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1 Engineering mannitol biosynthesis in *Escherichia coli*  
2 and *Synechococcus* sp. PCC 7002 using a green algal  
3 fusion protein

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10 **Abstract**

11 The genetic engineering of microbial cell factories is a sustainable alternative to the chemical  
12 synthesis of organic compounds. Successful metabolic engineering often depends on  
13 manipulating several enzymes, requiring multiple transformation steps and selection markers,  
14 as well as protein assembly and efficient substrate channeling. Naturally occurring fusion  
15 genes encoding two or more enzymatic functions may offer an opportunity to simplify the  
16 engineering process and to generate ready-made protein modules, but their functionality in  
17 heterologous systems remains to be tested. Here we show that heterologous expression of a  
18 fusion enzyme from the marine alga *Micromonas pusilla*, comprising a mannitol-1-phosphate  
19 dehydrogenase and a mannitol-1-phosphatase, leads to synthesis of mannitol by *Escherichia*  
20 *coli* and by the cyanobacterium *Synechococcus* sp. PCC 7002. Neither of the heterologous  
21 systems naturally produces this sugar alcohol, which is widely used in food, pharmaceutical,  
22 medical and chemical industries. While the mannitol production rates obtained by single-gene  
23 manipulation were lower than those previously achieved after pathway optimization with

24 multiple genes, our findings show that naturally occurring fusion proteins can offer simple  
25 building blocks for the assembly and optimization of recombinant metabolic pathways.

26 **Keywords**

27 Synthetic biology, Fusion Protein, Mannitol, Cyanobacteria, *Micromonas*.

28

29 Microbial cell factories, particularly photosynthetic chassis that consume carbon dioxide as  
30 their sole carbon source, are an attractive alternative to chemical synthesis <sup>1</sup>. They present a  
31 sustainable approach to producing an array of substances with usages in food,  
32 pharmaceutical, nutraceutical and cosmetic industries.

33 Biological production platforms struggle to compete economically, particularly for the  
34 production of low value commodity chemicals and materials <sup>2</sup>. A recurring problem is  
35 performance, which is measured as titer, yield and productivity. Artificial metabolic pathways  
36 inherently generate imbalances in pathway flux resulting in (toxic) intermediate and side  
37 product accumulation, growth inhibition, and ultimately low product yield <sup>3</sup>. Therefore,  
38 individual modifications (e.g. the introduction of a single enzyme or pathway) are not sufficient  
39 to achieve industrially relevant titers. As a result, cell factory development requires the  
40 introduction of several genes to synthesize the desired product, the overproduction of  
41 precursors, and the deletion of competing pathways <sup>4-5</sup>.

42 The use of fusion genes, generated from previously separate genes, can help to simplify the  
43 biological engineering process <sup>6</sup>. Firstly, reducing the number of genes required for a  
44 synthetic pathway simplifies the design and assembly of synthetic expression constructs as  
45 well as the transformation and selection of producing strains. Secondly, reducing the number  
46 of enzymes simplifies assembly of protein complexes encoded by separate genes and  
47 stoichiometric optimization to balance pathway flux. Furthermore, catalytic sites are brought in  
48 closer proximity, thus enhancing substrate channeling and reducing intermediate  
49 accumulation and/or loss to competing pathways <sup>7</sup>. Additionally, transit times for intermediate  
50 products between catalytic sites are significantly reduced thus improving biosynthetic  
51 efficiency.

52 D-Mannitol is a sugar alcohol, or polyol, ubiquitous in prokaryotes and eukaryotes, except for  
53 Archaea and animals. It has desirable properties for food, pharmaceutical, medical and  
54 chemical industries <sup>8</sup>. Based on a recent analysis of mannitol markets  
55 (<http://www.grandviewresearch.com/industry-analysis/mannitol-market>; last accessed on  
56 01/11/2017), demand for this polyol, driven by an increasing use in the food and

57 pharmaceutical sectors, is expected to reach 35.9 million kg representing a market of USD  
58 418.3 million by 2024.

59 Mannitol can be produced by extraction, chemical synthesis or biosynthesis. Traditionally,  
60 mannitol was harvested from plant material which was seasonal and yields highly variable <sup>9</sup>.  
61 To stabilize mannitol supplies, commercial production now mostly occurs through chemical  
62 hydrogenation of fructose-glucose syrups. However, poor selectivity of the nickel catalyst  
63 results in a mixture of mannitol and sorbitol which is relatively difficult to separate and thus  
64 costly <sup>10</sup>. Chemical synthesis can be improved by altering the substrate, e.g. isomerizing  
65 glucose to fructose by enzymatic conversion; however, enzyme availability and added costs  
66 of additional steps prevent this from being economical.

67 The chemical industry is now looking to bio-based production methods to reduce costs and  
68 environmental impact. The most successful approach to mannitol biosynthesis currently uses  
69 heterofermentative lactic acid bacteria. Under anaerobic conditions, these organisms reduce  
70 fructose using the native enzyme mannitol dehydrogenase. This approach requires an  
71 external sugar supply that is a) predominantly obtained from traditional crops, e.g. corn and b)  
72 a major cost for biosynthesis of commodities such as mannitol. External carbon sources and  
73 associated costs can be eliminated from the bio-production pipeline by using photosynthetic  
74 organisms, which assimilate atmospheric carbon dioxide into sugars via the Calvin cycle <sup>11</sup>.

75 Cyanobacteria represent an incredibly diverse phylum of phototrophic prokaryotes that are  
76 being developed for photosynthetic bio-production <sup>12-14</sup>. One particularly attractive chassis is  
77 the unicellular euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 due to its reported  
78 fast growth and tolerance of high salt, light and temperature <sup>15-17</sup>. Importantly, *Synechococcus*  
79 is amenable to natural transformation <sup>18-19</sup> and its genome is fully sequenced  
80 (<http://genome.microbedb.jp/cyanobase/SYNPCC7002>). Some molecular tools to engineer  
81 *Synechococcus* have been developed including transformation vectors, promoters to control  
82 transcription and ribosome binding sites to control translation <sup>20-22</sup>.

83 *Synechococcus* has previously been engineered to produce mannitol from fructose-6-  
84 phosphate, an intermediate of the Calvin cycle <sup>23</sup>. This recombinant mannitol biosynthetic

85 pathway involved heterologous expression of two enzymes encoded by separate genes: (1)  
86 an *Escherichia coli* gene encoding mannitol-1-phosphate dehydrogenase (M1PDH), which  
87 catalyzes the reduction of fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P), and  
88 (2) an *Eimeria tenella* gene encoding phosphohistidine mannitol-1-phosphatase (M1Pase),  
89 which hydrolyses M1P to mannitol.

90 In recent years, new insights have been gained on the mannitol biosynthetic pathway, notably  
91 in algae. The first algal genes involved in mannitol production were identified in the model  
92 brown alga *Ectocarpus*<sup>24</sup>, and biochemical characterization of the recombinant enzymes  
93 confirmed M1PDH and M1Pase function<sup>25-27</sup>. Analysis of the distribution and evolution of  
94 these mannitol biosynthetic genes across algal lineages showed that mannitol synthesis is  
95 more widely spread and diverse than initially thought<sup>28</sup>. Interestingly, several fusion genes  
96 combining modules for M1PDH and M1Pase activities were identified, notably in marine  
97 green algae. For instance, Mipuc10g00620  
98 (<http://bioinformatics.psb.ugent.be/orcae/annotation/Mipuc/current/Mipuc10g00620>) of  
99 *Micromonas pusilla* strain CCMP1545 is predicted to encode an enzyme expected to  
100 transform F6P directly into mannitol.

