metabolomics approach was used to detect expected intermediates in bacteria grown on
216 Y_{5%}SQ media. Detected intermediates were identified based on their LC-MS/MS retention
217 time and mass spectra with authentic reference standards of the sulfo-EMP and sulfo-ED
218 pathway that were synthesized in-house. Sulfogluconate (SG) was synthesized by oxidation
219 of SQ with iodine (26) (Fig. S9), while SQ, SF, SFP, DHPS, SLA and SL were prepared as
220 previously reported (27). *Rl-SRDI565* was grown to mid-log phase in Y_{5%}M or Y_{5%}SQ ,
221 metabolically quenched and extracted polar metabolites analyzed by LC/MS-MS. SQ-grown
222 bacteria contained SQ, SF, SG, SL and DHPS, while SFP and SLA could not be detected
223 (Fig. 4a-e). The detection of SG is characteristic for a sulfo-ED pathway, and presumably
224 arises from the action of the putative SQ dehydrogenase and SGL lactonase. The
225 identification of DHPS and SF was unexpected, as these intermediates/products of the sulfo-
226 EMP pathway (12). BLAST analysis of the genome of *Rl-SRDI565* did not identify putative
227 genes for the sulfo-EMP pathway. SF may therefore be formed by the action of
228 phosphoglucose isomerase (PGI), while DHPS could be the product of a promiscuous
229 aldehyde reductase. *Rl-SRDI565* was unable to utilize DHPS or SL as sole carbon source in
230 Y_{5%} medium, supporting the absence of an alternative pathway of sulfoglycolysis that utilizes
231 these intermediates. Unexpectedly, cytosolic levels of DHPS were 20-fold higher than SL,
232 suggesting that cells may lack a membrane transporter to export accumulated DHPS, in
233 contrast to the SL transporter.

234 NMR and LC-MS/MS analysis of the culture supernatant of both unlabeled and
235 ($^{13}\text{C}_6$)-labelled SQ-cultivated *Rl-SRDI565* confirmed that the substrate is almost completely
236 consumed by the time bacteria reach stationary growth (final concentration of 0.006 ± 0.001
237 mM compared to 5.0 ± 0.5 mM SQ in starting medium) (Fig. S10). Using a highly sensitive
238 cryoprobe ^{13}C NMR spectroscopic analysis revealed that both DHPS and SG were present in
239 culture supernatant of $^{13}\text{C}_6$ -SQ-cultivated *Rl-SRDI565*. Quantitative LC-MS/MS analysis
240 showed that consumption of SQ was associated with production of SL (5.70 ± 0.12 mM), and
241 low levels of DHPS (0.081 ± 0.010 mM), (SG 0.172 ± 0.006 mM) and SF (0.002 ± 0.0001 mM)
242 (Table 1). This experiment was repeated to assess the effect of growth of *Rl-SRDI565* but
243 using SQGro as carbon source. As noted previously, *Rl-SRDI565* grows inconsistently on
244 SQGro and complete consumption of SQGro could not be achieved. However, the results of
245 partial consumption broadly agreed with the results for growth on SQ, namely that SL is the
246 major terminal metabolite detected in the culture media, with much lower amounts of SF, SG
247 and DHPS (Table 1).

248

249 Discussion

250 We demonstrate here that *Rl-SRDI565* has a functional sulfo-ED pathway that allows these
251 bacteria to utilize SQ as their major carbon source. Catabolism of SQ is primarily or
252 exclusively mediated by a sulfo-ED pathway with production of SL as the major end-product,
253 similar to the situation in *P. putida* SQ1, the only other experimentally described exemplar of
254 this pathway (13). In contrast to *P. putida* SQ1, *Rl-SRDI565* also produces trace amounts of
255 DHPS which could reflect the presence of enzymes which exhibit promiscuous activities
256 similar to those in the conventional sulfo-EMP pathway. This observation is reminiscent of
257 *Klebsiella* sp. strain ABR11 isolated from soil (28) that is also able to grow on SQ with
258 production of both SL and DHPS. *Klebsiella* sp. strain ABR11 possesses an NAD⁺-specific
259 sulfoquinovose-dehydrogenase activity (29), suggesting it has an operative sulfo-ED
260 pathway.

261 Various bacteria that can metabolize SQ have been isolated from soil including
262 *Agrobacterium* sp., (29) *Klebsiella* sp., (29) and *Flavobacterium* sp., (30) as well as *P. putida*
263 SQ1 (13), which was isolated from a freshwater littoral sediment. These bacteria may work
264 cooperatively with species such as *Paracoccus pantotrophus* NKNCYSA that can convert SL
265 to mineral sulfur, leading to stoichiometric recovery of sulfite/sulfate (14). Together these
266 bacterial communities achieve the complete mineralization of SQ to sulfate, which is
267 available for use by plants.

268 Proteomic and biochemical evidence suggests that the sulfo-ED pathway is
269 constitutively expressed in *Rl-SRDI565*, with only relatively small increases in protein
270 expression as shown by statistically significant increases in only KDSG aldolase and SQase.
271 in the presence of SQ. As *Rl-SRDI565* in the soil is likely to be oligotrophic, constitutive
272 expression of the sulfo-ED pathway may allow simultaneous usage of multiple non-
273 glycolytic substrates without requirement for significant transcriptional changes. Consistent

274 with this view, the proteomic abundance of the putative LacI-type regulator
275 WP_017967302.1 was unchanged between mannitol and SQ grown *Rl-SRDI565*. The sulfo-
276 ED operon in *Rl-SRDI565* differs from that described for *P. putida* SQ1 through the absence
277 of a putative SQ mutarotase. SQ undergoes mutarotation with a half-life of approximately 6
278 h, which is much slower than for the glycolytic intermediate Glc-6-P, which has a half-life of
279 just seconds (21). Aldose mutarotases are often relatively non-specific and possibly a
280 constitutive mutarotase not in the sulfo-ED operon expressed by the cell provides this
281 catalytic capacity. Alternatively, the SQ dehydrogenase may not be stereospecific, with the
282 ability to act on both anomers of SQ, or even that it acts on α -SQ (the product released from
283 SQGro by an SQase) at a high rate such that mutarotation to β -SQ is of insignificant
284 importance. A second difference in the sulfo-ED operon lies in the presence of an ABC
285 transporter cassette. ABC transporter cassettes are the most common solute transporters, and
286 can translocate their substrates in either a forward or reverse direction (31). While we
287 propose that the ABC transporter cassette operates in the forward direction, based on the
288 presence of a signal sequence in the putative solute binding domain targeting it to the
289 periplasm, and consistent with a wide range of sugar import systems, the directionality of
290 transport and thus the choice of substrate (SQ/SQGro versus SL) may depend on the relative
291 abundance of these metabolites intra and extracellularly.

