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

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Abstract

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

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

Keywords

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

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

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

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

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

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

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

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

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

Synechococcus has previously been engineered to produce mannitol from fructose-6-phosphate, an intermediate of the Calvin cycle [23]. This recombinant mannitol biosynthetic

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

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

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

Results

Mpusfus is functional in E. coli

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

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

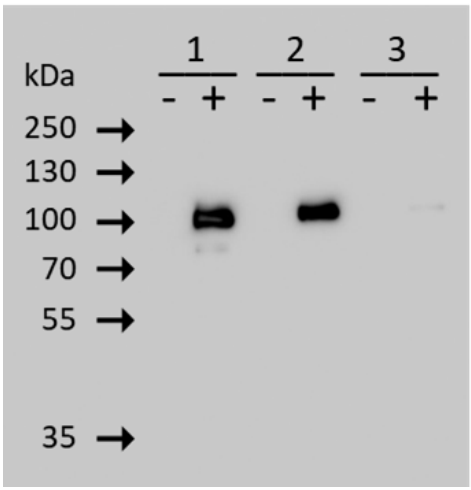

Figure 1. Expression of the M1PDH/M1Pase fusion protein in E. coli

Western-blot analysis of His-tagged M1PDH/M1Pase in soluble fractions of protein extracts from E. coli transformed with mpusfus. Lanes marked 1, 2, and 3 contain protein extracts from three independent clones after culture in absence (-) and in presence (+) of IPTG. Numbers at the left of the panel indicate size (kDa) of proteins in the ladder.

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

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

BioBricks represent the largest collection of standardized parts for genetic engineering [29]. While a number of genetic tools have been developed for Synechococcus [20-22], none of them are compatible with BioBrick parts. In order to develop a BioBrick-compatible integration vector for Synechococcus, we reengineered an existing expression vector, pAQ1EX [30]. This vector targets the transgene to a neutral site in the native, high copy pAQ1 plasmid of Synechococcus. BioBrick prefix and suffix sequences containing the restriction enzyme recognition sites required for gene assembly were synthesized and inserted into the integrative region of the pAQ1EX vector. For ease of cloning, domestication of DNA parts is important whereby BioBrick restriction sites do not occur outside of the prefix and suffix sequences. An XbaI site occurring directly upstream of the selective aadA gene was therefore replaced with an XhoI site. Finally, the phycocyanin promoter of Synechocystis sp. PCC 6803, $P_{cpcB594}$, which has been reported to be a strong promoter in cyanobacteria, was inserted into the BioBrick cloning site [31]. The resulting vector called pAQ1BB provides a convenient tool for transformation of Synechococcus sp. PCC 7002 (Figure 2). The target integration site (“landing pad”) can be modified by replacing homologous sequences Flank A and Flank B using NsiI/EcoRI and SalI/SphI restriction sites respectively. The marker gene for selection can be modified using XhoI and SalI restriction sites.

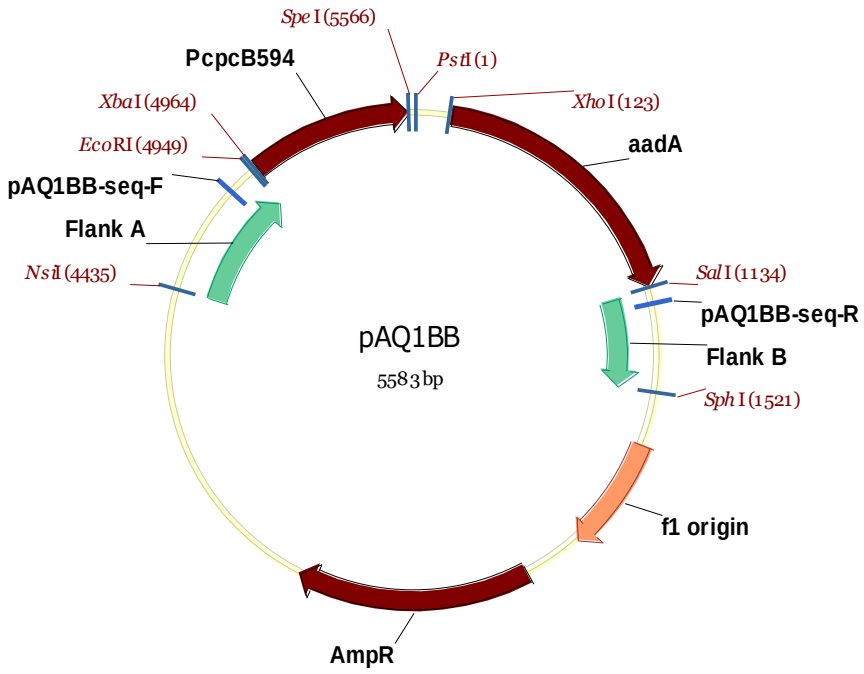

Figure 2. New BioBrick vector for transformation of Synechococcus sp. PCC 7002.