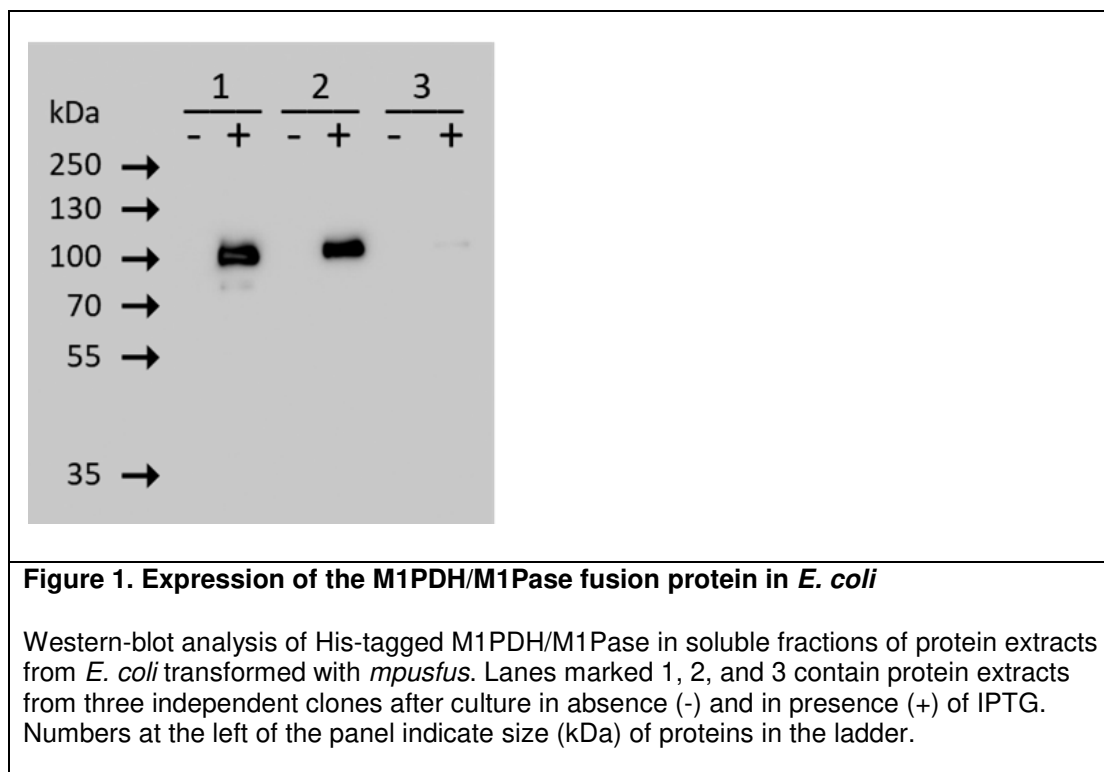
101 In this study, we explored whether the M1PDH/M1Pase fusion gene of *M. pusilla* strain  
102 CCMP1545 is functional when heterologously expressed in heterotrophic (*E. coli*) or photo-  
103 autotrophic (*Synechococcus*) bacteria. To facilitate the engineering of *Synechococcus* we  
104 generated BioBrick-compatible molecular tools (vectors and ribosome binding sites). We  
105 found that both *E. coli* and *Synechococcus* produced mannitol when transformed with the  
106 fusion gene. The one-step mannitol production pathway provides an excellent starting point  
107 for further optimization of sustainable mannitol production in cyanobacteria.

## 108 **Results**

### 109 ***Mpusfus* is functional in *E. coli***

110 The M1PDH/M1Pase fusion gene of *Micromonas pusilla* strain CCMP1545 was codon  
111 optimized for expression in *Synechococcus* (see Methods) and we called the resulting

112 sequence *mpusfus*. To test expression and function in heterologous systems, *mpusfus* was  
 113 first introduced into *E. coli* using the pFO4 vector that carries an IPTG-inducible expression  
 114 system. A six-histidine tag was added to the 5' end of the gene. Western-blot of protein  
 115 extracts from IPTG-induced cells revealed a band of the expected size while no band was  
 116 detected in the controls without IPTG (Figure 1). The size of the primary band was close to  
 117 the value of 94.8 kDa calculated for the full-length predicted amino acid sequence. This  
 118 showed that a single fusion protein was produced from the fusion gene.



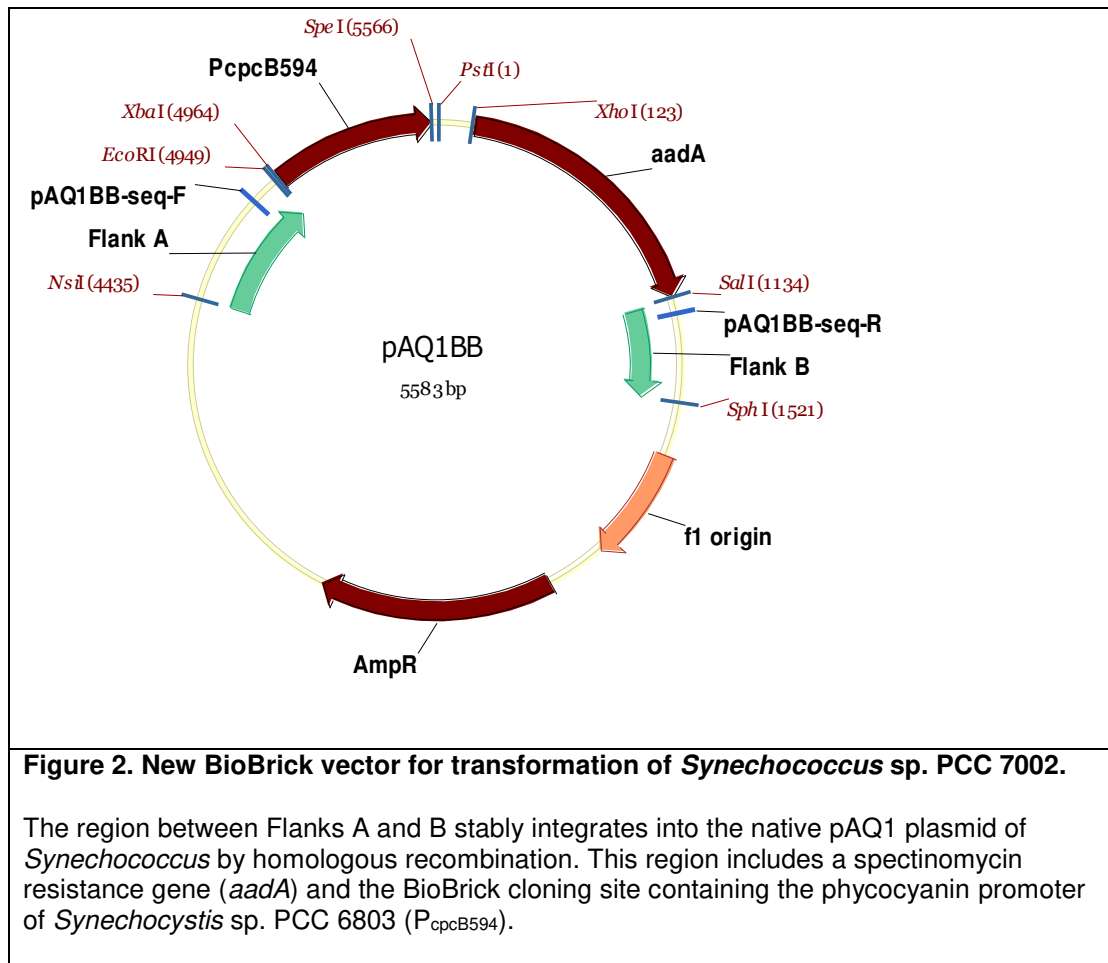
119

120 Mannitol was measured both in the culture media and in the cells after 20 hours of incubation  
 121 in absence and in presence of IPTG. In 5 ml cultures with a final OD<sub>600</sub> of 2.5-3.0 we found 1  
 122  $\pm 0.05$  mg of mannitol in the media and  $0.08 \pm 0.008$  mg of mannitol inside the cells (n = 3  
 123 clones) after incubation with IPTG. Extrapolated to a 1-litre culture, total mannitol production  
 124 was  $218 \pm 11.9$  mg/L, of which  $202 \pm 10.7$  mg (93 %) were exported into the medium and  $16$   
 125  $\pm 1.6$  mg (7 %) were retained inside the cells. No mannitol was detected in cultures grown  
 126 without IPTG. The results showed that the algal fusion gene produces a single protein in *E.*  
 127 *coli* that catalyses the biosynthesis of mannitol.