292 Sulfoglycolysis in *Rl-SRDI565* leads to production of pyruvate and the excretion of
293 the C3-organosulfonate SL (Fig. 5). In order to satisfy the demands of the pentose phosphate
294 pathway and cell wall biogenesis, sulfoglycolytic cells must synthesize glucose-based
295 metabolites such as glucose-6-phosphate and glucose-1-phosphate. Gluconeogenesis has been
296 studied in *Rhizobium leguminosarum* strain MNF3841, and operates through a classical
297 pathway involving fructose bisphosphate aldolase (32). Action of phosphoglucose isomerase
298 on SQ might lead to production of SF, thereby explaining the observation of this metabolite

299 in *Rl*-SRDI565. This is not likely to be consequential, as the reversibility of this reaction will
300 ultimately allow complete consumption of any SF through isomerization back to SQ. The
301 formation of DHPS may result from a promiscuous aldehyde reductase. Analysis of spent
302 culture media reveals that the production of DHPS is minor in terms of total carbon balance.
303 However, within the cytosol, DHPS accumulates to levels much higher than SL, presumably
304 because of the absence of a dedicated exporter for the former. Possibly, reduction of SLA to
305 DHPS is reversible and enables conversion of this metabolite to SL and subsequent excretion
306 from the cell. The observation of SG, SF and DHPS in the spent culture media at low levels
307 is suggestive of low levels of leakage of these metabolites from the cell, either through cell
308 lysis or leaky export systems.

309 Given that SQ contains a significant portion of organic sulfur within plants, the
310 pathways of SQ catabolism leading to release of its sulfur may be important to enable
311 recycling of this important macronutrient. Plants can only use sulfate, which is poorly
312 retained by most soils. Biomineralization of organic sulfur to sulfate is important to allow
313 plants to access this element. As one of just two known pathways for the catabolism of SQ,
314 the sulfo-ED pathway is likely to be an important part of environmental breakdown of SQ
315 and may contribute to the persistence of symbiotic rhizobia within the pedosphere. The
316 present work lays the groundwork for a more detailed investigation of sulfoglycolysis in a
317 well-characterized bacterium with an established capability for symbiosis of a leguminous
318 plant host.

319

320 **Materials and Methods**

321 **Reagents**

322 SQ, (¹³C₆)-SQ, SF, SFP, SLA, SL, and DHPS were chemically and chemoenzymatically
323 synthesized as described previously (27). IFG-SQ was chemically synthesized as described
324 (24).

325 **Bacteria and culture conditions**

326 *Rhizobium leguminosarum* bv. *trifolii* SRDI565 was a gift from Dr Ross Ballard (South
327 Australian Research and Development Institute, Adelaide, South Australia). Minimal salts
328 media consists of 0.5 g·L⁻¹ K₂HPO₄, 0.2 g·L⁻¹ MgSO₄, 0.1 g·L⁻¹ NaCl, 1 M CaCl₂ 3 mL·L⁻¹,
329 adjusted to pH 7.0. YM media consists of minimal salts media plus 1 g·L⁻¹ yeast extract, 10
330 g·L⁻¹ mannitol. Y_{5%}M consists of minimal salts media plus 50 mg·L⁻¹ yeast extract, 5 mM
331 mannitol. Y_{5%}SQ consists of minimal salts media plus 50 mg·L⁻¹ yeast extract, 5 mM SQ.

332

333 Growth curves were determined in a MicrobeMeter built in-house according to published
334 plans (33) and blueprints available at <https://humanetechnologies.co.uk/> The MicrobeMeter
335 was calibrated by performing serial 2-fold dilutions across the detection range of the
336 MicrobeMeter (0-1023 units), starting with an OD₆₀₀ approx. 1 culture of *Rl*-SRDI565. OD₆₀₀
337 measurements were made with a UV/Vis spectrophotometer and plotted against the reading
338 of the MicrobeMeter. The data was fit to a polynomial to obtain a calibration curve.

339

340 **Proteomic sample preparation:** Cells were washed 3 times in PBS and collected by
341 centrifugation at 10,000 x g at 4°C then snap frozen. Frozen whole cell samples were
342 resuspended in 4% SDS, 100 mM Tris pH 8.0, 20 mM DTT and boiled at 95°C with shaking
343 at 2000 rpm for 10 min. Samples were then clarified by centrifugation at 17,000 × g for 10
344 min, the supernatant collected, and protein concentration determined by bicinchoninic acid

345 assay (Thermo Scientific Pierce). 100 µg of protein from each sample was cleaned up using
346 SP3 based purification according to previous protocols (34). Briefly, reduced samples were
347 cooled and then alkylated with 40 mM 2-chloroacetamide (CAA) for 1 hour at RT in the
348 dark. The alkylation reactions were then quenched with 40 mM DTT for 10 min and then
349 samples precipitated onto SeraMag Speed Beads (GE Healthcare, USA) with ethanol (final
350 concentration 50% v/v). Samples were shaken for 10 min to allow complete precipitation
351 onto beads and then washed three times with 80% ethanol. The precipitated protein-covered
352 beads were resuspended in 100 mM ammonium bicarbonate containing 2 µg trypsin (1/50
353 w/w) and allowed to digest overnight at 37 °C. Upon completion of the digests, samples were
354 centrifuged at 14000 g for 5 min to pellet the beads and the supernatant collected and
355 desalted using homemade C18 stage tips (35). The eluted material was dried and stored, until
356 analysed by LC-MS.

357

358 **Proteomics analysis using reversed phase LC-MS:** Purified peptides prepared were re-
359 suspended in Buffer A* (2% ACN, 0.1% CF₃CO₂H) and separated using a two-column
360 chromatography set-up composed of a PepMap100 C18 20 mm × 75 µm trap and a PepMap
361 C18 500 mm × 75 µm analytical column (Thermo Fisher Scientific). Samples were
362 concentrated onto the trap column at 5 µL/min for 5 min and infused into an Orbitrap Elite™
363 (Thermo Fisher Scientific). 120 min gradients were run altering the buffer composition from
364 1% buffer B (80% ACN, 0.1% formic acid) to 28% B over 90 min, then from 28% B to 40%
365 B over 10 min, then from 40% B to 100% B over 2 min, the composition was held at 100% B
366 for 3 min, and then dropped to 3% B over 5 min and held at 3% B for another 10 min. The
367 Elite Orbitrap Mass Spectrometers was operated in a data-dependent mode automatically
368 switching between the acquisition of a single Orbitrap MS scan (120,000 resolution) and a
369 maximum of 20 MS-MS scans (CID NCE 35, maximum fill time 100 ms, AGC 1*10⁴).

