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Systematic approaches to efficiently produce 2,3-butanediol in a marine cyanobacterium

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Abstract

Cyanobacteria have attracted significant interest as a platform for renewable production of fuel and feedstock chemicals from abundant atmospheric carbon dioxide by way of photosynthesis. While great strides have been made in developing this technology in freshwater cyanobacteria, logistical issues remain in scale-up. Use of the cyanobacterium *Synechococcus* sp. PCC 7002 (7002) as a chemical production chassis could address a number of these issues given the higher tolerance to salt, light, and heat as well as the fast growth rate of 7002 in comparison to traditional model cyanobacteria such as *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803. However, despite growing interest, the development of genetic engineering tools for 7002 continues to lag behind those available for model cyanobacterial strains. In this work we demonstrate the systematic development of a 7002 production strain for the feedstock chemical 2,3-butanediol (23BD). We expand the range of tools available for use in 7002 by identifying and utilizing new integration sites for homologous recombination, demonstrating the inducibility of theophylline riboswitches, and screening a set of isopropyl β -D -1-thiogalactopyranoside (IPTG) inducible promoters. We then demonstrate improvements of 23BD production with the systematic screening of different conditions including: operon arrangement and copy number, light strength, inducer concentration, cell density at the time of induction, and nutrient concentration. Final production tests yielded titers of 1.6 g/L 23BD after 16 days at a rate of 100 mg/L/day. This work represents great strides in the development of 7002 as an industrially relevant production host.

Key words

Cyanobacteria, Metabolic engineering, 2,3-Butanediol, Marine cyanobacteria

Introduction

Rising CO_2 levels have brought carbon dioxide sequestration to the forefront of global concern. The desire for a platform capable of capturing waste CO_2 for removal from the atmosphere and converting it to a usable chemical form has brought much research interest to cyanobacteria.[1] A number of successful cyanobacterial production systems in model cyanobacteria such as *Synechococcus elongatus* PCC 7942 (hereafter 7942) and *Synechocystis* PCC 6803 (hereafter 6803) have been established for small molecules[1] ranging from ethylene[2] to 2,3-butanediol[3] to fatty acids.[4] While the primary reason for the appeal of cyanobacteria is the simplicity of their nutrient requirements (CO_2, sunlight, and water), the need to provide significant quantities of fresh water to these model organisms for large-scale production cultures is not ideal from an environmental or economic standpoint.[5] Though the use of seawater presents its own unique challenges such as limited locations for production facilities, transportation of seawater, and growing and production challenges associated with increased salt concentrations; given the vast excess of saltwater available (salt water makes up about 97.5% of all of the water on Earth[6]), the use of a salt-tolerant production strain in addition to the freshwater strains already in use could provide additional options for cyanobacterial use and prevent further strain on sources of freshwater. Unlike model cyanobacterial production hosts such as 7942 and 6803, the strain *Synechococcus* sp. PCC 7002 (hereafter 7002) is not only tolerant to a range of salt conditions[7], but also has improved thermal stability and photo stability[8] which makes 7002 better able to tolerate strong light[9] and

therefore increases options for scale up locations relying on natural lighting. Additionally, 7002 has demonstrated a significantly increased growth rate over most model cyanobacterial species with a doubling time of 2.6 hours under ideal growth conditions.[10] These qualities combined with a fully sequenced genome and natural transformability[11, 12] makes 7002 an attractive candidate for industrial chemical production.

Despite the many advantages of 7002 as a host strain there are still a number of challenges to overcome before it can become a widely used host strain. One of the primary challenges is that the tools available for 7002 genetic manipulation are still limited. However, recent work by a handful of groups has made great strides in establishing a range of inducible promoter systems,[13, 14] new sites for gene integration via homologous recombination,[8, 15, 16] and the successful use of CRISPRi[17] in 7002. Still, the main drawback in using 7002 remains that, unlike more model host organisms such as *Escherichia coli*, 7002 lacks the breadth of genetic tools necessary for precise control of gene expression and metabolism. Expansion of this genetic toolbox is essential for broader utilization of 7002. Regardless of the challenges presented there have been a few successful demonstrations of chemical production in 7002 reported thus far, including the production of ethanol,[18] lactate,[17] poly-3-hydroxybutyrate and poly3-hydroxybutyrate-co-4-hydroxybutyrate,[19] fatty acids,[20] limonene and bisabolene,[21] and mannitol.[22] Of particular note are the production of ethanol, which achieved a final titer of 1.97 g/L after 30 days,[18] and lactate, which achieved a production rate of 180 mg/L/day.[17]

Previous work in our group has established engineered production of 2,3-butanediol (hereafter 23BD) from the model cyanobacterium 7942.[3] 23BD is a valuable industrial feedstock chemical used in the manufacturing of plasticizers, inks, fumigants, explosives, polymers, liquid fuel additives, and industrial solvents.[3] Given our expertise with 23BD production, this system was used as a starting point to approach engineered production from 7002.

We found that directly transferring the pathway design from 23BD production in 7942[3] to 7002 was not a successful strategy. Engineered production of 23BD in 7002 required the screening and tailoring of designs specifically for use in this new host. In this work we demonstrate further expansion of the genetic engineering toolbox for 7002 with the development of new integration sites for homologous recombination, promoter strength comparisons, and demonstration of riboswitch function. In addition, we also demonstrate the systematic development of culturing conditions for a new host leading to improved production of a chemical of interest. Production strains were screened for improved induction timing, inducer concentration, lighting strength, and nutrient concentration. Minor alterations to these conditions improved growth, titer, and the stability of production strains over time, leading to a final titer of 1.6 g/L 23BD after 16 days under enriched CO_2 conditions. This systematic condition improvement suggests that further optimization of growth conditions for 7002 could improve its industrial relevance significantly.