The region between Flanks A and B stably integrates into the native pAQ1 plasmid of Synechococcus by homologous recombination. This region includes a spectinomycin resistance gene (aadA) and the BioBrick cloning site containing the phycocyanin promoter of Synechocystis sp. PCC 6803 ($P_{cpcB594}$).

Using established protocols for natural transformation [30], transformation efficiency of Synechococcus with pAQ1BB was initially poor. Increasing both the amount of DNA and incubation time prior to plating onto selective media significantly enhanced transformation efficiency (Figure S1). The optimized transformation protocol can be found in the Methods section.

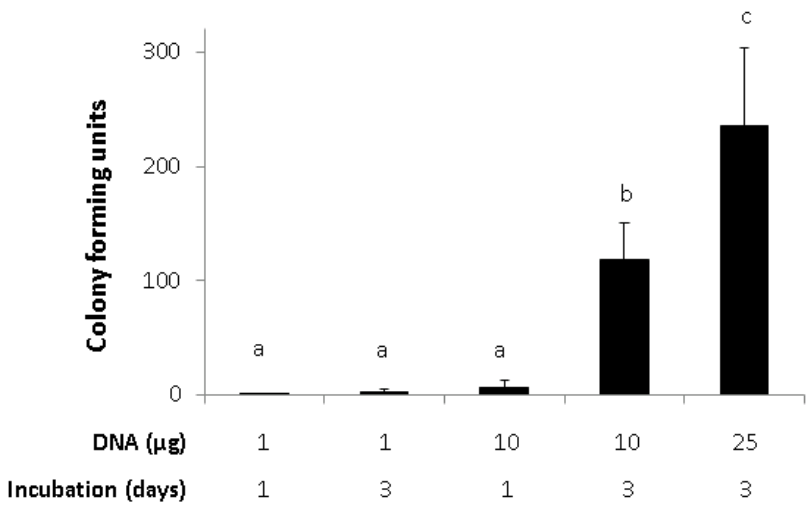

Figure S1. Optimization of Synechococcus sp. PCC 7002 natural transformation.

Synechococcus sp. PCC 7002 cultures were grown to an OD_{730} of 1, transformed with variable amounts of DNA, and incubated for different lengths of time (indicated along the x-axis) before plating onto selective media. Colonies were counted after 12 days of incubation at 30 ℃, 16 h/8 h day-night cycle with 150 µmol photons m^{-2} s^{-1} light intensity. Bars represent means ± S.E.M. of three independent experiments. Letters indicate statistical differences determined by Tukey's test following ANOVA ($p<0.005$).

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

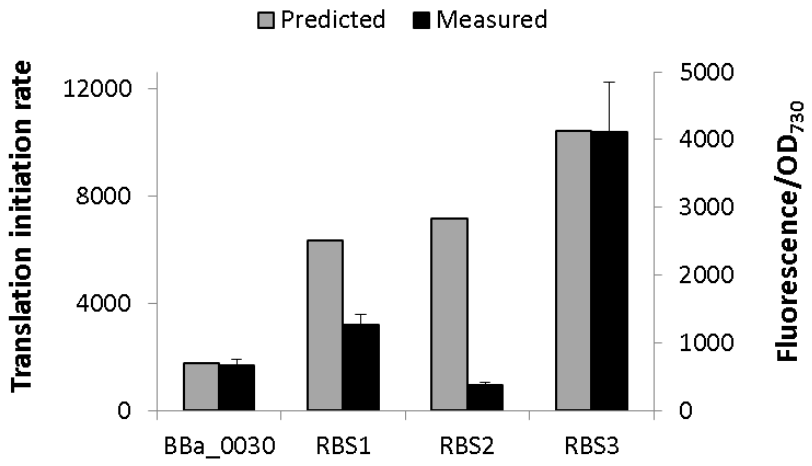

Figure 3. Predicted and measured strength of synthetic ribosome binding sites for Synechococcus sp. PCC 7002.