370

371 **Mass spectrometry data analysis.** Proteomic comparison of growth with and without
372 sulfoquinovose was accomplished using MaxQuant (v1.5.5.1) (36). Searches were performed
373 against *Rhizobium leguminosarum* *bv. trifolii* SRDI565 (NCBI Taxonomy ID: 935549,
374 downloaded 01-08-2019, 6404 entries) with carbamidomethylation of cysteine set as a fixed
375 modification. Searches were performed with Trypsin cleavage allowing 2 miscleavage events
376 and the variable modifications of oxidation of methionine and acetylation of protein N-
377 termini. The precursor mass tolerance was set to 20 parts-per-million (ppm) for the first
378 search and 10 ppm for the main search, with a maximum false discovery rate (FDR) of 1.0%
379 set for protein and peptide identifications. To enhance the identification of peptides between
380 samples the Match Between Runs option was enabled with a precursor match window set to 2
381 min and an alignment window of 10 min. For label-free quantitation, the MaxLFQ option
382 within MaxQuant (37) was enabled in addition to the re-quantification module. The resulting
383 peptide outputs were processed within the Perseus (v1.4.0.6)(38) analysis environment to
384 remove reverse matches and common protein contaminants with missing values imputed. The
385 mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium
386 via the PRIDE partner repository with the dataset identifier PXD015822.

387

388 **Enzyme kinetics of *RISQase***

389 **Michaelis Menten plot.** Kinetic analysis of *RISQase** was performed using PNPSQ as
390 substrate, using a UV/visible spectrophotometer to measure the release of the 4-
391 nitrophenolate ($\lambda = 348$ nm). Assays were carried out in 50 mM sodium phosphate, 150 mM
392 NaCl, pH 7.2 at 30 °C using 212 nM *RISQase** at substrate concentrations ranging from 0.05
393 μ M to 4 mM. Using the extinction coefficient for 4-nitrophenolate of $5.125 \text{ mM}^{-1} \text{ cm}^{-1}$,
394 kinetic parameters were calculated using Prism.

395 **pH profile.** For the determination of pH profile, specific activities of *RISQase** were
396 monitored by measuring absorbance changes at $\lambda = 348$ nm in the presence of the following
397 buffers: sodium acetate buffer (pH 5.6, sodium phosphate buffer (pH 6.0–8.5), and glycine
398 NaOH buffer (pH 8.8–9.2). The assays were performed at 30 °C in duplicates and specific
399 activities determined using extinction coefficient of PNP $5.125 \text{ mM}^{-1} \text{ cm}^{-1}$ at the isobestic
400 point (348 nm). One unit of SQase activity is defined as the amount of protein that releases 1
401 μmol PNP per min.

402

403 **Cloning, expression and kinetic analysis of *RI-SRDI565* sulfoquinovosidase (*RISQase**)**

404 The gene sequence coding for *RISQase** SERp mutant was synthesised with codon
405 optimisation for expression in *E. coli* and was cloned within a pET-28a(+) vector with C-
406 terminal His-tag through GenScript. The plasmid His₆-*RISQase**-pET-28a(+) containing the
407 gene for target *RISQase** was transformed into *E. coli* BL21(DE3) cells for protein
408 expression. Pre-cultures were grown in LB-medium (5 mL) containing 30 $\mu\text{g/mL}$ for 18 h at
409 37 °C, 200 rpm. Cultures (1 L LB-medium supplemented with kanamycin 30 $\mu\text{g/mL}$) were
410 inoculated with the pre-culture (5 mL) and incubated at 37 °C, 200 rpm until an OD₆₀₀ of 0.6-
411 0.8 was achieved. Protein expression was induced by addition of IPTG (1 mM) and shaking
412 was continued overnight (20-22 h) at 18 °C, 200 rpm. The cells were harvested by
413 centrifugation (5000 rpm, 4 °C, 20 min), resuspended in 50 mM Tris, 300 mM NaCl pH 7.5
414 buffer and were subjected to further cell lysis. Cells were disrupted using French press under
415 20 k Psi pressure and the lysate was centrifuged at 50,000 g for 30 min.

416 The N-terminal His₆-tagged protein was purified by immobilized metal ion affinity
417 chromatography, followed by size exclusion chromatography (SEC) (Fig. S8). The lysate was
418 loaded onto a pre-equilibrated Ni-NTA column, followed by washing with load buffer (50
419 mM Tris-HCl, 300 mM NaCl, 30 mM imidazole pH 7.5). The bound protein was eluted using

420 a linear gradient with buffer containing 500 mM imidazole. Protein containing fractions were
421 pooled, concentrated and loaded onto a HiLoad 16/600 Superdex 200 gel filtration column
422 pre-equilibrated with 50 mM Tris-HCl, 300 mM NaCl pH 7.5 buffer. The protein was
423 concentrated to a final concentration of 60 mg mL⁻¹ using a Vivaspin® 6 with a 300 kDa
424 MW cut-off membrane for characterization and enzyme assays.

425

426 **SEC-MALS analysis**

427 Experiments were conducted on a system comprising a Wyatt HELEOS-II multi-angle light
428 scattering detector and a Wyatt rEX refractive index detector linked to a Shimadzu HPLC
429 system (SPD-20A UV detector, LC20-AD isocratic pump system, DGU-20A3 degasser and
430 SIL-20A autosampler). Experiments were conducted at room temperature (20 ± 2°C).
431 Solvents were filtered through a 0.2 µm filter prior to use and a 0.1 µm filter was present in
432 the flow path. The column was equilibrated with at least 2 column volumes of buffer (50 mM
433 Tris, 300 mM NaCl pH 7.5) before use and buffer was infused at the working flow rate until
434 baselines for UV, light scattering and refractive index detectors were all stable. The sample
435 injection volume was 100 µL *RISQase** at 6 mg/mL in 50 mM Tris buffer, 300 mM NaCl pH
436 7.5. Shimadzu LC Solutions software was used to control the HPLC and Astra V software for
437 the HELEOS-II and rEX detectors (Fig. S8). The Astra data collection was 1 min shorter than
438 the LC solutions run to maintain synchronisation. Blank buffer injections were used as
439 appropriate to check for carry-over between sample runs. Data were analysed using the Astra
440 V software. Molar masses were estimated using the Zimm fit method with degree 1. A value
441 of 0.158 was used for protein refractive index increment (dn/dc).

442

443 **Detection of SQase activity in cell lysates**

444 *Rl*-SRDI565 was grown in 50 mL Y_{5%}M and Y_{5%}SQ media at 30 °C to mid log phase,
445 approximately OD₆₀₀ = 0.2, measured using a Varian Cary50 UV/visible spectrophotometer.
446 Cells were harvested by adding 3× volume of ice-cold PBS to metabolically quench the
447 samples then centrifuged at 2000 g, 4 °C for 10 min. The supernatant was discarded and the
448 cells were washed 3 times with ice-cold PBS, with each wash involving resuspension and
449 centrifugation at 2000 g, 4 °C for 10 min. The cells were collected once more by
450 centrifugation at 10,000 g, 4 °C, for 1 min then snap frozen in liquid nitrogen and stored at -
451 80 °C.