Results

Our engineered 23BD production pathway consists of three enzymatic steps from the central metabolite pyruvate (Figure 1A).[3] First, two molecules of pyruvate are condensed by an acetolactate synthase (ALS) to form 2-acetolactate. 2-acetolactate is subsequently decarboxylated by an acetolactate decarboxylase (ALDC) to form acetoin. Acetoin is then reduced by a secondary alcohol dehydrogenase (ADH) to form 23BD (Figure 1A). To establish a baseline for 23BD production in 7002 we first tested the toxicity of 23BD and its direct precursor acetoin to which 7942 displayed acute toxicity.[3] Cultures were assessed by amount of growth from a starting OD_{730} of 0.1. 7002 was able to maintain growth at g/L concentrations of both acetoin and 23BD, although growth was diminished compared to samples grown in the absence of acetoin or 23BD (Figure 1B). At 2 g/L of added acetoin, 7002 growth was reduced by about a third as compared to the control. At 5 g/L of added acetoin, 7002 growth was reduced by half as compared to the control. No growth was observed at 12.5 g/L (Figure 1B). No significant difference in growth was observed at 20 g/L of added 23BD as compared to the control. At 40 g/L of added 23BD, 7002 growth was reduced by about two thirds as compared to the control. At 60 g/L of added 23BD, an OD_{730} increase of only 0.04 was observed (Figure 1B).

Encouraged by these results, we then moved forward with our construction of an expression system for the 23BD production pathway in 7002. We first explored different locations for the integration of the heterologous 23BD production pathway genes. In addition to targeting the endogenous high copy

plasmid pAQ1, used previously for homologous recombination,[8] two additional sites on the chromosome were identified as targets for transformation: gene SYNPCC7002_A1001 encoding a restriction endonuclease with similarity to the type IV restriction endonuclease Mrr and gene SYNPCC7002_A1189 encoding a type II site-specific deoxyribonuclease associated with the type II restriction enzyme AquI. We will refer to these sites as Mrr and AquI respectively for simplicity. Knocking out restriction endonuclease genes generally improves transformation efficiency by preventing DNA cleavage of heterologous constructs and they serve as a new site for the integration of non-native genes.[23, 24] The sequences used as homology arms for integration into these sites are listed in Table S1. Expression from these integration sites was tested using superfolder green fluorescent protein (sfGFP)[25] as a reporter for activity under the previously established constitutive promoter P_{cpcB}.[8] Only a very small growth difference was observed compared to wild type (Figure S1) in using Mrr and AquI sites, and sfGFP was determined to be active (Figure 2), suggesting that both sites are promising targets for homologous recombination in future work with this strain. The level of sfGFP fluorescence for pAQ1 was an order of magnitude higher than that for AquI and Mrr, which corresponds well to the reported copy number ratio of the endogenous pAQ1 plasmid to the 7002 chromosome, about 50 to 6[8] (Figure 2).

Any engineered production system hinges on tight control of inserted genes, so we next sought a reliable system for inducible gene expression to replace the constitutive promoter $P_{cpcB.}$ After unsuccessful attempts to utilize the

expression control designs from 23BD production in 7942,[3] we next pursued a solution specific to 7002.[3] A system that represses gene expression until an optimal growth phase is reached allows for better balancing of cell growth and chemical production.[26] Previously, the use of theophylline dependent riboswitches has been demonstrated for the control of gene expression in 7942.[27] We sought to adapt these riboswitches for use in 7002 as an inducible system to control 23BD production. We built and screened a series of riboswitch constructs using *sfGFP* as a reporter gene in 7002 (Figure 3). We first explored the use of the theophylline inducible riboswitches A-E created by Topp et al. for use in non-model strains in conjunction with P_{cpcB}, as well as a modified version of E (E*) (Figure S2).[28] E* contains the consensus prokaryotic RBS sequence and was created as an alternative riboswitch for use in Gram-positive bacteria.[28] Riboswitches E and E* did show successful induction in 7002, but only at low levels (Figure S2). To improve induction levels, we next explored further adaptations of the constant leader sequence upstream of the riboswitch aptamer. The importance of a constant leader sequence upstream of the riboswitch aptamer has been identified in various organisms.[27, 28] We first tested two previously identified leader sequences[27, 28] (Riboswitch 1 & 2, Figure 3A). The difference in leader sequence had little effect (Figure 3B). We then explored the replacement of P_{cpcB} with P_{trc}, and $P_{cLac143}$ (described below) (Riboswitch 3 & 4, Figure 3A). The change in the promoter sequence significantly improved induction levels with Riboswitch 3 demonstrating the best performance (Figure 3B).

Given our previous success in the use of the *Escherichia coli*-derived isopropyl β -D -1-thiogalactopyranoside (IPTG) inducible promoter P_{LlacO1} to tightly control expression of the 23BD pathway in 7942,[26] we next explored the use of IPTG-inducible promoters for gene expression control in an effort to further increase induction levels. Markley et al. developed a series of IPTG-inducible promoters in 7002[13]; we therefore chose to compare one promoter from their work with the best fold induction, $P_{cLac143}$, with the common IPTG-inducible *E. coli* promoters P_{LlacO1} and P_{trc} to control expression of *sfGFP* in 7002 (Figure 4). We also explored the use of a single amino acid substitution in the LacI repressor to improve repression as used by Markley et al.[13] For each promoter, we explored its behavior in the presence and absence of the point mutation in *lacI* (W220F). Interestingly, in our hands the $lacI^{W220F}$ mutation did not result in greater repression before induction, but rather in greater induction after the addition of IPTG (Figure 4). For both of the best performing promoters P_{trc} and $P_{cLac143}$ the strain containing $lacI^{W220F}$ displayed significantly higher fluorescence than the strain lacking the mutation. With or without $lacI^{W220F}$, these strains significantly outperformed our best performing riboswitch construct (Figures 3&4). Interestingly, strains under control of P_{LlacO1}, the promoter used for our previous production in 7942,[3] performed quite poorly in 7002 in comparison to the riboswitch strain and the other two promoters. Although the strain under the control of P_{trc} with $lacI^{W220F}$ demonstrated the highest fluorescence upon induction, this strain demonstrated greater leaky expression in the absence of inducer than the next closest strain by an order of magnitude. This leaky

expression resulted in only a 24-fold increase in fluorescence upon induction, whereas the strain under the control of $P_{cLac143}$ with *lacI*^{W220F} displayed tight repression in the absence of inducer resulting in a 145-fold increase in fluorescence upon induction (Figure 4). We therefore moved forward with subsequent strain construction using $P_{cLac143}$ with the point mutation (*lacI*^{W220F}).