128 ***New molecular tools and protocols to engineer Synechococcus sp. PCC 7002***

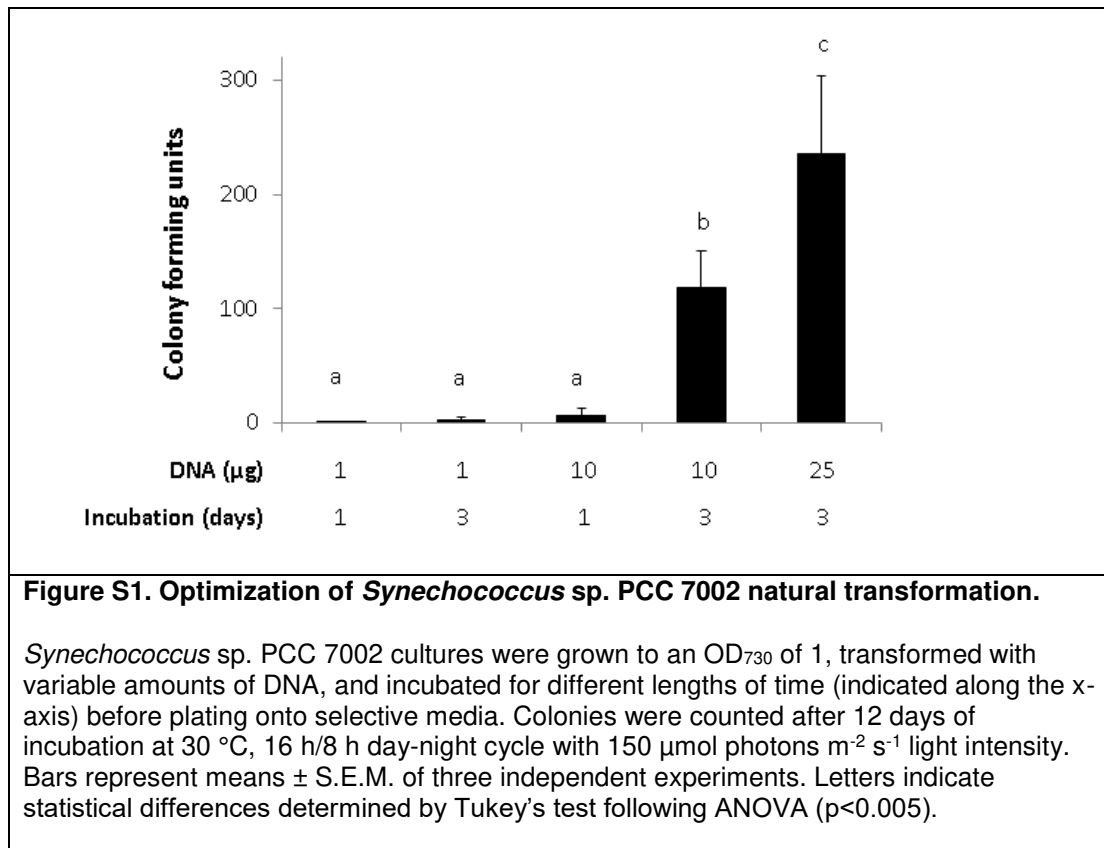
129 BioBricks represent the largest collection of standardized parts for genetic engineering <sup>29</sup>.  
130 While a number of genetic tools have been developed for *Synechococcus* <sup>20-22</sup>, none of them  
131 are compatible with BioBrick parts. In order to develop a BioBrick-compatible integration  
132 vector for *Synechococcus*, we reengineered an existing expression vector, pAQ1EX <sup>30</sup>. This  
133 vector targets the transgene to a neutral site in the native, high copy pAQ1 plasmid of  
134 *Synechococcus*. BioBrick prefix and suffix sequences containing the restriction enzyme  
135 recognition sites required for gene assembly were synthesized and inserted into the  
136 integrative region of the pAQ1EX vector. For ease of cloning, domestication of DNA parts is  
137 important whereby BioBrick restriction sites do not occur outside of the prefix and suffix  
138 sequences. An *Xba*I site occurring directly upstream of the selective *aadA* gene was therefore  
139 replaced with an *Xho*I site. Finally, the phycocyanin promoter of *Synechocystis* sp. PCC  
140 6803, P<sub>opcB594</sub>, which has been reported to be a strong promoter in cyanobacteria, was  
141 inserted into the BioBrick cloning site <sup>31</sup>. The resulting vector called pAQ1BB provides a  
142 convenient tool for transformation of *Synechococcus* sp. PCC 7002 (Figure 2). The target  
143 integration site (“landing pad”) can be modified by replacing homologous sequences Flank A  
144 and Flank B using *Nsi*I/*Eco*RI and *Sal*I/*Sph*I restriction sites respectively. The marker gene for  
145 selection can be modified using *Xho*I and *Sal*I restriction sites.





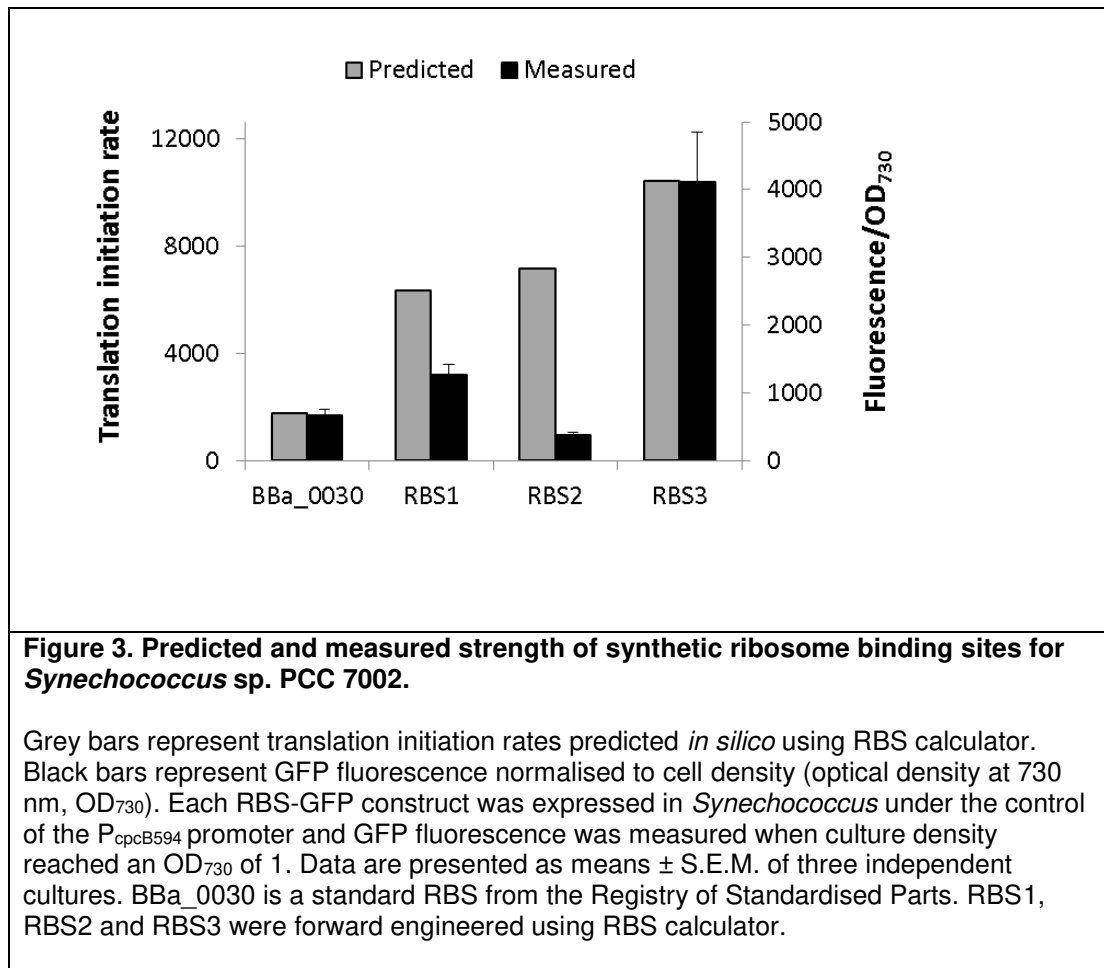
146

147 Using established protocols for natural transformation<sup>30</sup>, transformation efficiency of  
 148 *Synechococcus* with pAQ1BB was initially poor. Increasing both the amount of DNA and  
 149 incubation time prior to plating onto selective media significantly enhanced transformation  
 150 efficiency (Figure S1). The optimized transformation protocol can be found in the Methods  
 151 section.