452 Cells were lysed by addition of 1000 µL pre-chilled PBS, 1 µL RNaseA, 1 µL DNase,
453 1 µL 100 mg·mL⁻¹ hen egg white lysozyme (Sigma), and a 1× final concentration of
454 cOmplete EDTA-free protease inhibitor cocktail (Roche) to the cell pellet. The cells were
455 gently resuspended and mixed at 4 °C for 10 min. The suspension was placed on ice and
456 irradiated with a Sonoplus HD3200 MS 73 sonicator probe (Bandelin) at a frequency of 20
457 kHz, 20% amplitude, pulse 2s on 8s off, repeated for a total time of the sonication to 150 s,
458 then incubated on ice for 5 min. The suspension was clarified by centrifuging at 14,000 g, 4
459 °C for 1 min and the supernatant was filtered through a Nanosep mini centrifugal spin
460 column with a 0.2 µm filter (Pall) into a 1.5mL Eppendorf tubes and stored at 4 °C. Protein
461 concentration was determined using a BCA assay.

462 SQase activity was measured in triplicate using PNPSQ and an Agilent Cary UV
463 Workstation (G5191-64000) at 30°C. Reactions contained buffer consisted of 50 mM NaPi
464 and 150 mM NaCl, pH=7.4, and 2.5 mM PNPSQ. Reactions were initiated by addition of
465 SQ- or mannitol-derived lysate to a final concentration of 43.7 µg·mL⁻¹ protein, and
466 absorbance was monitored at 400 nm for 3 h. After 3 h, IFGSQ was added to a final
467 concentration of 6.25 mM to the SQ-lysate sample, and absorption monitored for 3 h.

468

469 **Metabolite analysis of *Rhizobium leguminosarum* cell extracts**

470 **Metabolic quenching and extraction.** *Rl*-SRDI565 was grown on Y_{5%}SQ or Y_{5%} containing
471 35 mM glucose to mid-logarithmic phase (approx. 0.15), as calculated based on the OD₆₀₀
472 measured by Cary 50 UV/visible spectrophotometer, and were rapidly quenched in a
473 prechilled 15 mL Falcon tube containing phosphate buffered saline (PBS) at 4 °C. Ice-cold
474 PBS (11 mL) was infused into cell culture media (4 mL). The Falcon tubes were mixed by
475 inversion and incubated in ice/water slurry for 5 min then were centrifuged at 2000 × *g* at 1
476 °C for 10 min. The supernatant was removed by aspiration and cell pellets were washed twice
477 with 1 mL of ice-cold PBS (with resuspension each time) and transferred into 1.5 mL
478 Eppendorf tubes. Cells were pelleted by centrifugation at 14000 rpm and residual solvent was
479 carefully removed. Cell pellets were stored at -80°C until extraction. Cells were extracted in
480 200 µL of extraction solution (methanol/water, 3:1 v/v) containing an internal standard, 5 µM
481 ¹³C₄-aspartate (Cambridge Isotopes), and subjected to 10 freeze-thaw cycles to facilitate cell
482 lysis (30 s in liquid nitrogen, followed by 30 s in dry ice/ethanol bath). Debris was pelleted
483 by centrifugation at 14000 rpm, 5 min, 1°C and cell lysate was transferred into a HPLC vial
484 insert for LC/MS analysis.

485 **LC/MS analysis and identification of sulfonate metabolites.** Separation and detection of
486 polar metabolites was performed using an Agilent Technologies 1200 series high
487 performance liquid chromatography (HPLC) coupled to a quadrupole time-of-flight mass
488 spectrometer (6545 QTOF, Agilent Technologies) using a method modified from
489 Masukagami *et al.* (39). Metabolite extracts were transferred into 2 mL auto sampler vials
490 with glass inserts and placed in the auto sampler kept at 4 °C prior to analysis. Metabolite
491 separation was performed by injecting 7 µL of the extract into a SeQuant® ZIC-pHILIC
492 PEEK coated column (150 mm × 4.6 mm, 5 µm polymer, Merck Millipore) maintained at
493 25°C, with a gradient of solvent A (20 mM ammonium carbonate, pH 9.0, Sigma-Aldrich)

494 and solvent B (100% acetonitrile, Hypergrade for LCMS LiChrosolv, Merck) at a flow rate
495 of 0.3 mL/min. A 33.0 min gradient was setup with time (t) = 0 min, 80% B; t = 0.5 min,
496 80% B; t = 15.5 min, 50% B; t = 17.5 min, 30% B; t = 18.5 min, 5% B; t = 21.0 min, 5% B; t
497 = 23.0 min, 80% B.

498 The LC flow was directed into an electrospray ionization (ESI) source with a capillary
499 voltage of 2500 V operating in negative ionization mode. Drying nitrogen gas flow was set to
500 10 L/min, sheath gas temperature and nebulizer pressure were set to 300 °C and 20 psi,
501 respectively. The voltages of fragmentor and skimmer were set at 125 V and 45 V,
502 respectively. Data was acquired in MS and MS/MS mode, with a scan range of 60 to 1700
503 m/z and 100 to 1700 m/z respectively, at a rate of 1.5 spectra/sec. MS/MS acquisition was
504 performed with four collision energies (0, 10, 20 and 40 V). The mass spectrometer was
505 calibrated in negative mode prior to data acquisition and mass accuracy during runs was
506 ensured by a continuous infusion of reference mass solution at a flow rate of 0.06 mL/min
507 (API-TOF Reference Mass Solution Kit, Agilent Technologies). Data quality was ensured by
508 multiple injections of standards (with 1.5 μ M concentration each) and pooled biological
509 sample (a composite of cell extracts) used to monitor the instrument performance. Samples
510 were randomized prior to metabolite extraction and LC/MS analysis.

511 **Standard preparation.** Standards of selected metabolites (Supplementary Table 1) were
512 prepared at 10 μ M in 80% acetonitrile (Hypergrade for LCMS LiChrosolv, Merck) and
513 injected separately into a column connected to mass spectrometer interface. Retention time
514 and detected molecular ion were used to create a targeted MS/MS acquisition method. The
515 spectra, mass to charge (m/z) and retention time (RT) were imported into a personal
516 compound database and library (PCDL Manager, version B.07.00, Agilent Technologies)
517 used in data processing workflow.

518 **Data analysis.** Data were analysed using MassHunter Qualitative and Quantitative Analysis
519 software (version B.07.00, Agilent Technologies). Identification of metabolites was
520 performed in accordance with metabolite identification (Metabolomics Standard Initiative,
521 MSI) level 1 based on the retention time and molecular masses matching to authentic
522 standards included in the personal database and library. Peak integration was performed in
523 MassHunter quantitative software (version B.07.00, Agilent Technologies) on the spectra
524 from identified metabolites.