After selecting an induction system to control gene expression in the strain we used it to screen different gene combinations for 23BD production adapted from the construct used in 7942 for 23BD production[3, 26] (Figure 5A). For these various designs we utilized the endogenous high copy plasmid pAQ1 (~50 copies) integration site[29] as well as the chromosomal integration site, Mrr (6 copies)[8] (Figure 5A). Additionally, given that expression of a heterologous *alsS* gene is not essential for 23BD production, we explored several designs incorporating the *alsS* gene from *Bacillus subtilis* as in our previous work[3], overexpression of the endogenous *alsS*, and relying entirely on endogenous expression levels of the native *alsS*. As in our previous work we utilized the *alsD* gene from *Aeromonas hydrophila* and the *adh* gene from *Clostridium beijerinckii*[3]. Four different gene combinations were tested for 23BD production as shown in Figure 5A. All reported 23BD concentrations refer to the amount measured extracellularly in the supernatant. We first compared *alsD* and *adh* alone (Strain 1) to the three gene design from our 7942 production (Strain 2).[26] Both designs were integrated into the high copy endogenous plasmid pAQ1 (Figure 5A). The 23BD production in Strain 1 was very similar with that in Strain 2 relying on native *alsS* expression levels, both producing between 500 and 600

mg/L of 23BD after 4 days (Figure 5B), although the ALS activity in Strain 2 was much higher than that in Strain 1 (Figure 5D). Next we tested a slightly lower level of ALS activity. We replaced the highly active *alsS* gene from *B. subtilis* with the endogenous *alsS* gene from 7002 on pAQ1 (Strain 3). This strain produced less than 100 mg/L of 23BD after two days, far behind the rate of production for Strain 1 and Strain 2, and so we did not continue with further measurements (Figure 5B). Interestingly this strain displayed a significantly enhanced growth rate as compared to any of the other strains included in this screen (Figure 5C). We also moved the *B. subtilis alsS* gene to the chromosomal neutral site Mrr in order to decrease its copy number (Strain 4). This strain accumulated about 300 mg/L 23BD by day 2 when production ceased (Figure 5B). The ALS activities in Strain 3 and Strain 4 were about 50% and 25% of that in Strain 2, respectively (Figure 5D). We chose to move forward with Strain 1 given its higher 23BD titers as compared to Strains 3 and 4, and given the lack of benefit of the overexpressed *B. subtilis alsS* in Strain 2.

After identifying a good design for the 23BD pathway, we sought to improve production conditions to probe the possible capabilities of our system. We first investigated the time of induction by comparing 23BD production over a 4-day period between cultures induced at different optical densities at 730 nm (OD_{730}) (Figure 6A). Cultures were induced at an OD_{730} of 0.4, 1.6, and 4. While initial 23BD titers were similar between OD_{730} 1.6 and OD_{730} 4 cultures at 24 hours, cultures induced at OD_{730} 1.6 continued to produce over subsequent days while those induced at OD_{730} 4 did not. 23BD levels from cultures induced at

OD_{730} 0.4 remained low throughout testing. Using induction timing at an OD_{730} of 1.6, we then screened different concentrations of the IPTG inducer (Figure 6B). In a comparison test of 23BD production levels over 5 days induced with 5, 1, and 0.1 mM IPTG we found the best performing IPTG concentration for production to be 0.1 mM, which produced about 500 mg/L 23BD after 5 days. Higher IPTG concentrations produced less than 300 mg/L 23BD. After having observed some slight discoloration and cell stress after several days of production, we tested 23BD production under lower light (300 μmol photons/m^2/sec) conditions (we were originally running tests at 600 μmol photons/m^2/sec). These lower light conditions not only reduced discoloration but also increased total 23BD production (Figure 6C). The control strains lacking any pathway genes also grew better under lower light, however, interestingly Strain 1 did not, despite its reduced discoloration (Figure 6D). It should be noted that the light conditions explored in this study are stronger than those used in previous work in 7002, which have ranged from 60 to 250 μmol photons/m^2/sec.[8, 13, 16, 21] Given that published media conditions for many strains are not optimized for chemical production, some previous work has explored media optimization for production strains.[30] After testing several different media modifications (data not shown), we also found that reducing the concentration of magnesium in the A+ medium to 1.25 g/L $MgSO_4 \cdot 7H_2O$, about 25% of the published concentration,[31] was beneficial for 7002 growth. Strains displayed more than double the growth rate with the modified magnesium concentration compared to those grown using the published magnesium concentration (Figure 6E).

After identifying improved production conditions, we sought to further improve production through the supplementation of an additional carbon source. Glycerol is a relatively inexpensive source of carbon that can be obtained as a byproduct of biodiesel formation.[32] While some cyanobacterial strains like 7942 need genetic modifications in order to utilize glycerol,[33] 7002 can naturally utilize glycerol after an adaptation period in order to supplement growth from CO_2.[8] Therefore, we decided to test if the supplementation of glycerol as an additional source of carbon could improve 23BD production (Figure 7). We first carried out a toxicity screen in 7002 in order to determine glycerol tolerance. A small growth defect is seen even with the addition of only 1 g/L glycerol. This growth detriment becomes severe at concentrations higher than 5 g/L (Figure 7A). Glycerol concentration was therefore monitored throughout the subsequent tests to ensure continued availability of glycerol while at the same time keeping levels below 5 g/L (Figure 7D). Upon the supplementation of glycerol in our production strains, we observed that strains given the added glycerol consistently outperformed strains not given glycerol in terms of growth (Figure 7B). However, while we initially observed an improved 23BD titer with the addition of glycerol (410 mg/L after 48h vs. 334 mg/L), after 7 days the strains without glycerol supplementation caught up to and surpassed those with glycerol supplementation (Figure 7C). This suggests that while carbon availability for production may be limiting, glycerol supplementation in the long term may have a toxic effect on cultures. We observed that the cultures supplemented with glycerol turn yellow over the course of the production experiment. Further study

into glycerol utilization pathways in 7002 may help to elucidate the mechanism of toxicity.