152

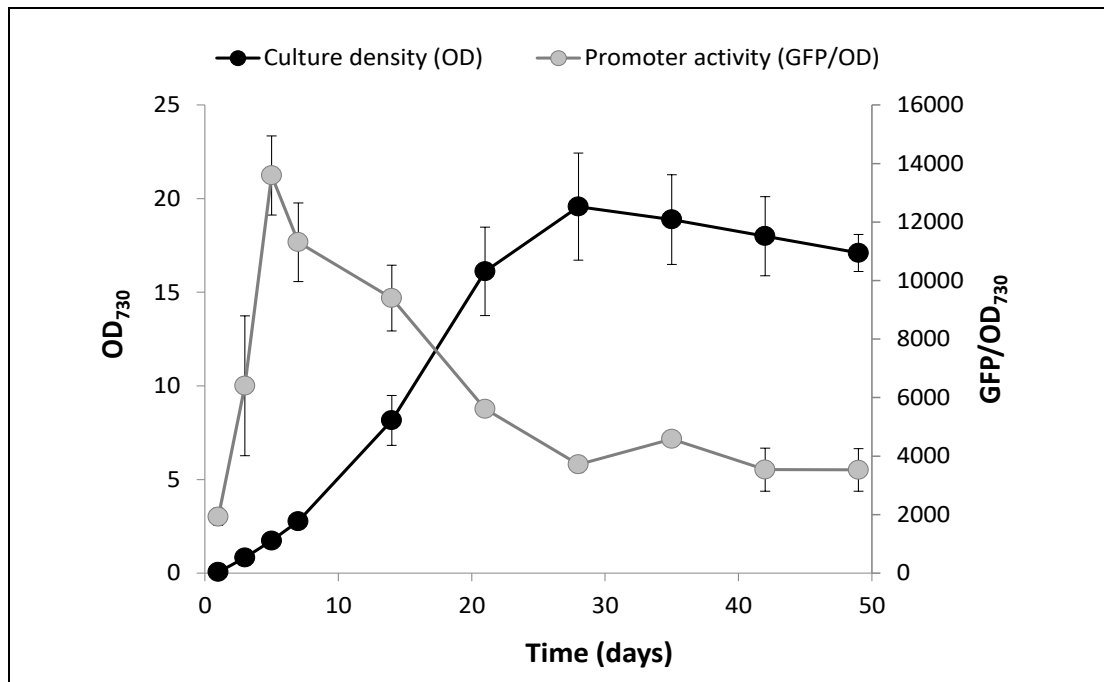
153 Ribosome binding sites (RBSs) play an essential role in translation initiation during protein  
 154 synthesis. Software have been developed to design synthetic RBSs considering factors such  
 155 as secondary mRNA structure influenced by flanking nucleotide sequences, the Shine-  
 156 Dalgarno (SD) sequence complementary to the 16S ribosomal RNA, spacing between the SD  
 157 and start codon, and the sequence of the start codon itself <sup>32</sup>. We used a web-based RBS  
 158 calculator <sup>33</sup> to design RBS for *Synechococcus* (sequences in Table S2) and assessed the  
 159 suitability of three potential RBS for producing green fluorescent protein (GFP). RBS-GFP  
 160 were cloned into pAQ1BB and used to transform *Synechococcus*. Similar to a previous report  
 161 <sup>21</sup> we observed a moderate correlation between predicted and actual translation rates (Figure  
 162 3). Both the RBS calculator and the GFP-assay identified RBS3 as the strongest RBS and we  
 163 therefore used RBS3 to express *mpusfus*.



164

165 To assess promoter activity in combination with RBS3, we monitored GFP fluorescence in the  
 166 P<sub>cpcB594</sub>:RBS3-GFP expressing cells over the course of culture growth. The experiment  
 167 showed that the promoter is most active in the early exponential phase (Figure 4) as  
 168 previously suggested<sup>30</sup>.

169



**Figure 4. Promoter activity over the course of culture growth**

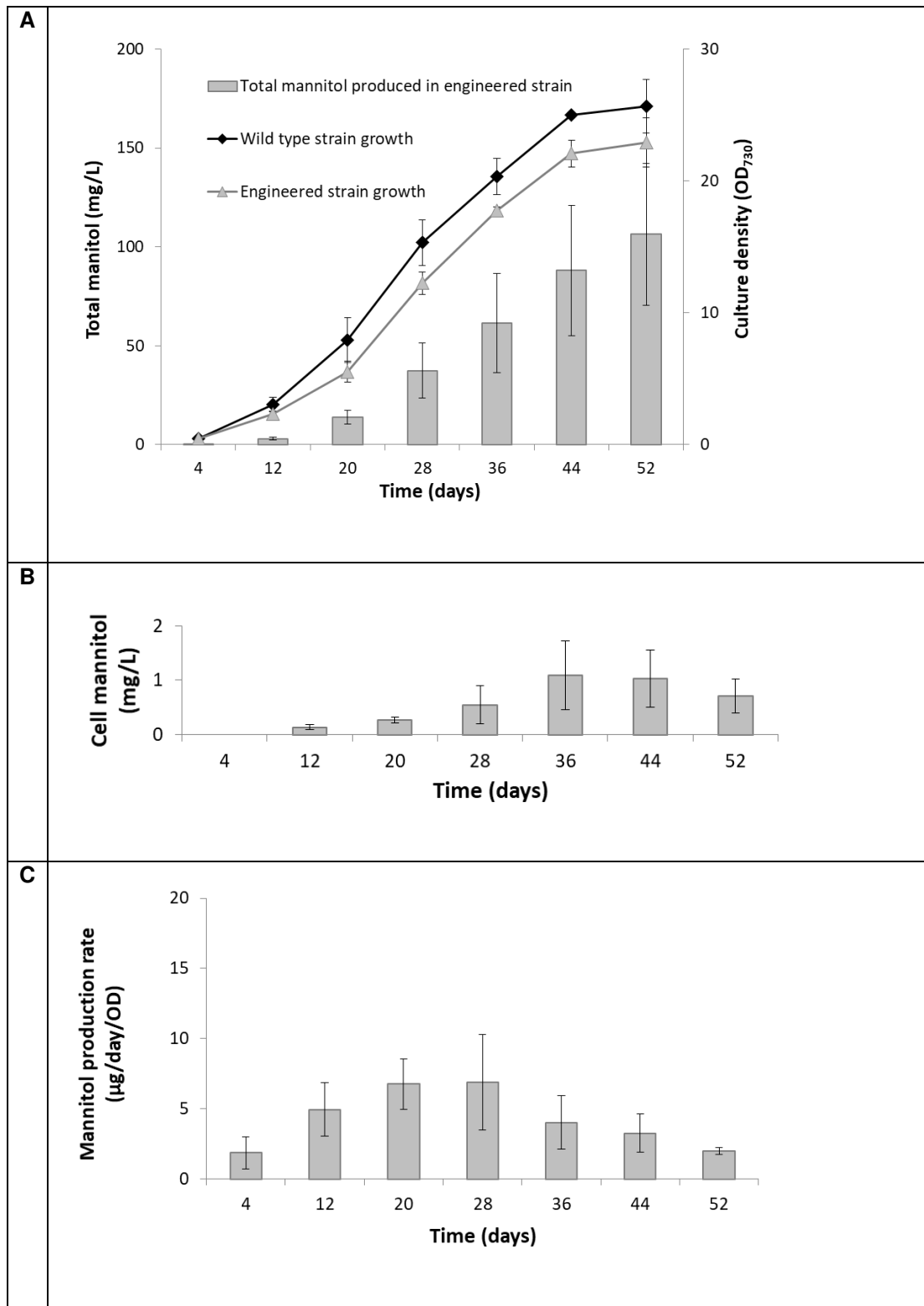
Black points represent culture density (OD<sub>730</sub>). Grey points represent GFP fluorescence normalized to cell density (GFP/OD<sub>730</sub>). RBS3-GFP was expressed in *Synechococcus* under the control of the P<sub>cpcB594</sub> promoter and GFP fluorescence was measured over the course of culture growth. Data are presented as means ± S.E.M. of three independent cultures.