525

526 **Chemical synthesis of 6-deoxy-6-sulfo-D-gluconate (SG)**

527 NaOH in methanol (4% w/v, 4 mL) was added dropwise to a stirred solution of
528 sulfoquinovose (100 mg, 0.410 mmol) and iodine (209 mg, 1.65 mmol) in water (1 mL) and
529 methanol (4 mL) held at 40 °C. As the sodium hydroxide was added the color of iodine
530 dissipated. The solvent was evaporated under reduced pressure and the crude residue was
531 subjected to flash chromatography (EtOAc/MeOH/H₂O, 4:2:1 to 2:2:1, then water) to give
532 the 6-deoxy-6-sulfogluconate sodium salt (89.2 mg). An aqueous solution of the sodium salt
533 was eluted through a column of Amberlite IR120 (H⁺ form) resin. The acidic eluate was
534 collected and concentrated under reduced pressure give SG (71.3 mg, 67%). ¹H NMR (400
535 MHz, D₂O): δ 4.23–4.15 (1 H, m, H2), 4.13 (1 H, d, *J* = 3.3 Hz, H3), 4.05 (1 H, t, *J* = 3.2 Hz,
536 H5), 3.74 (1 H, dd, *J* = 6.5, 3.4 Hz, H4), 3.35 (1 H, d, *J* = 14.6 Hz, H6a), 3.05 (1 H, dd, *J* =
537 14.6, 9.7 Hz, H6b); ¹³C{¹H} NMR (100 MHz, D₂O) δ 178.7 (C1), 74.2 (C4), 73.8 (C2), 70.8
538 (C3), 67.8 (C5), 53.4 (C6); HRMS (ESI⁻) calcd for C₆H₁₁O₉S [M⁻] 259.0129, found
539 259.0131.

540

541 **Quantitation of metabolite levels in spent culture media**

542 The metabolites (DHPS, SF, SQ, SL and SG) present in spent culture media were quantified
543 against standard solutions of pure metabolites by HPLC-ESI-MS/MS. Quantification was
544 done with the aid of calibration curves generated by dissolving the pure standards in spent
545 media from *Rl-SRDI565* grown on Y_{5%}M. Spiked spent media was diluted 100-fold with
546 water and then analysed by LC-MS/MS with α -MeSQ as internal standard. For experimental
547 determination of metabolites, spent culture media from *Rl-SRDI565* grown in Y_{5%}SQ or
548 Y_{5%}SQGro were diluted 100-fold with water and analysed by LC-MS/MS with α -MeSQ as
549 internal standard.

550 HPLC-ESI-MS/MS analysis was performed using a TSQ Altis triple quadrupole mass
551 spectrometer (Thermo Fisher Scientific) coupled with a Vanquish Horizon UHPLC system
552 (Thermo Fisher Scientific). The column was a ZIC-HILIC column (5 μ m, 50 \times 2.1 mm;
553 Merck). The HPLC conditions were: from 90% B to 40% B over 15 min; then 40% B for 5
554 min; back to 90% B over 1 min (solvent A: 20 mM NH₄OAc in 1% acetonitrile; solvent B:
555 acetonitrile); flow rate, 0.30 ml min⁻¹; injection volume, 1 μ l. The mass spectrometer was
556 operated in negative ionization mode. Quantitation was done using the MS/MS selected
557 reaction monitoring (SRM) mode using Thermo Scientific XCalibur software and normalized
558 with respect to the internal standard, α -MeSQ. Prior to analysis, for each analyte, the
559 sensitivity for each SRM-MS/MS transition was optimized.

560 DHPS: ESI-MS/MS m/z of [M-H]⁻ 155, product ions 137, 95; retention time: 4.91 min

561 α -MeSQ (internal standard): ESI-MS/MS m/z of [M-H]⁻ 257, product ions 166, 81;

562 retention time: 6.31 min

563 SF: ESI-MS/MS m/z of [M-H]⁻ 243, product ions 207, 153; retention time: 6.81 min

564 SQ: ESI-MS/MS m/z of [M-H]⁻ 243, product ions 183, 123; retention time: 7.58 and 7.89

565 min for α / β

566 SL: ESI-MS/MS m/z of [M-H]⁻ 169, product ions 107, 71; retention time: 9.26 min

567 SG: ESI-MS/MS m/z of $[M-H]^-$ 259, product ions 241, 161; retention time: 9.66 min

568 SQGro: ESI-MS/MS m/z of $[M-H]^-$ 317, product ions 225, 165; retention time: 7.15 min

569

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689 **Figure 1: Proposed sulfoglycolytic Entner-Doudoroff (sulfo-ED) pathway in *Rhizobium***
690 ***leguminosarum* bv. *trifolii* SRDI565.** (a) Operon encoding sulfo-ED pathway. (b) Proposed
691 sulfo-ED pathway. (c) Comparison with the Entner-Doudoroff pathway.

692

693 **Figure 2: Growth of *Rhizobium leguminosarum* bv. *trifolii* SRDI565 on SQ produces SL**
694 **as the major terminal metabolite.** a) Growth of *Rl*-SRDI565 on 5% yeast extract media
695 containing 5 mM SQ (●) or 5 mM mannitol (○). This data is representative of 2 independent
696 experiments. b) ¹³C NMR (126 MHz) spectra of (top) SQ, (middle) 5 mM SQ in 5% yeast
697 extract media and (bottom) spent culture media from growth of *Rl*-SRDI565 on 5 mM SQ. c)
698 ¹³C NMR (126 MHz) spectrum of spent culture media from growth of *Rl*-SRDI565 on 5 mM
699 (¹³C₆)-SQ. The signal at δ 38.7 ppm is present in control experiments of *Rl*-SRDI565 grown
700 on mannitol and is believed to derive from yeast extract. d) Tabulated ¹³C NMR (126 MHz)
701 data for ¹³C₃-SL from (c). All samples contain 10% D₂O, added to allow frequency lock. e)
702 Quantitative proteomics was undertaken to identify proteins associated with sulfoquinovose
703 catabolism versus mannitol. Examination of proteins observed to increase in abundance
704 greater than four-fold revealed 17 proteins including alpha-dehydro-beta-deoxy-D-glucarate
705 aldolase (WP_017967308.1). f) Growth in sulfoquinovose leads to the increase of multiple
706 proteins associated with the TCA cycle including NAD(P)-dependent oxidoreductase
707 (WP_017965793.1), NADH-quinone oxidoreductase subunit NuoH (WP_017963854.1),
708 NAD-dependent succinate-semialdehyde dehydrogenase (WP_017967313.1) and citrate
709 synthase/methylcitrate synthase (WP_017964386.1) highlighted in blue.

710

711 **Figure 3: *Rhizobium leguminosarum* SRDI565 produces a functional sulfoquinovosidase**
712 **that can be detected in cell lysates.** a) pH profile of *Rl*SQase*. Specific activities were
713 determined for hydrolysis of PNPSQ at the isosbestic point, 348 nm. b) Michaelis Menten

714 plot of kinetic parameters for *RI*SQase* for hydrolysis of PNPSQ at 400 nm. c) Analysis of
715 sulfoquinovosidase activity of *RI*-SRDI565 lysate grown on sulfoquinovose and mannitol.
716 Cell lysates of soluble proteins derived from growth on SQ or mannitol was standardized for
717 equal protein and SQase activity measured using the chromogenic substrate PNPSQ at 400
718 nm. SQase activity was confirmed by inhibition by the azasugar inhibitor SGIFG. Error bars
719 denote standard error of the mean.