Finally, to establish the production potential as well as the stability of our best strain under enriched CO_2 conditions we carried out a long-term production experiment using our optimized conditions (OD_{730} 1.6 for induction with 0.1mM IPTG, 300µmol photons/m^2/sec light, 1.25 g/L $MgSO_4 \cdot 7H_2O$). After 16 days of production we achieved a maximum titer of 1.6 g/L 23BD at an average rate of 100 mg/L/day (Figure 8A). We subsequently carried out *in vitro* enzyme assays to confirm the overexpression of *alsD* and *adh* in Strain 1 as compared to wild type (WT) (Figures 8B and 8C respectively). A comparison of our engineered strain's photosynthetic rate to that of WT was also carried out by measuring oxygen evolution normalized by chlorophyll A content, however we did not observe a significant difference between the strains (Figure 8D).

Discussion

This work represents a systematic approach to construct an efficient 23BD production strain using 7002. 7002 has attractive properties for photosynthetic chemical production such as its increased salt tolerance, photo and thermal stability, increased growth rate, and, as demonstrated in this study, significantly increased tolerance to certain chemicals of interest. Compared to our previous tests in 7942,[3] 7002 demonstrates an increased tolerance to the 23BD pathway intermediate acetoin by an order or magnitude and about twice the tolerance to 23BD (Figure 1) which had previously been chosen for production from 7942

partially for its low toxicity.[3] This increased tolerance greatly raises the ceiling of possibility for 23BD titers from 7002 that could be achieved after further strain development.

Despite these advantages, the use of 7002 as a production host has remained limited, due in part to the limited genetic tools available for the strain. In this work, we expand the scope of genetic tools available for use in 7002 by successfully demonstrating the use of a series of theophylline inducible riboswitches as an alternative means of gene expression control (Figure 3). While our riboswitch constructs did not turn out to be the best performing choice for 23BD production, they still have great potential to be useful in cases where a lower level of induction is needed. Theophylline inducible riboswitches are particularly attractive systems as they do not require the expression of any accessory proteins, such as the LacI repressor, and theophylline is relatively cheap compared to more traditional inducers such as IPTG.[28] We also identified two new sites for gene integration on the genome, which we have named Mrr and AquI, and demonstrated successful integration and expression of heterologous genes at both sites (Figure 2). These sites were chosen with a desire to knock out restriction enzyme genes in an effort to ease transformation. In addition to the new tools we developed for future use in 7002, we built upon previous tool development work by Markley et al.[13] with the demonstration of chemical production under the control of their IPTG inducible promoter $P_{cLac143}$ created for use in 7002 (Figure 8A).

Our production experiments with different gene combinations as well as those with glycerol supplementation both illustrate potential areas for optimization of carbon flux towards chemical production (Figures 5 and 7). The performance of Strain 1 versus Strain 2 (Figure 5B) suggests that increasing levels of ALS in an effort to divert more pyruvate away from central metabolism does not result in a boost to 23BD production in all cases. Analysis of production strains via metabolomics could reveal potential areas for strain engineering to relieve bottlenecks, and allow for greater carbon flux to 23BD production while maintaining balance in central metabolism. Such experiments could also shed light on the behavior of Strain 3 and Strain 4. The dramatic decrease in 23BD production coupled with the dramatic growth increase observed for Strain 3 (Figures 5B and 5C) suggests that the endogenous ALS may prefer substrates other than pyruvate in 7002 metabolism and that its overexpression leads to unintended changes in metabolism. For Strain 4 we expected to see 23BD production and growth on par with that seen for Strain 1 and Strain 2, however in both cases Strain 4 performed significantly worse (Figures 5B and 5C). Based on these results it is possible that while only a small growth defect was observed in our initial integration site screening with *sfGFP* (Figure S1), that in a strain modified with the increased burden of production genes impaired performance could be revealed. Further characterization of the Mrr integration site would be required to explore this theory further.

Recent work in 7942 has shown that the incorporation of glucose into metabolism can increase titers significantly and allow for production under both

light and dark conditions.[34] Additionally, this work demonstrated that diversion of high carbon flux to the CO_2 fixation step through the oxidative pentose phosphate pathway and deletion of the regulatory protein Cp12, was beneficial to increasing the amount of carbon sequestered by RuBisCO.[30] It would be of interest to explore whether these same modifications would be beneficial in 7002. Rather than incorporating glucose to boost production, which requires a glucose transporter gene, we chose to supplement glycerol which 7002 has been demonstrated to utilize naturally.[8] While our carbon supplementation strategy did not ultimately improve our 23BD production levels (Figure 7C), it did consistently lead to improved strain growth (Figure 7B). Further exploration of the mechanism of glycerol utilization in 7002 could shed light on potential engineering strategies for the redirection of supplemented carbon to chemical production rather than biomass. Employing strategies to reduce glycerol toxicity could also assist in improving the titer and stability of photomixotrophic production in 7002. Overexpressing downstream genes in the glycerol utilization pathway to drive flux away from potentially toxic metabolites has been a successful strategy in 7942,[33] and a similar approach could improve glycerol utilization in 7002. Since we observed an immediate benefit upon glycerol addition to 7002, we did not carry our strains through an adaptation period as others have reported.[8] However, doing so in future studies may assist in alleviating potential toxicity issues.

Our production strain achieved a maximum titer of 1.6 g/L after 16 days and an overall rate of 100 mg/L/d under enriched CO_2 conditions (Figure 8A).

Our results demonstrate that 7002 can support chemical production while still maintaining a high growth rate (Figure 8A), and are comparable to the high titers and fast rates observed in 7002 by other groups[17, 18] as well as to the 2.4 g/L of 23BD that we achieved previously after 21 days of production in 7942.[3] While these titers represent an important first step towards industrial viability, we believe that full condition and strain optimization would provide further improvements for 7002 chemical production. This work also demonstrates the necessity of adapting each engineered production design to the host strain of interest. As interest in 7002 as a new chassis for renewable chemical production continues to grow, this work provides another step in the direction of moving 7002 from uncharacterized host to model organism.