170

171 ***Mpusfus* is functional in *Synechococcus* sp. PCC 7002**

172 *Synechococcus* was transformed with the plasmid containing P<sub>cpcB594</sub>: RBS3-*mpusfus*. Initial  
 173 growth rates were slightly impaired in the engineered *Synechococcus* and the densities of  
 174 stationary phase cultures were slightly lower than in wild type cells (Figure 5A). Mannitol was  
 175 measured in the media and in the cell pellet of wild type- and *mpusfus*-expressing cultures.  
 176 No mannitol was detected in wild type cultures, confirming a previous report that  
 177 *Synechococcus* does not naturally produce mannitol<sup>23</sup>. By contrast, mannitol was detected  
 178 both in the cellular fraction and in the media of *mpusfus*-expressing cultures, indicating that  
 179 the fusion protein is functional in *Synechococcus*. Total mannitol steadily increased over the  
 180 course of culture growth to around 100 mg/L in early stationary phase (Figure 5A). Mannitol in  
 181 the cellular fraction of the culture increased with culture growth (Figure 5B), but the majority of  
 182 mannitol produced was exported into the medium. At the end of the experiment over 99% of  
 183 the total mannitol was found in the culture medium.

184 Mannitol production rates were calculated by dividing the amount of mannitol produced  
185 between two time points by time and by mean cell number ( $OD_{730}$ ) in this time period. As  
186 shown in Figure 5C the production rate increased to  $7 \mu\text{g/day/OD}$  on day 28 (mid exponential  
187 phase) and then decreased.



**Figure 5. Mannitol production in *mpusfus*-expressing *Synechococcus* cultures.**

A: Line graphs represent culture growth as optical density at 730 nm (OD<sub>730</sub>) measured over time in wild type (black symbols) and engineered *Synechococcus* (grey triangles) expressing *mpusfus* under the control of P<sub>cpcB594</sub> and RBS3. Grey bars represent total mannitol (sum of mannitol measured in the cellular fraction and in the medium).

B: Amount of mannitol (per 1 litre culture) present in the cellular fraction of the engineered *Synechococcus* culture.

C: Mannitol production rate in the engineered *Synechococcus* culture normalised to OD<sub>730</sub>.

All data are means  $\pm$  S.E.M. of three independent cultures.

188

189 In summary, the results show that expression of a single fusion gene encoding two enzyme  
190 functions results in the production of mannitol in heterologous systems that do not naturally  
191 synthesize mannitol,

192 **Discussion**

193 ***Use of fusion genes for biotechnology***

194 Fusion genes encode more than one enzymatic function in one gene. They have potential  
195 benefits for biotechnology because they would reduce the number of constructs and  
196 transformations required for engineering metabolic pathways. Furthermore, it is hoped that, in  
197 a naturally evolved fusion protein, occurrence of adjacent modules catalyzing consecutive  
198 steps in a metabolic process will prevent loss of intermediate through efficient substrate  
199 channeling. However, functionality of fusion genes/proteins in heterologous systems  
200 remained to be proven. In this study, we show that a fusion gene from the green alga  
201 *Micromonas pusilla* is functional and sufficient to achieve production of mannitol in prokaryotic  
202 systems, namely *E. coli* and *Synechococcus* sp. PCC 7002.

203 *M. pusilla* strain CCMP1545 is a unicellular marine green alga that is a potentially valuable  
204 source of fusion genes for biotechnology. A high quality draft of its 22 Mb genome is available  
205 and resolution continues to improve around the telomeric regions of its 19 chromosomes<sup>34-35</sup>.  
206 Bioinformatic analyses have identified a number of fusion genes in the CCMP1545 genome  
207 encoding enzymes involved in a variety of cellular processes including pigment production<sup>36</sup>,

208 polyamine biosynthesis<sup>37</sup>, DNA double-strand break repair<sup>38</sup> and carbon fixation<sup>35</sup>. Some  
209 *Micromonas* genes have been characterized by heterologous expression to date<sup>39-42</sup>, but  
210 none of them encode fusion proteins. Therefore, the potential of the *Micromonas* fusion  
211 genes for metabolic engineering remained to be explored. The gene employed in this study  
212 combined a M1PDH and an M1Pase module and was tested for producing mannitol in  
213 prokaryotic model systems.

214 We first expressed the *mpusfus* fusion gene in *E. coli*. This organism has been previously  
215 engineered to develop whole-cell biotransformation systems for mannitol synthesis from  
216 glucose<sup>43-44</sup>, resulting in mannitol molar yield of 80%<sup>43</sup> and 87%<sup>44</sup>. We found that *E. coli*  
217 expressing the single *mpusfus* fusion gene produced a single recombinant protein of  
218 approximately 100 kDa and produced mannitol, most of which was exported into the medium.  
219 This shows that the fusion protein is functional in *E. coli*. However, the achieved mannitol  
220 concentration of 218 mg/L under our experimental conditions (molar yield of 2% on glucose)  
221 was considerably lower than in the previously engineered strains<sup>43-44</sup>. Additional  
222 manipulations addressing codon usage, metabolic flux towards substrate, substrate transport  
223 and feeding could now be attempted to increase mannitol titres in *E. coli*.

224 Photosynthetic bacteria provide an opportunity to produce organic compounds from CO<sub>2</sub>  
225 without the need of feeding sugars. We were therefore interested to test whether the *mpusfus*  
226 fusion gene can be used to produce mannitol in a photo-autotrophic system. *Synechococcus*  
227 sp. PCC 7002 is one of the model systems for metabolic engineering of cyanobacteria and  
228 has previously been engineered to produce mannitol from F6P using two separate genes  
229 (M1PDH from *E. coli* and M1Pase from *Eimeria tenella*)<sup>23</sup>. As *E. coli*, *Synechococcus* does  
230 not possess an endogenous pathway for mannitol production or breakdown. To facilitate the  
231 engineering of *Synechococcus*, we first generated a BioBrick compatible vector and a  
232 synthetic ribosomal binding site. The new tools in combination with a previously identified  
233 'super-strong' promoter were confirmed by GFP-assay and are available to the scientific  
234 community.

235 Successful functional expression of *mpusfus* in *Synechococcus* was proven by the  
236 appearance of mannitol in the transgenic cultures. As before<sup>23</sup>, the majority of mannitol

237 produced was exported into the media where it can easily be harvested. Since  
238 *Synechococcus* does not naturally produce mannitol it is likely that the export occurs through  
239 non-specific transport proteins for other compounds. *Synechococcus* and other cyanobacteria  
240 have been shown to release low-molecular-weight metabolites when subjected to hypo-  
241 osmotic stress <sup>45-46</sup>, but the exact transport pathways remain to be identified.

242 The total amount of mannitol produced in this report is considerably lower than in the previous  
243 study <sup>23</sup>, namely around 0.1 g/L compared to 0.6 g/L. Usage of a glycogen-deficient strain  
244 helped to increase titres <sup>23</sup>, but the main differences between the two studies lies in the  
245 growth rate of the cultures. The highest mannitol-producing strain reported previously <sup>23</sup>  
246 reached a maximal OD<sub>730</sub> of around 10 within 150 h and achieved the aforementioned  
247 mannitol concentration in 300 h. By contrast, our strain grew much more slowly and required  
248 50 days to produce 0.1 g/L mannitol albeit reaching a higher OD<sub>730</sub> of 25. It is likely that  
249 protein turnover over such a long period of time prevents the accumulation and maintenance  
250 of substantial amounts of recombinant protein. Protein synthesis then becomes very sensitive  
251 to promoter activity, which was maximal during early culture growth (see Fig. 4). A  
252 combination of low promoter activity and protein turnover would explain why production rates  
253 were very low and decreased even before the cultures entered stationary phase (see Fig.  
254 5C). It can therefore be expected that usage of new promoter(s), which are active during the  
255 late stages of growth, and changes in growth conditions, *e.g.* fed-batch cultivation to keep  
256 cultures in the production stage for longer, could increase mannitol productivity by engineered  
257 *Synechococcus* sp. PCC 7002.