720

721 **Figure 4: Detection of sulfoglycolytic intermediates and end-products in cytosolic**
722 **extracts of *RI*-SRDI565.** *RI*-SRDI565 was grown on Y_{5%}SQ media and metabolically-
723 quenched by rapid cooling to 4 C, followed by extraction of cellular metabolites and lc/ms
724 analysis. Detection of sulfoglycolytic and glycolytic/neoglucogenic intermediates A) SQ, B)
725 SG, C) SL, D) SF, E) DHPS. In each case the upper panel corresponds to the collision-
726 induced dissociation mass spectrum of chemically-synthesized standard, while the lower
727 panel is the equivalent mass spectrum for the metabolite identified in the cytosolic extract. F)
728 Relative mass spectrometric intensities of metabolites from cells grown on Glc or SQ.

729

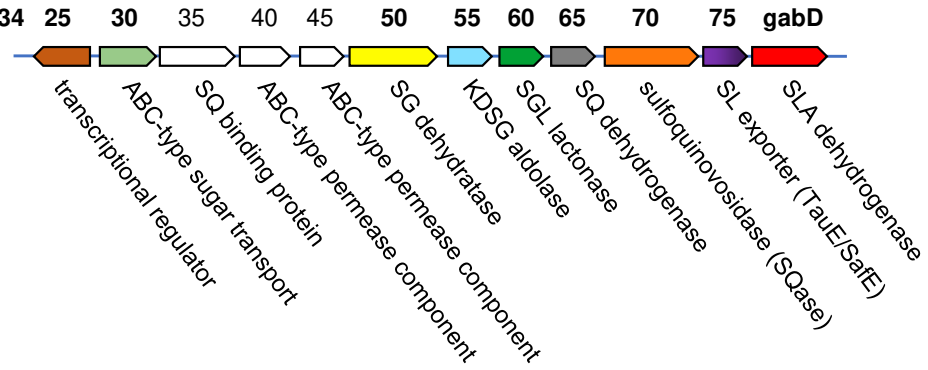
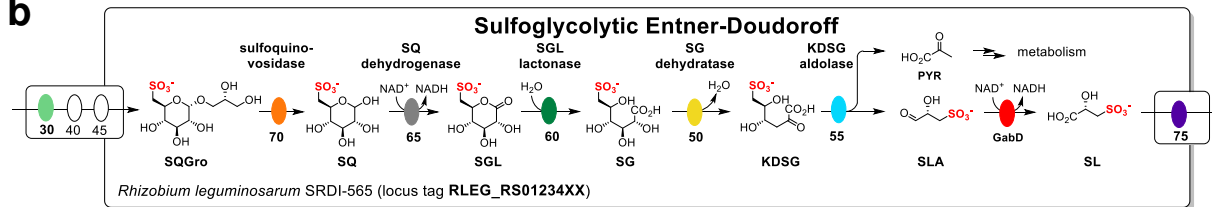
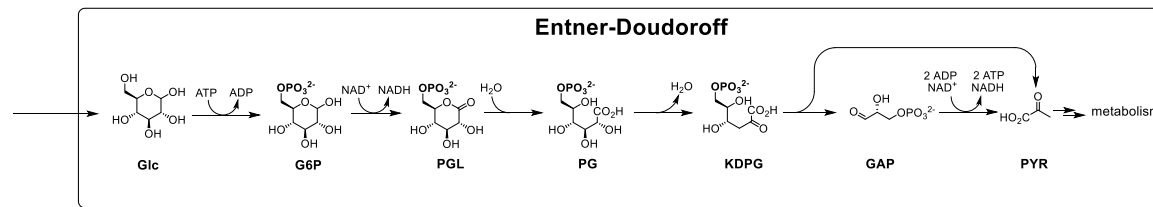
730 **Figure 5: Proposed pathway for SQ metabolism in *Rhizobium leguminosarum* SRDI565.**

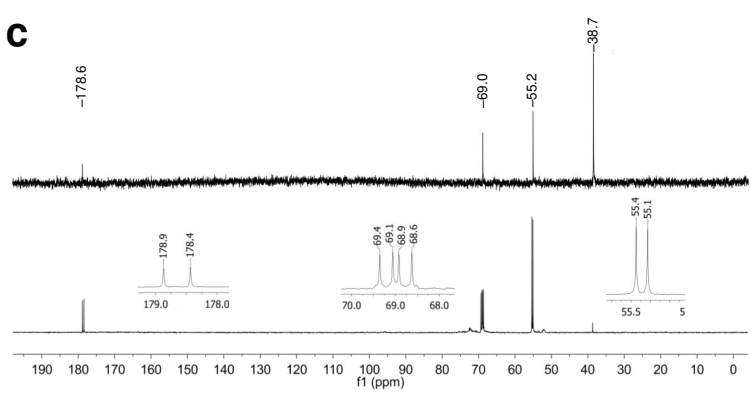
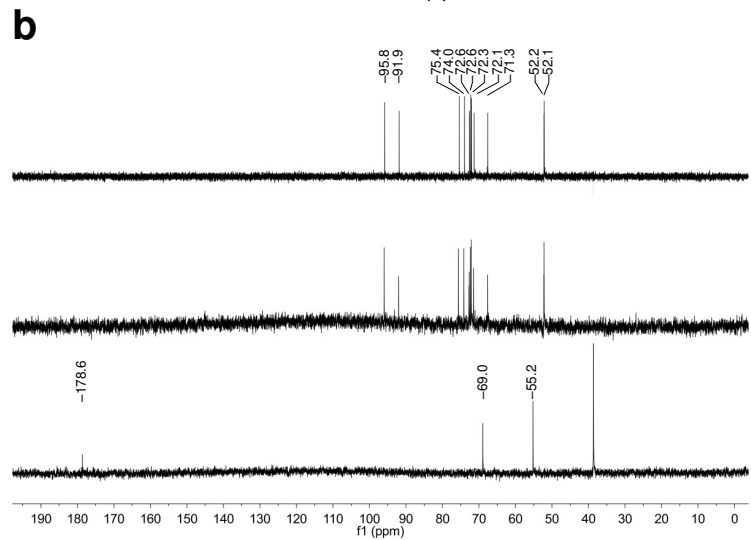
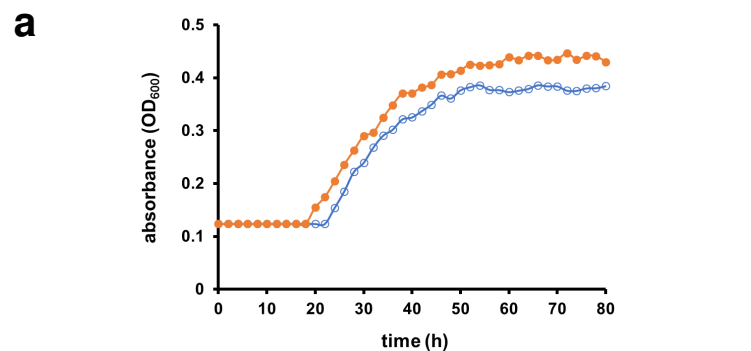
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732 **Table 1.** Analysis of sulfonate metabolites detected in spent culture media of *RI*-SRDI565
733 grown on 5.0±0.5 mM SQ or SQGro (standard error estimate). Measurements were
734 performed in triplicate using LC/MS-MS. Errors listed in the table are standard error mean.