Methods

Reagents

Oligonucleotides were synthesized by Eurofins Genomics and Integrated DNA Technologies (IDT). Phusion and T4 DNA polymerases were purchase from New England Biolabs. Gentamicin was purchased from Teknova. Spectinomycin was purchased from MP Biomedicals. Kanamycin, isopropyl-*β*-D-thiogalactoside (IPTG), silicon oil, and glycerol were purchased from Fisher Scientific. 23BD, acetoin, and thiamine pyrophosphate were purchased from Sigma Aldrich. Pyruvic acid sodium salt was obtained from Acros Organics. NADPH was obtained from Calbiochem. Noble Agar was purchased from Affymetrix.

Culture Conditions

7002 strains were cultured in 15 mL of A+ media[31] in a 38°C water bath in 30 mL culture tubes with under a light strength of 600 µmol photons/m^2/sec (measured used an Apogee Instruments Model MQ-200 quantum meter) for high light experiments or 300 µmol photons/m^2/sec for low light experiments. ViaVolt T5 High Output Copper Fluorescent Grow Light Fixtures in the blue spectrum were used for all experiments. All experiments were performed under continuous light. Cultures were agitated by bubbling air enriched with 3-6% CO_2 through steel gauge needles (180 mm length and 1.2 mm outer diameter). 5 microliters of silicon oil was added to each culture to prevent foaming. Antibiotics were added to cultures as appropriate: 100 µg/mL for kanamycin, 50 µg/mL for gentamicin, and 50 µg/mL for spectinomycin. CO_2 percentage was monitored using a CO_2 analyzer from PP Systems. Cell growth was monitored by measuring OD_{730} using a Microtek Synergy H1 plate reader (BioTek).

For superfolder green fluorescent protein (sfGFP) measurements, cultures were grown under high light conditions with appropriate antibiotics. Each strain was inoculated in triplicate or quadruplicate at an OD_{730} of 0.1 and allowed to grow for 3 hours. After 3 hours *sfGFP* expression was induced either through the addition of 2 mM theophylline for riboswitch strains or 5 mM IPTG for strains containing IPTG inducible promoters. Florescence was measured after 20 hours, 488 nm was used for excitation and emission was measured at 530 nm using a Microtek Synergy H1 plate reader (BioTek).

For 23BD production and enzyme assays in 7002, cultures with the appropriate antibiotics were diluted to OD_{730} 0.2 and grown overnight at the specified light strength to an OD_{730} of 1.6 (or as noted). At an OD_{730} of 1.6, 0.1 mM IPTG was added to each culture unless otherwise specified. Every 24 hours culture volume lost to evaporation was replenished with sterile water prior to sampling. Any culture volume removed for sampling was replaced with fresh media containing the appropriate IPTG and antibiotics concentrations.

Plasmid and Strain Construction

Strains used and constructed are listed in Table S2. Plasmids used and constructed are listed in Table S3. All plasmids were constructed using sequence and ligation independent cloning (SLIC)[35] in *E. coli* strain XL1-Blue (Agilent Technologies). Primers used for construction are listed in Table S4. Construction designs are listed in Tables S5 and S6.

Transformation of 7002

For transformation of 7002 strains, 15 mL cultures in exponential phase were spun down at 2,683 x g for 10 minutes and resuspended in 5 mL of fresh A+ media without antibiotics. This wash step was repeated twice and cells were subsequently resuspended in a final volume of 500 µL of fresh A+ media without antibiotics. 200 µL of concentrated culture was then mixed with 1 to 2 µg of intact plasmid designed for targeted homologous recombination in a 1.5 mL tube. The tube was subsequently shaken in the dark, overnight, on a rotary shaker (100

rpm) and then plated on Petri dishes made with A+ media, 15-18 g/L Noble Agar, appropriate antibiotics (see above culture conditions), and topped with nitrocellulose transfer membranes (Merck Millipore HATF08250). Plates were incubated at 30^{o}C under a light strength of about 24 µmol photons/m^{2}/sec. Chromosomal segregation for the introduced fragments was achieved through propagation of multiple generations on a selective agar plate and verified by colony PCR. Correct recombinants were confirmed by PCR to verify integration of target genes into the chromosome and then sequenced. The strains used and constructed are listed in Table S2.

Acetoin Quantification

Acetoin was quantified by the method of Voges and Proskauer[36] as optimized by Westerfield[37] and adapted to small volume on 96-well plates. Samples were diluted in water to 100 µL initial volume. To this mixture 100 µL of a solution, prepared at the time of use, consisting of one part 5% (wt/vol) naphthol dissolved in 2.5 N NaOH and one part 0.5% (wt/vol) creatine in water was added. Readings were taken after 30 minutes using a Microtek Synergy H1 plate reader (BioTek). Triplicate measurements of no fewer than three standards, including at least one value each above, below, and within the desired range, were included in every assay.

Quantification of 23BD

23BD was quantified in cell supernatant using a Shimadzu (GC-2010) gas chromatograph (GC) equipped with a flame-ionization detector. Separation was achieved using an Agilent J&W HP-5 column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness). For each analysis GC oven temperature was increased from 70^{o}C to 150^{o}C with a gradient of 40^{o}C min^{-1} and then held at 150^{o}C for 2 minutes. Ultrahigh purity helium was used as the carrier gas. The temperatures of the injector and detector were set at 280 and 330^{o}C respectively. A set of standards of known concentration containing standards higher in concentration, lower in concentration, and within the range of the samples were run with each batch to accurately determine 23BD concentration.

Enzyme Assays

7002 cultures were grown for 24 hours after induction unless otherwise specified. For cell lysis cultures were spun down at 4^{o}C at 2,683 x g for 10 minutes and washed twice with 50 mM phosphate buffer pH *8* and resuspended in 1 mL of the same buffer. Crude extracts were prepared with 200 μL of 0.1 mm glass beads and shaken 4 times for 45 seconds with 1 minute on ice in between rounds using a Mini-bead beater (Biospec Products). The total protein determination was performed using Advanced Protein Assay Reagent from Cytoskeleton.

Acetolactate synthase (ALS) and acetolactate decarboxylase (ALDC) activities were determined as previously described[38]. The concentration of acetoin produced was measured by a standard curve using pure acetoin. One

specific unit of ALS activity corresponds to the formation of 1 nmol of acetoin per milligram of protein per minute.