258 Despite the ability of cyanobacteria to use atmospheric carbon for industrial bioproduction,  
259 carbon availability is a key limiting factor in polyol production. The use of fast-growing strains  
260 and the development of efficient photo-bioreactors will be crucial to move toward industrial  
261 scale production systems that can compete with the traditional sugar-fed cultures. Our  
262 demonstration that an algal fusion gene is functional in cyanobacteria presents an important  
263 step towards simplifying the generation of recombinant metabolic pathways, and can now be  
264 combined with the usual metabolic engineering strategies to overcome metabolic bottlenecks.



265 Metabolic network models have become available for *Synechococcus*<sup>47</sup>, which should  
266 facilitate this task.

267

## 268 **Methods**

### 269 ***Construction of pAQ1BB vector for transformation of Synechococcus sp. PCC 7002***

270 The integrative vector pAQ1EX for transformation of *Synechococcus* sp. PCC 7002<sup>30</sup> was  
271 modified to accommodate BioBrick assembly. Synthetic oligonucleotides were annealed to  
272 generate BioBrick prefix and suffix sequences flanked by restriction site overhangs for  
273 insertion into pAQ1EX using *EcoRI/NcoI* and *NdeI/BamHI* restriction sites respectively (Table  
274 S1). To prevent interference with the BioBrick assembly, an *XbaI* restriction site at the 5' end  
275 of the spectinomycin resistance gene *aadA* was replaced with an *XhoI* restriction site by site-  
276 directed mutagenesis using primers listed in Table S1. Modifications were confirmed by  
277 sequencing and the resulting vector was designated pAQ1BB. To drive transgene expression,  
278 the 594 bp phycocyanin promoter of *Synechocystis* sp. PCC 6803, P<sub>cpcB594</sub><sup>30</sup> was amplified  
279 by PCR from genomic DNA using forward primer 5'-  
280 GAATTCGCGGCCGCTTCTAGAGTTCGTTATAAAATAAACTTAACAAATCTATAC-3' and reverse primer 5'-  
281 CTGCAGCGGCCGCTACTAGTAGGAATTAATCTCCTACTTGACTTTATG-3', and inserted into the pAQ1BB  
282 BioBrick cloning site.

### 283 ***Generation of codon-optimized mpusfus***

284 The 863 aa M1PDH/M1Pase fusion protein of *Micromonas pusilla* strain CCMP1545  
285 (<http://bioinformatics.psb.ugent.be/orcae/annotation/Mipuc/current/Mipuc10g00620>) contains  
286 codons that may reduce translation efficiency in cyanobacteria. Codon optimization for  
287 expression in *Synechococcus* sp. PCC 7002 was therefore performed (GenScript USA Inc.)  
288 using the OptimumGene™ algorithm, which takes into consideration a variety of parameters  
289 important for gene expression efficiency. These include, but are not limited to, codon usage  
290 bias, GC content, mRNA secondary structure, internal ribosome binding sites and restriction

291 sites. Codon usage bias is scored as a codon adaptation index (CAI), where CAI 1.0 is  
292 optimal in the desired expression organism, and CAI > 0.8 is regarded as good in terms of  
293 high expression. Following codon optimization, the CAI was improved from 0.66 in the native  
294 gene to 0.96 in the codon-optimized gene (Supplementary data file 1, Supplemental Figure  
295 1a). GC content and distribution was also optimized to prolong the half-life of the mRNA, and  
296 stem-loop secondary structures were broken to improve mRNA stability and ribosomal  
297 binding. Five internal ribosome binding sites were removed to prevent any aberrant  
298 translation from within the coding sequence. Finally, six restriction sites involved in BioBrick  
299 assembly were removed. The resulting codon-optimized gene was designated *mpusfus*.  
300 Details of codon optimization, native and optimized sequences can be found in  
301 Supplementary data file 1.

### 302 ***Generation of transgenic Synechococcus sp. PCC 7002 strains***

303 Synthetic ribosome binding sites were designed using the Salis lab RBS calculator<sup>33</sup> and  
304 added directly upstream of the transgene during PCR amplification (primer sequences can be  
305 found in Table S2). Following sequence confirmation in the pGEM-T® Easy (Promega, UK)  
306 vector, the amplified DNA (RBS + gene) was cloned into the pAQ1BB vector, downstream of  
307 the P<sub>cpcB594</sub> promoter. The synthetic expression constructs were integrated into the  
308 *Synechococcus* genome by natural transformation. Transformation efficiency was optimised  
309 by varying either amount of DNA (1-25 µg) or incubation time (1-3 days) prior to plating on  
310 selective media (Figure S1) and the following optimised transformation protocol was used: 1.5  
311 mL culture (OD<sub>730</sub> 1) was combined with 10 µg circular plasmid DNA and incubated for 72 h  
312 under standard growth conditions with minimal sparging. Cells were plated on solid A+  
313 medium with 1.5% w/v agar and 50 µg/ml spectinomycin. Single colonies appeared after 5-7  
314 days. Individual colonies were isolated and grown for characterization. Genomic DNA was  
315 isolated using phenol-chloroform extraction<sup>48</sup>, and the correct insertion of the synthetic  
316 expression constructs were verified by PCR amplification using primers pAQ1BB-seq-F (5'-  
317 CACATGAGAATTTGTCCAG-3') and pAQ1BB-seq-R (5'-CCTTTCGGGCTTTGTTAG-3') and  
318 sequencing.

**319 *Synechococcus sp. PCC 7002 growth***

320 *Synechococcus sp. PCC 7002* cultures were grown in A+ medium<sup>18</sup> (containing 300 mM  
321 NaCl) at 30 °C with photoperiod 16 h/8 h light/dark, light intensity 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$   
322 and sparged with humidified ambient air (standard conditions). Cultures of transformed  
323 *Synechococcus* were supplemented with 25  $\mu\text{g/ml}$  spectinomycin. For mannitol analyses,  
324 cultures were grown to OD<sub>730</sub> 1-3, diluted to OD<sub>730</sub> 1 and 1.3 ml was inoculated to 400 ml A+  
325 medium. At regular intervals throughout culture growth, OD<sub>730</sub> was measured and 20 ml of  
326 culture was harvested by centrifugation at 4,000 *g* for 15 min. Supernatant were transferred to  
327 fresh tubes and stored at -20 °C. Cells were resuspended in 3 ml fresh Medium A+,  
328 transferred to a fresh 15 ml Falcon tube, centrifuged at 4,000 *g* for 15 min at 4 °C,  
329 supernatants were discarded and pellets were frozen at -20 °C. For subsequent analysis,  
330 cells were freeze-dried overnight at -50 °C and 0.13 millibar, and re-suspended in 1 ml of  
331 Tris-HCl 10 mM pH 8.0. After five freeze-thaw cycles, cell suspensions were sonicated (5 x  
332 30 sec) using an ultrasonic processor VC50 sonicator (Jencons) and cell debris pelleted by  
333 centrifugation 15 min at .12,500 *g*. Aliquots of the cell lysates were used for determination of  
334 intracellular mannitol concentration.

**335 *Generation of transgenic E. coli strain***

336 *Mpusfus* DNA without the initial start codon was amplified from the plasmid carrying the  
337 codon-optimized gene with the forward primer  
338 5'-GGGGGGGGATCCAATAGTGCCGTGACCATCAGCC-3' (*Bam*HI restriction site underlined) and the  
339 reverse primer 5'-CCCCCGAAATTCTTAGCGGGGATTGGGATCTTC-3' (*Eco*RI restriction site  
340 underlined). The PCR fragment obtained was cloned into the vector pFO4 as previously  
341 described<sup>49</sup>, producing the plasmid pMPUSFUS and allowing the recombinant proteins to be  
342 fused to a six-histidine tag at their N-terminus. This plasmid was subsequently transformed in  
343 *E. coli* expression strain BL21 (DE3). For gene induction and functional assays, three distinct  
344 recombinant clones were pre-cultured in 5 ml of M9 medium supplemented with 10 g/L of  
345 glucose and 0.1 g/L ampicillin overnight at 37 °C and 200 rpm. The following day, new  
346 cultures were started at OD<sub>600</sub> 0.1 under the same conditions. When OD<sub>600</sub> 0.5 was attained,  
347 cultures were divided into two tubes, and isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was

348 added in one of them at a final concentration of 1 mM. Incubation was carried out for 20 h at  
349 25 °C and 200 rpm, and cultures reached a final OD<sub>600</sub> of 2.5-3.0 in both induced and non-  
350 induced conditions. Samples were then harvested by centrifugation at 3,500 *g* for 10 min.  
351 Supernatants and cell pellets were frozen individually at -20 °C for downstream analyses. To  
352 determine intracellular mannitol concentration, cells were re-suspended in 600 µl of buffer  
353 Tris-HCl 25 mM pH 7.5 and NaCl 200 mM, and disrupted by four pulses of 15 sec of  
354 sonication using an ultrasonic processor VC50 sonicator (Jencons). Cell debris were pelleted  
355 by centrifugation at 14,000 *g* and 4 °C for 20 min; supernatants were used for mannitol  
356 analysis and considered as soluble fractions for protein gel and Western-blot analysis.  
357 Proteins were quantified using the Pierce™ Coomassie Plus Assay Kit (ThermoFischer  
358 Scientific) according to manufacturer's instructions. Three µg of proteins of each fractions were  
359 loaded on a 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad). After separation,  
360 proteins were transferred to nitrocellulose membrane using the iBlot 2 Dry blotting System  
361 (ThermoFischer Scientific). Recombinant proteins were visualized with a conjugated  
362 monoclonal anti-polyhistidine-peroxidase antibody (Sigma-Aldrich) detected by the  
363 SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFischer Scientific).