a*Rhizobium leguminosarum* SRDI565

Locus tag RLEG16_RS01234

**b****c****Figure 1**



d

¹³ C chemical shift (δ ppm)	coupling (Hz)	assignment
53.2	¹ J _{C1,C2} = 37.1	C3
64.7	¹ J _{C2,C3} = 54.8 ¹ J _{C1,C2} = 37.1	C2
178.6	¹ J _{C2,C3} = 54.8	C1

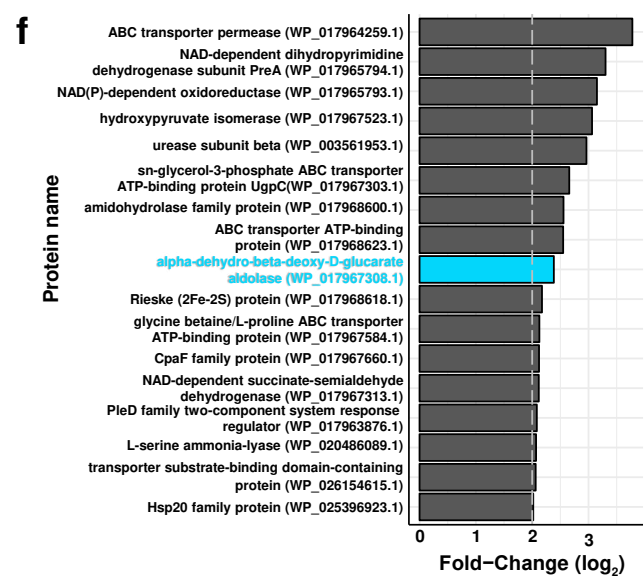
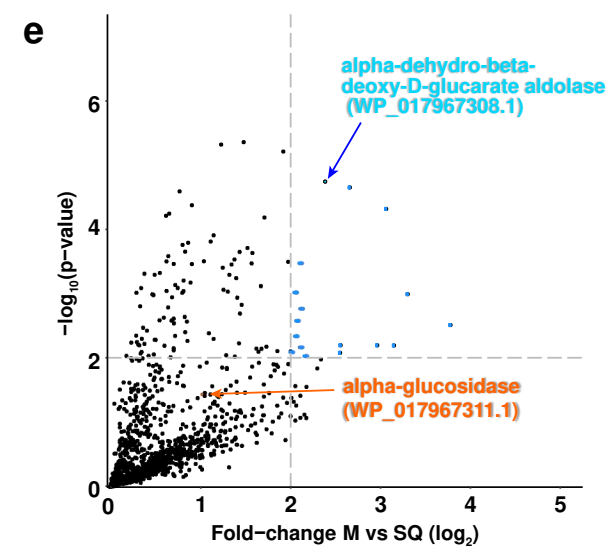


Figure 2

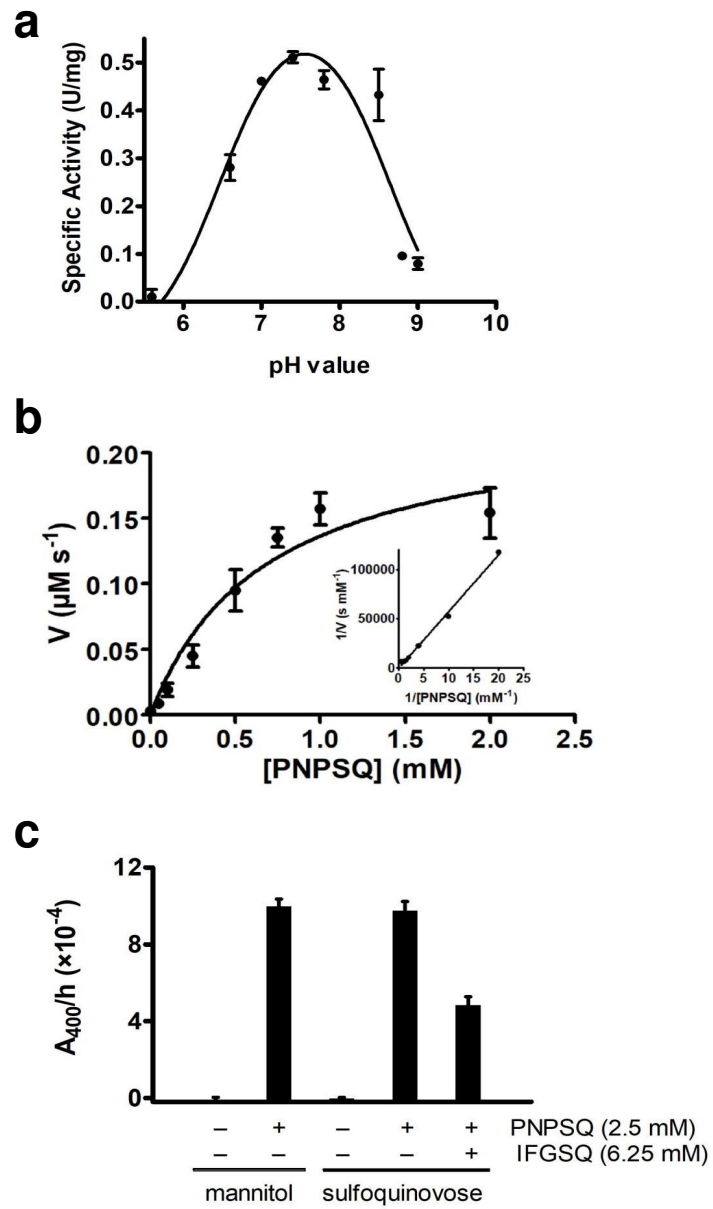
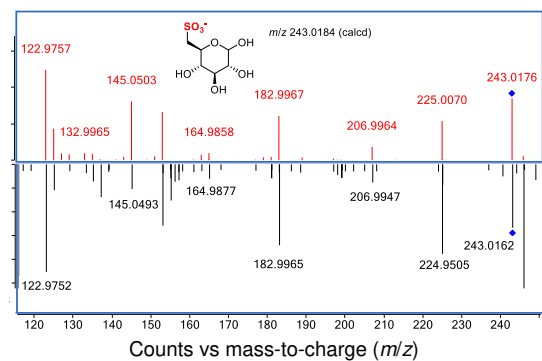
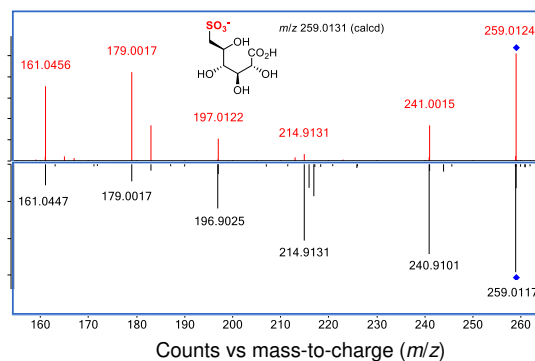