Alcohol dehydrogenase (ADH) activity was determined as previously described[3]. One specific unit of ADH activity corresponds to the oxidation of 1 nmol of NAD(P)H per minute per milligram of protein.

O_2 Evolution

Evolution of O_2 was measured using a Clark-type electrode with the Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK) as previously described[3]. Water was circulated in the surrounding water jacket and set at 37°C. Fresh cells were collected and resuspended to a final OD_{730} of 2 in 500 µL of A+ media bubbled with 3-6% CO_2. This suspension was transferred to a 4 mL borosilicate glass chamber. Cells were stirred at 100 rpm using a magnetic flea, subjected to excess light (300 µmol photons/m^2/sec), and allowed to equilibrate until a constant rate of O_2 evolution could be measured (3−5 min). Oxygen evolution rates were normalized by intracellular chlorophyll content.

Chlorophyll content

100 µL culture broth was centrifuged at 10,000 x g for 10 minutes and resuspended in the same volume of distilled water. 900 µL methanol was added and mixed thoroughly by vortexing. The mixture was incubated at room temperature for 10 minutes in darkness. The supernatant was obtained by centrifugation at 10,000 x g for 5 minutes and transferred to a 96 well plate

(360μL per well) for chlorophyll measurement. An absorbance spectrum was measured between 630nm and 780nm. Chlorophyll A content was calculated using the method of Porra et al. and normalized by OD_{730}.[39]

Glycerol quantification

Glycerol was quantified using the Sigma Aldrich glycerol assay kit (Catalog number MAK117) colorimetric assay according to the supplier's instructions. Samples were diluted using sterile water to a detectible concentration within the standard curve.

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Author Contributions

N.E.N., A.E.C., A.L.C., and S.A. designed research; N.E.N., A.E.C., and A.L.C. performed the experiments; N.E.N., A.E.C., A.L.C., and S.A. analyzed data; N.E.N., A.E.C., A.L.C., and S.A. wrote the paper.

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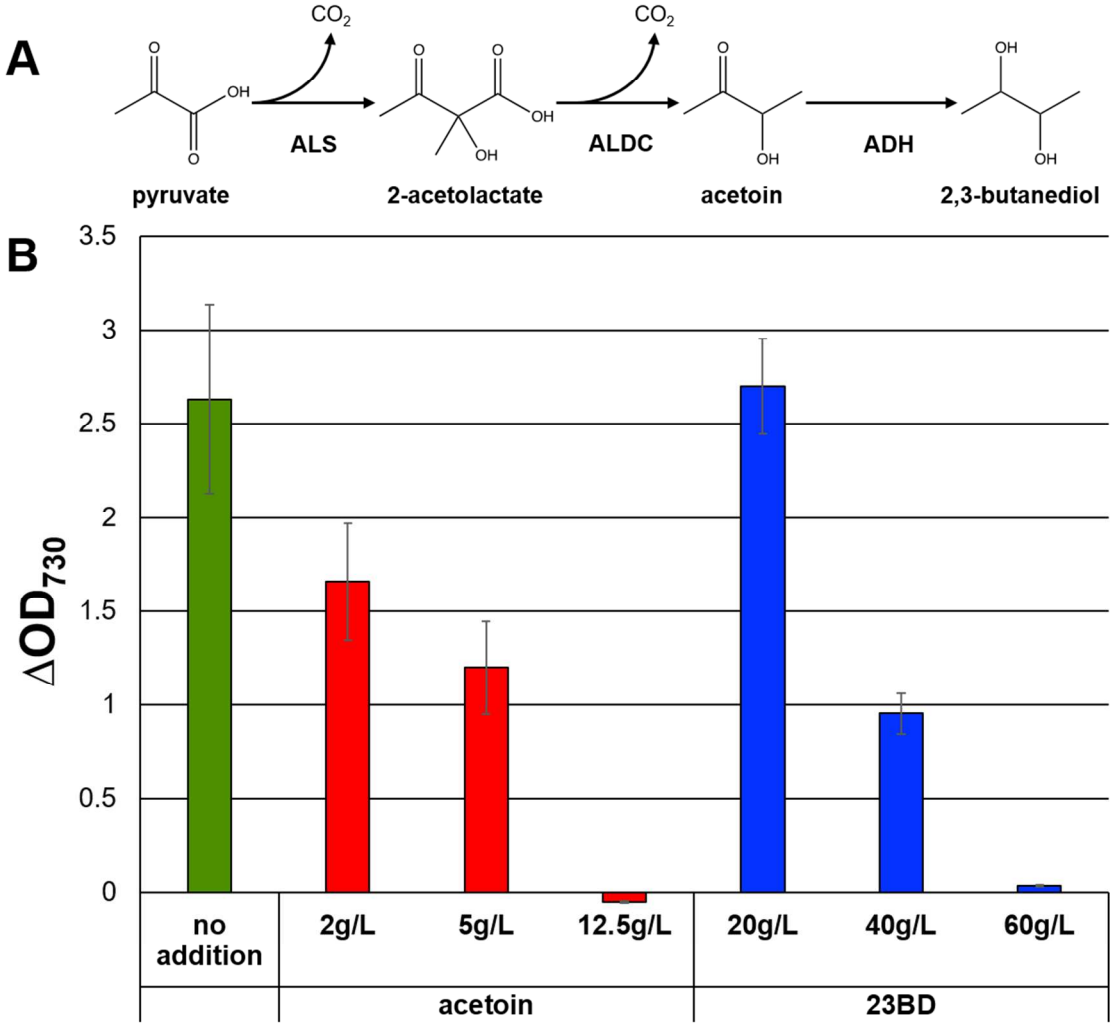


Figure 1. Pathway for 23BD production in 7002
(A) Heterologous pathway for the conversion of pyruvate to 23BD. **(B)** The toxicity limit of the intermediate acetoin and desired product 23BD were determined over 24 hours of growth. Change in OD_{730} from an initial starting value of 0.1 reported. The concentration of chemical added to each culture is listed below each bar. Acetoin in red, 23BD in blue, and green for no chemical addition. Error bars indicate SD for a least 3 biological replicates.