#### 364 ***Measurement of GFP***

365 RBS and promoter activity was assessed in transgenic cyanobacteria using a GFP reporter  
366 encoded by BioBrick part BBa\_E0040. Cultures were harvested for GFP measurements at  
367 the same point during the diurnal cycle and, when appropriate, growth stage. Optical density  
368 was adjusted to OD<sub>730</sub> 0.25-0.30 in fresh A+ media and fluorescence was measured using a  
369 LS 55 Luminescence Spectrophotometer (PerkinElmer, UK) using 480 nm excitation and 514  
370 nm emission wavelengths.

#### 371 ***Mannitol analysis***

372 To determine mannitol production, 50-200 µl aliquots of culture media or bacterial extracts were  
373 analyzed with the D-Mannitol/L-Arabitol assay kit (K-MANOL, Megazyme) according to the  
374 manufacturer's instructions.

375

376 **Associated content**

377 ***Supporting information***

378 Details for codon optimization of *Micromonas pusilla* M1PDH/M1Pase fusion gene (PDF)

379 List of primers used for pAQ1BB construction and to generate RBS+gene constructs (XLSX)

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388 ***Author contributions***

389 MAM, AA, and TT conceived the study. Experimental data were generated by MAM, SS, and

390 TT. All authors analyzed data. MAM, AA, and TT wrote the manuscript.

391 ***Notes***

392 The authors declare no competing financial interest.

393

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401 **References**

- 402 1. Singh, R., et al., Parihar, P., Singh, M., Bajguz, A., Kumar, J., Singh, S., Singh, V.P., and  
403 Prasad, S.M. (2017) Uncovering potential applications of cyanobacteria and algal metabolites  
404 in biology, agriculture and medicine: Current status and future prospects.  
405 *Front. Microbiol.* 8, 515.
- 406 2. Gustavsson, M., and Lee, S.Y. (2016) Prospects of microbial cell factories developed  
407 through systems metabolic engineering. *Microb. Biotechnol.* 9, 610-617.
- 408 3. Jeschek, M., Gerngross, D., and Panke, S. (2017) Combinatorial pathway optimization for  
409 streamlined metabolic engineering. *Curr. Opin. Biotechnol.* 47, 142-151.
- 410 4. Chae, T.U., Choi, S.Y., Kim, J.W., Ko, Y.S., and Lee, S.Y. (2017) Recent advances in  
411 systems metabolic engineering tools and strategies. *Curr. Opin. Biotechnol.* 47, 67-82.
- 412 5. Nielsen, J., Archer, J., Essack, M., Bajic, V.B., Gojobori, T., and Mijakovic, I. (2017)  
413 Building a bio-based industry in the Middle East through harnessing the potential of the Red  
414 Sea biodiversity. *Appl. Environ. Microbiol.* 101, 4837-4851.
- 415 6. Jensen, M.K., and Keasling, J.D. (2015) Recent applications of synthetic biology tools for  
416 yeast metabolic engineering. *FEMS Yeast Res.* 15, 1-10.
- 417 7. Miles, E.W., Rhee, S., and Davies, D.R. (1999) The molecular basis of substrate  
418 channeling. *J. Biol. Chem.* 274, 12193-12196.
- 419 8. Dai, Y., Meng, Q., Mu, W., and Zhang, T. (2017) Recent advances in the applications and  
420 biotechnological production of mannitol. *J. Funct. Foods* 36, 404-409.
- 421 9. Oddo, E., Saiano, F., Alonzo, G., Bellini, E. (2002) An investigation of the seasonal pattern  
422 of mannitol content in deciduous and evergreen species of the oleaceae growing in northern  
423 Sicily. *Ann. Bot.* 90, 239-243.
- 424 10. Saha, B.C., and Racine, F.M. (2011) Biotechnological production of mannitol and its  
425 applications. *Appl. Microbiol. Biotechnol.* 89, 879-891.
- 426 11. Hays, S.G., and Ducat, D.C. (2015) Engineering cyanobacteria as photosynthetic  
427 feedstock factories. *Photosynth. Res.* 123, 285-295.
- 428 12. Hagemann, M. and Hess, W.R., (2018) Systems and synthetic biology for the  
429 biotechnological application of cyanobacteria. *Curr. Opin. Biotechnol.* 49, 94-99.

- 430 13. Cassier-Chauvat, C., Dive, V., and Chauvat, F. (2017) Cyanobacteria: photosynthetic  
431 factories combining biodiversity, radiation resistance, and genetics to facilitate drug discovery.  
432 *Appl. Environ. Microbiol.* 101, 1359-1364.
- 433 14. Al-Haj, L., Lui, Y.T., Abed, R.M., Gomaa, M.A., and Purton, S. (2016) Cyanobacteria as  
434 chassis for industrial biotechnology: Progress and prospects. *Life* 6, E42.
- 435 15. Minas, K., Karunakaran, E., Bond, T., Gandy C., Honsbein, A., Madsen, M., Amezaga, J.,  
436 Amtmann, A., Templeton, M.R., Biggs, C.A., and Lawton, L. (2015) Biodesalination: an  
437 emerging technology for targeted removal of Na<sup>+</sup> and Cl<sup>-</sup> from seawater by cyanobacteria.  
438 *Desalin. Water Treat.* 55, 2647-2668.
- 439 16. Nomura, C.T., Sakamoto, T., and Bryant, D.A. (2006) Roles for heme-copper oxidases in  
440 extreme high-light and oxidative stress response in the cyanobacterium *Synechococcus* sp.  
441 PCC 7002. *Arch. Microbiol.* 185, 471-479.
- 442 17. Van Baalen, C. (1962) Studies on marine blue-green algae. *Bot. Mar.* 4, 129-139.
- 443 18. Stevens, S.E., and Porter, R.D. (1980) Transformation in *Agmenellum quadruplicatum*.  
444 *Proc. Natl. Acad. Sci. U. S. A.* 77, 6052-6056.
- 445 19. Frigaard, N.U., Sakuragi, Y., and Bryant, D.A. (2004) Gene inactivation in the  
446 cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium*  
447 *tepidum* using in vitro-made DNA constructs and natural transformation. *Methods Mol. Biol.*  
448 274, 325-340.
- 449 20. Ruffing, A.M., Jensen, T.J., and Strickland, L.M. (2016) Genetic tools for advancement of  
450 *Synechococcus* sp. PCC 7002 as a cyanobacterial chassis. *Microb. Cell Fact.* 15, 190.
- 451 21. Markley, A.L., Begemann, M.B., Clarke, R.E., Gordon, G.C., and Pflieger, B.F. (2015)  
452 Synthetic biology toolbox for controlling gene expression in the cyanobacterium  
453 *Synechococcus* sp. strain PCC 7002. *ACS Synth. Biol.* 4, 595-603.
- 454 22. Zess, E.K., Begemann, M.B., and Pflieger, B.F. (2016) Construction of new synthetic  
455 biology tools for the control of gene expression in the cyanobacterium *Synechococcus* sp.  
456 strain PCC 7002. *Biotechnol. Bioeng.* 113, 424-432.
- 457 23. Jacobsen, J.H., and Frigaard N.U. (2014) Engineering of photosynthetic mannitol  
458 biosynthesis from CO<sub>2</sub> in a cyanobacterium. *Metab. Eng.* 21, 60-70.