Grey bars represent translation initiation rates predicted in silico using RBS calculator. Black bars represent GFP fluorescence normalised to cell density (optical density at 730 nm, OD_{730}). Each RBS-GFP construct was expressed in Synechococcus under the control of the $P_{cpcB594}$ promoter and GFP fluorescence was measured when culture density reached an OD_{730} of 1. Data are presented as means ± S.E.M. of three independent cultures. BBa_0030 is a standard RBS from the Registry of Standardised Parts. RBS1, RBS2 and RBS3 were forward engineered using RBS calculator.

To assess promoter activity in combination with RBS3, we monitored GFP fluorescence in the $P_{cpcB594}$:RBS3-GFP expressing cells over the course of culture growth. The experiment showed that the promoter is most active in the early exponential phase (Figure 4) as previously suggested [30].

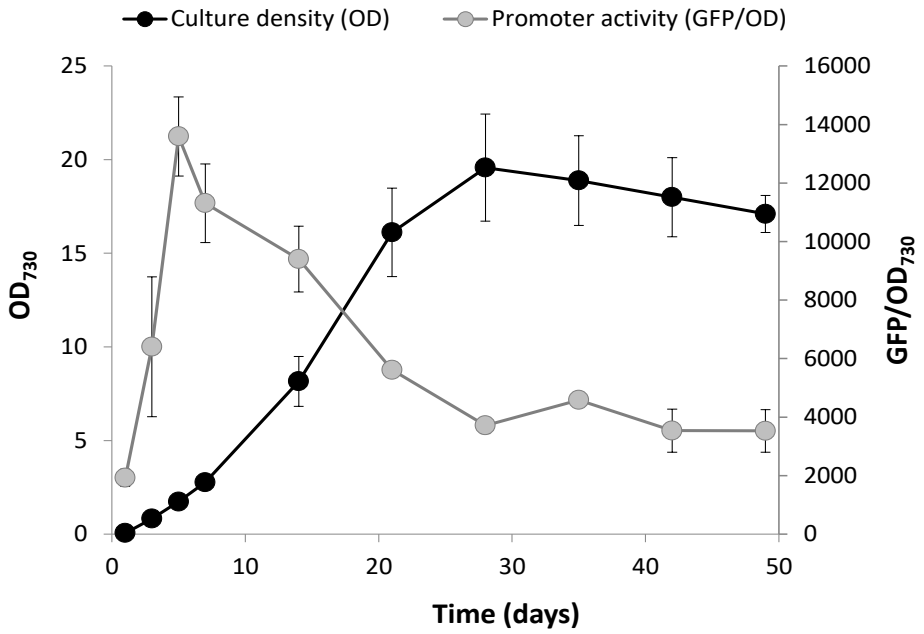

Figure 4. Promoter activity over the course of culture growth

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

Mpusfus is functional in Synechococcus sp. PCC 7002

Synechococcus was transformed with the plasmid containing $P_{cpcB594:}$ RBS3-mpusfus. Initial growth rates were slightly impaired in the engineered Synechococcus and the densities of stationary phase cultures were slightly lower than in wild type cells (Figure 5A). Mannitol was measured in the media and in the cell pellet of wild type- and mpusfus-expressing cultures. No mannitol was detected in wild type cultures, confirming a previous report that Synechococcus does not naturally produce mannitol [23]. By contrast, mannitol was detected both in the cellular fraction and in the media of mpusfus-expressing cultures, indicating that the fusion protein is functional in Synechococcus. Total mannitol steadily increased over the course of culture growth to around 100 mg/L in early stationary phase (Figure 5A). Mannitol in the cellular fraction of the culture increased with culture growth (Figure 5B), but the majority of mannitol produced was exported into the medium. At the end of the experiment over 99% of 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 μg/day/OD on day 28 (mid exponential
187 phase) and then decreased.