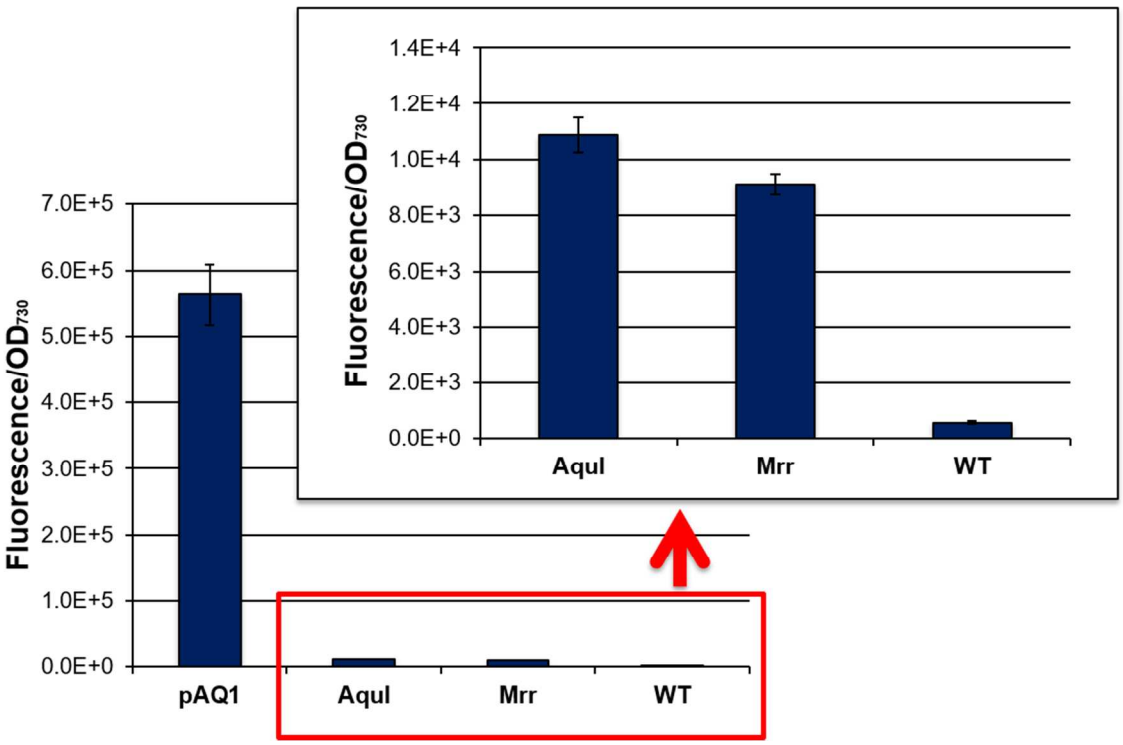


Figure 2. Expression of *sfGFP* from the integration sites

In addition to the previously identified site on the endogenous plasmid pAQ1, two additional sites for homologous recombination on the chromosome were identified: Mrr and AquI. Constitutive expression of *sfGFP* was used to confirm the expression of incorporated genes at the previously identified site on pAQ1, and at Mrr and AquI. WT = wild type 7002. Error bars indicate SD for a least 3 biological replicates.

A

Riboswitch	Promoter	Leader Sequence	Aptamer
1	P_{cpcB}	ATACGACTCACTATA	E*
2	P_{cpcB}	ATACGCTCACAATT	E*
3	P_{trc}	ATACGCTCACAATT	E*
4	$P_{cLac143}$	ATACGCTCACAATT	E*

B

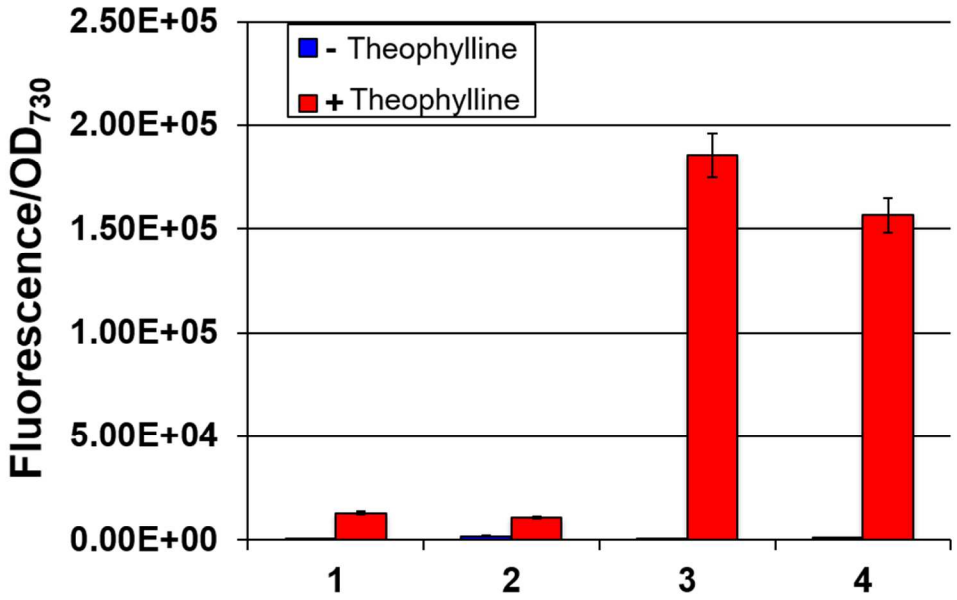


Figure 3. Riboswitch function in 7002
(A) Riboswitches tested with *sfGFP* distinguished by associated promoter, conserved leader sequence, and aptamer. (B) The relative activities of each riboswitch in the absence (blue) and presence (red) of 2 mM theophylline after 20 hours of induction. Error bars indicate SD for a least 3 biological replicates.