Figure 3

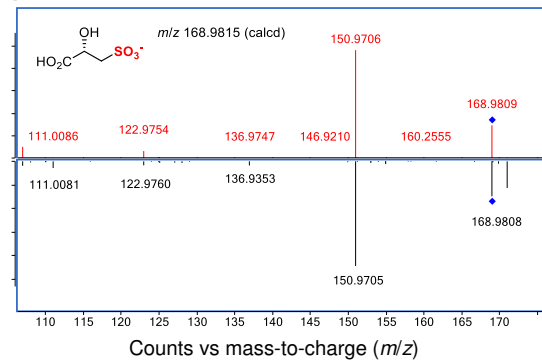
a) SQ



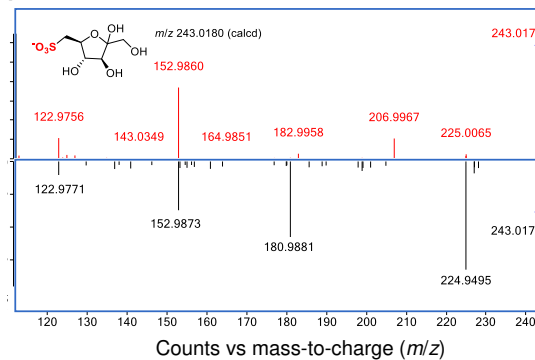
b) SG



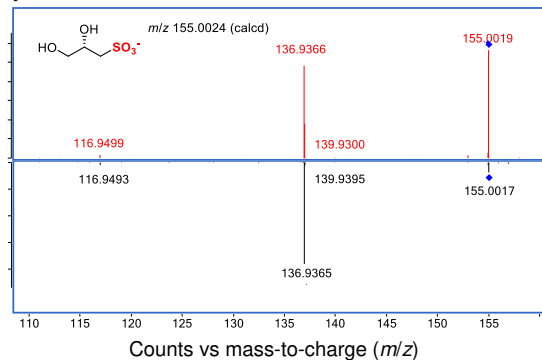
c) SL



d) SF



e) DHPS



f)

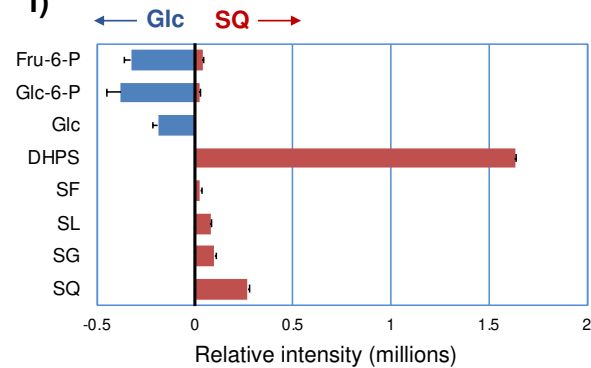


Figure 4

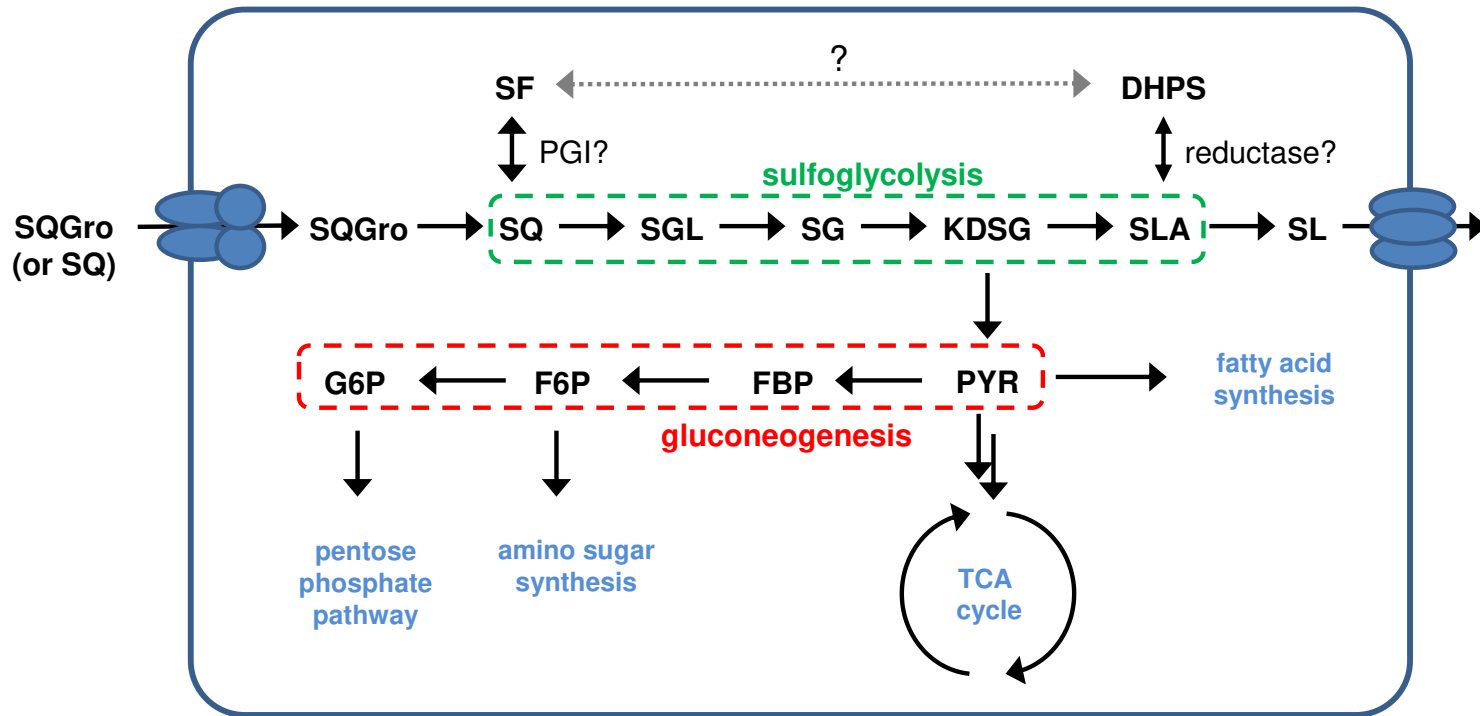


Figure 5