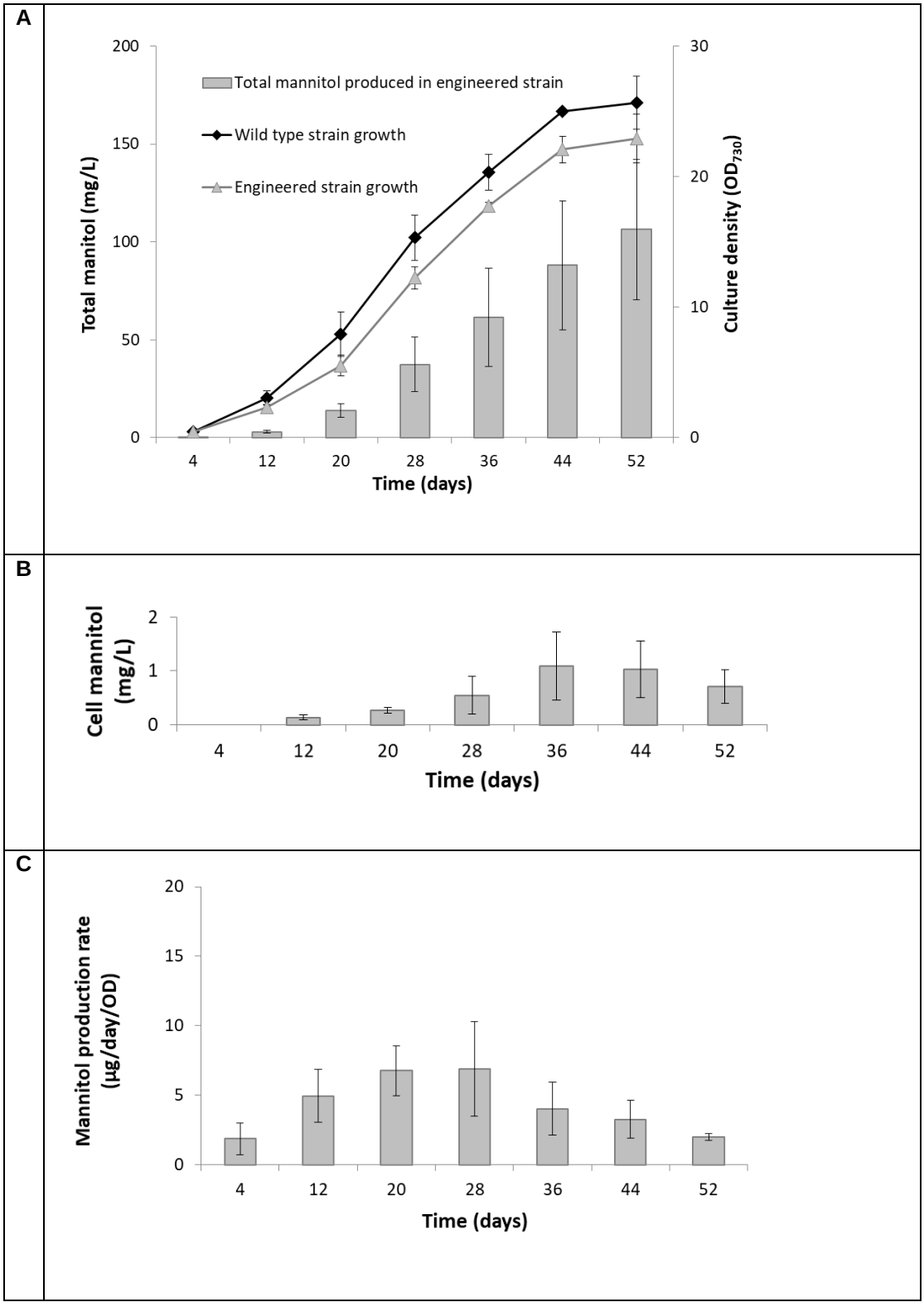

Figure 5. Mannitol production in mpusfus-expressing Synechococcus cultures.

A: Line graphs represent culture growth as optical density at 730 nm (OD_{730}) measured over time in wild type (black symbols) and engineered Synechococcus (grey triangles) expressing mpusfus under the control of $P_{cpcB594}$ 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_{730}.

All data are means ± S.E.M. of three independent cultures.