- 459 24. Michel, G., Tonon, T., Scornet, D., Cock, J.M., and Kloareg, B. (2010) Central and storage  
460 carbon metabolism of the brown alga *Ectocarpus siliculosus*: insights into the origin and  
461 evolution of storage carbohydrates in Eukaryotes. *New Phytol.* 188, 67-81.
- 462 25. Bonin, P., Groisillier, A., Raimbault, A., Guibert, A., Boyen, C., and Tonon T. (2015)  
463 Molecular and biochemical characterization of mannitol-1-phosphate dehydrogenase from the  
464 model brown alga *Ectocarpus* sp. *Phytochemistry* 117, 509-20.
- 465 26. Groisillier, A., Shao, Z., Michel, G., Goulitquer, S., Bonin, P., Krahulec, S., Nidetzky, B.,  
466 Duan, D., Boyen, C., and Tonon, T. (2014) Mannitol metabolism in brown algae involves a  
467 new phosphatase family. *J. Exp. Bot.* 65, 559-570.
- 468 27. Rousvoal, S., Groisillier, A., Dittami, S.M., Michel, G., Boyen, C., and Tonon, T. (2011)  
469 Mannitol-1-phosphate dehydrogenase activity in *Ectocarpus siliculosus*, a key role for  
470 mannitol synthesis in brown algae. *Planta* 233, 261-273.
- 471 28. Tonon, T., Li, Y., and McQueen-Mason, S. (2017) Mannitol biosynthesis in algae: more  
472 widespread and diverse than previously thought. *New Phytol.* 213, 1573-1579.
- 473 29. Shetty, R.P., Endy, D., and Knight, T.F.J. (2008) Engineering BioBrick vectors from  
474 BioBrick parts. *J. Biol. Eng.* 2, 5.
- 475 30. Xu, Y., Alvey, R.M., Byrne, P.O., Graham, J.E., Shen, G., and Bryant, D.A. (2011)  
476 Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for  
477 high-level gene expression in *Synechococcus* sp. PCC 7002. *Methods Mol. Biol.* 684, 273-  
478 293.
- 479 31. Zhou, J., Zhang, H., Meng, H., Zhu, Y., Bao, G., Zhang, Y., Li, Y., and Ma, Y. (2014)  
480 Discovery of a super-strong promoter enables efficient production of heterologous proteins in  
481 cyanobacteria. *Sci. Rep.* 4, 4500.
- 482 32. Reeve, B., Hargest, T., Gilbert, C., and Ellis, T. (2014) Predicting translation initiation  
483 rates for designing synthetic biology. *Front. Bioeng. Biotechnol.* 2, 1.
- 484 33. Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009) Automated design of synthetic ribosome  
485 binding sites to control protein expression. *Nat. Biotechnol.* 27, 946-950.
- 486 34. van Baren. M.J., Bachy, C., Reistetter, E.N., Purvine, S.O., Grimwood, J., Sudek, S., Yu,  
487 H., Poirier, C., Deerinck, T.J., Kuo, A., et al. (2016) Evidence-based green algal genomics  
488 reveals marine diversity and ancestral characteristics of land plants. *BMC Genomics* 17, 267.



- 489 35. Worden, A.Z., Lee, J.H., Mock, T., Rouzé, P., Simmons, M.P., Aerts, A.L., Allen, A.E.,  
490 Cuvelier, M.L., Derelle, E., Everett, M.V., et al. (2009) Green evolution and dynamic  
491 adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*. *Science* 324,  
492 268-272.
- 493 36. Blatt, A., Bauch, M.E., Pörschke, Y., and Lohr, M. (2015) A lycopene  $\beta$ -cyclase/lycopene  
494  $\epsilon$ -cyclase/light-harvesting complex-fusion protein from the green alga *Ostreococcus*  
495 *lucimarinus* can be modified to produce  $\alpha$ -carotene and  $\beta$ -carotene at different ratios. *Plant J.*  
496 82, 582-595.
- 497 37. Green, R., Hanfrey, C.C., Elliott, K.A., McCloskey, D.E., Wang, X., Kanugula, S., Pegg,  
498 A.E., and Michael, A.J. (2011) Independent evolutionary origins of functional polyamine  
499 biosynthetic enzyme fusions catalyzing de novo diamine to triamine formation. *Mol. Microbiol.*  
500 81, 1109-1124.
- 501 38. Yoshida, T., Claverie, J.-M., and Ogata, H. (2011) Mimivirus reveals Mre11/Rad50 fusion  
502 proteins with a sporadic distribution in eukaryotes, bacteria, viruses and plasmids. *Virology* 8,  
503 427-437.
- 504 39. Shi, H., Chen, H., Gu, Z., Zhang, H., Chen, W., and Chen, Y.Q. (2016) Application of a  
505 delta-6 desaturase with  $\alpha$ -linolenic acid preference on eicosapentaenoic acid production in  
506 *Mortierella alpina*. *Microb. Cell Fact.* 15, 117.
- 507 40. Duanmu, D., Bachy, C., Sudek, S., Wong, C.H., Jiménez, V., Rockwell, N.C., Martin, S.S.,  
508 Ngan, C.Y., Reistetter, E.N., van Baren M.J., et al. (2014) Marine algae and land plants  
509 share conserved phytochrome signaling systems. *Proc. Natl. Acad. Sci. U. S. A.* 111, 15827-  
510 15832.
- 511 41. Petrie, J.R., Shrestha, P., Mansour, M.P., Nichols, P.D., Liu, Q., and Singh, S.P. (2010)  
512 Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids into plants using an  
513 acyl-CoA  $\Delta 6$ -desaturase with  $\omega 3$ -preference from the marine microalga *Micromonas pusilla*.  
514 *Metab. Eng.* 12, 233-240.
- 515 42. Lada, A.G., Krick, C.F., Kozmin, S.G., Mayorov, V.I., Karpova, T.S., Rogozin, I.B., and  
516 Pavlov, Y.I. (2011) Mutator effects and mutation signatures of editing deaminases produced  
517 in bacteria and yeast. *Biochemistry (Moscow)* 76, 131-146.

- 518 43. Kaup, B., Bringer-Meyer, S., and Sahm, H. (2005) D-mannitol formation from D-glucose in  
519 a whole-cell biotransformation with recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.*  
520 *69*, 397-403.
- 521 44. Reshamwala, S. M., Pagar, S. K., Velhal, V. S., Maranholakar, V. M., Talangkar, V. G.,  
522 and Lali, A. M. (2014) Construction of an efficient *Escherichia coli* whole-cell biocatalyst for D-  
523 mannitol production. *J. Biosci. Bioeng.* *118*, 628-631.
- 524 45. Hagemann, M. (2011) Molecular biology of cyanobacterial salt acclimation. *FEMS*  
525 *Microbiol. Rev.* *35*, 87-123.
- 526 46. Xu, Y., Guerra, L. T., Li, Z., Ludwig, M., Dismukes, G. C., and Bryant, D. A. (2013) Altered  
527 carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC  
528 7002: Cell factories for soluble sugars. *Metab. Eng.* *16*, 56-67.
- 529 47. Gudmundsson, S., Nogales, J. (2015) Cyanobacteria as photosynthetic biocatalysts: a  
530 systems biology perspective. *Mol. BioSyst.* *11*, 60-70.
- 531 48. Tamagnini, P., Troshina, O., Oxelfelt, F., Salema, R., and Lindblad, P. (1997)  
532 Hydrogenases in *Nostoc* sp. strain PCC 73102, a strain lacking a bidirectional enzyme. *Appl.*  
533 *Environ. Microbiol.* *63*, 1801-1807.
- 534 49. Groisillier, A., Hervé, C., Jeudy, A., Rebuffet, E., Pluchon, P.F., Chevolut, Y., Flament, D.,  
535 Geslin, C., Morgado, I.M., Power, D., et al. (2010) MARINE-EXPRESS: taking advantage of  
536 high throughput cloning and expression strategies for the post-genomic analysis of marine  
537 organisms. *Microb. Cell Fact.* *9*, 45.

538