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

Discussion

Use of fusion genes for biotechnology

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

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

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

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

Photosynthetic bacteria provide an opportunity to produce organic compounds from CO_2 without the need of feeding sugars. We were therefore interested to test whether the mpusfus fusion gene can be used to produce mannitol in a photo-autotrophic system. Synechococcus sp. PCC 7002 is one of the model systems for metabolic engineering of cyanobacteria and has previously been engineered to produce mannitol from F6P using two separate genes (M1PDH from E. coli and M1Pase from Eimeria tenella) [23]. As E. coli, Synechococcus does not possess an endogenous pathway for mannitol production or breakdown. To facilitate the engineering of Synechococcus, we first generated a BioBrick compatible vector and a synthetic ribosomal binding site. The new tools in combination with a previously identified ‘super-strong’ promoter were confirmed by GFP-assay and are available to the scientific community.

Successful functional expression of mpusfus in Synechococcus was proven by the appearance of mannitol in the transgenic cultures. As before [23], the majority of mannitol

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

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

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

Metabolic network models have become available for Synechococcus [47], which should facilitate this task.

Methods

Construction of pAQ1BB vector for transformation of Synechococcus sp. PCC 7002

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

Generation of codon-optimized mpusfus

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

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

Generation of transgenic Synechococcus sp. PCC 7002 strains

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

Synechococcus sp. PCC 7002 growth

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

Generation of transgenic E. coli strain

Mpusfus DNA without the initial start codon was amplified from the plasmid carrying the codon-optimized gene with the forward primer 5’-GGGGGGGGATCCAATAGTGCCGTGACCATCAGCC-3’ (BamHI restriction site underlined) and the reverse primer 5’-CCCCCCGAATTCTTAGCGGGGATTGGGATCTTC-3’ (EcoRI restriction site underlined). The PCR fragment obtained was cloned into the vector pFO4 as previously described [49], producing the plasmid pMPUSFUS and allowing the recombinant proteins to be fused to a six-histidine tag at their N-terminus. This plasmid was subsequently transformed in E. coli expression strain BL21 (DE3). For gene induction and functional assays, three distinct recombinant clones were pre-cultured in 5 ml of M9 medium supplemented with 10 g/L of glucose and 0.1 g/L ampicillin overnight at 37 ℃ and 200 rpm. The following day, new cultures were started at OD_{600} 0.1 under the same conditions. When OD_{600} 0.5 was attained, cultures were divided into two tubes, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was

added in one of them at a final concentration of 1 mM. Incubation was carried out for 20 h at 25 ℃ and 200 rpm, and cultures reached a final OD_{600} of 2.5-3.0 in both induced and non-induced conditions. Samples were then harvested by centrifugation at 3,500 g for 10 min. Supernatants and cell pellets were frozen individually at -20 ℃ for downstream analyses. T o determine intracellular mannitol concentration, cells were re-suspended in 600 µl of buffer Tris-HCl 25 mM pH 7.5 and NaCl 200 mM, and disrupted by four pulses of 15 sec of sonication using an ultrasonic processor VC50 sonicator (Jencons). Cell debris were pelleted by centrifugation at 14,000 g and 4 ℃ for 20 min ; supernatants were used for mannitol analysis and considered as soluble fractions for protein gel and Western-blot analysis. Proteins were quantified using the Pierce™ Coomassie Plus Assay Kit (ThermoFischer Scientific) according to manufacter's instructions. Three µg of proteins of each fractions were loaded on a 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad). After separation, proteins were transferred to nitrocellulose membrane using the iBlot 2 Dry blotting System (ThermoFischer Scientific). Recombinant proteins were visualized with a conjugated monoclonal anti-polyhistidine-peroxidase antibody (Sigma-Aldrich) detected by the SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFischer Scientific).

Measurement of GFP

RBS and promoter activity was assessed in transgenic cyanobacteria using a GFP reporter encoded by BioBrick part BBa_E0040. Cultures were harvested for GFP measurements at the same point during the diurnal cycle and, when appropriate, growth stage. Optical density was adjusted to OD_{730} 0.25-0.30 in fresh A+ media and fluorescence was measured using a LS 55 Luminescence Spectrophotometer (PerkinElmer, UK) using 480 nm excitation and 514 nm emission wavelengths.

Mannitol analysis

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

Associated content

Supporting information

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

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

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Author contributions

MAM, AA, and TT conceived the study. Experimental data were generated by MAM, SS, and TT. All authors analyzed data. MAM, AA, and TT wrote the manuscript.

Notes

The authors declare no competing financial interest.

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