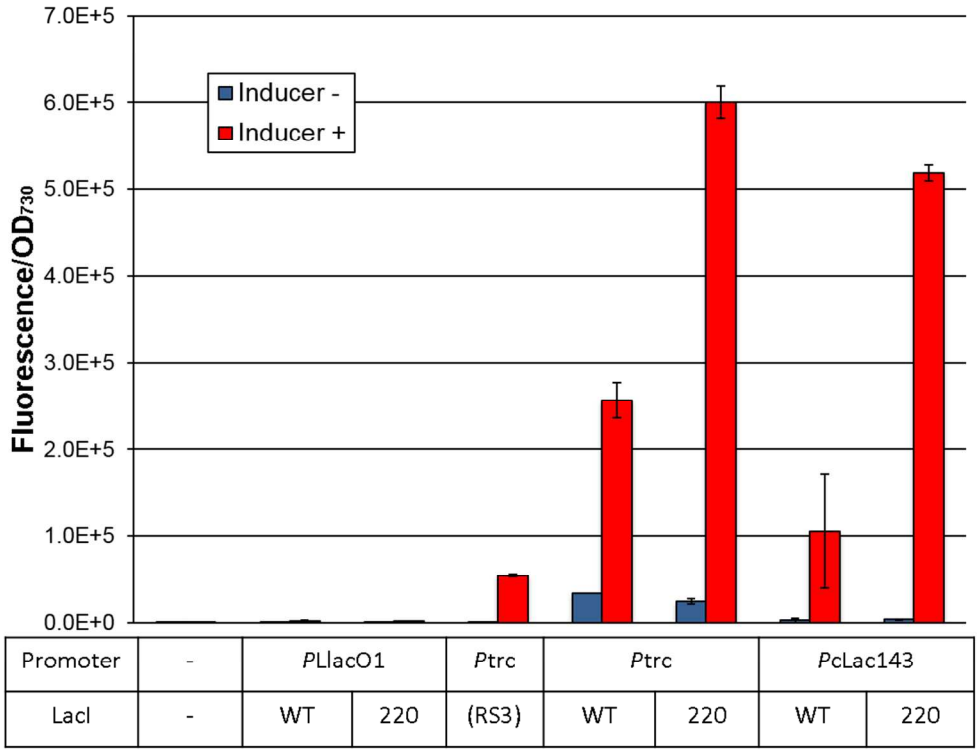


Figure 4. IPTG inducible promoter function in 7002

IPTG inducible promoters were used to control expression of *sfGFP* for screening in 7002. The *E. coli* promoters P_{LlacO1} and P_{trc} as well as $P_{cLac143}$, previously developed for use in 7002, were screened both with (WT) and without a point mutation ($lacI^{W220F}$) in LacI (220). The best performing riboswitch construct (Riboswitch 3 (RS3), Fig. 3) was also included for comparison. Measurements of sfGFP fluorescence were taken after 20 hours and normalized by OD_{730}. Samples were tested in the presence (red) and absence (blue) of 5 mM IPTG for IPTG-inducible promoters or 2 mM theophylline for the riboswitch construct. Error bars indicate SD for a least 3 biological replicates.

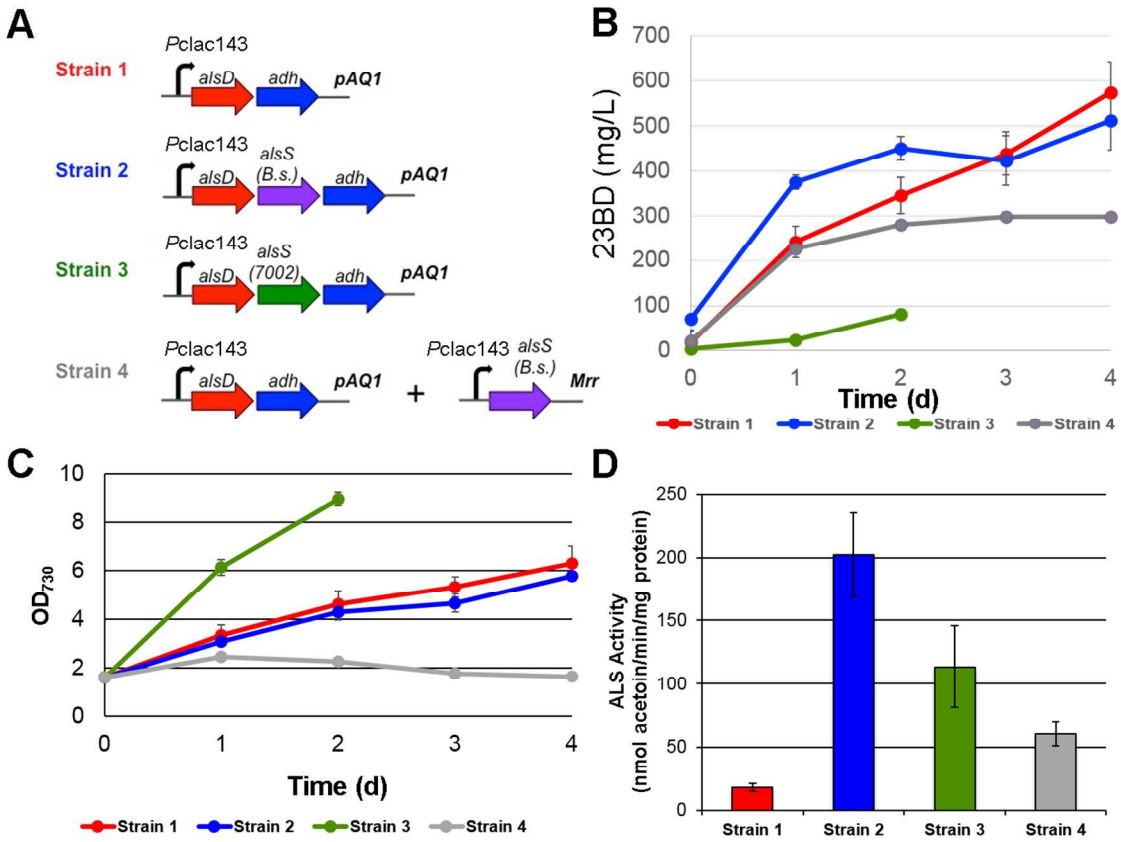


Figure 5. 23BD pathway arrangements

(A) Screening 23BD pathway arrangements. Four different gene arrangements were constructed with various expression levels of both the native *alsS* (7002) and *alsS* from *B. subtilis* (*B.s.*). These constructs were screened for 23BD production and stability over time **(B)** and for cell growth **(C)**. Relative ALS activity of each construct was measured **(D)**. Error bars indicate SD for a least 3 biological replicates.

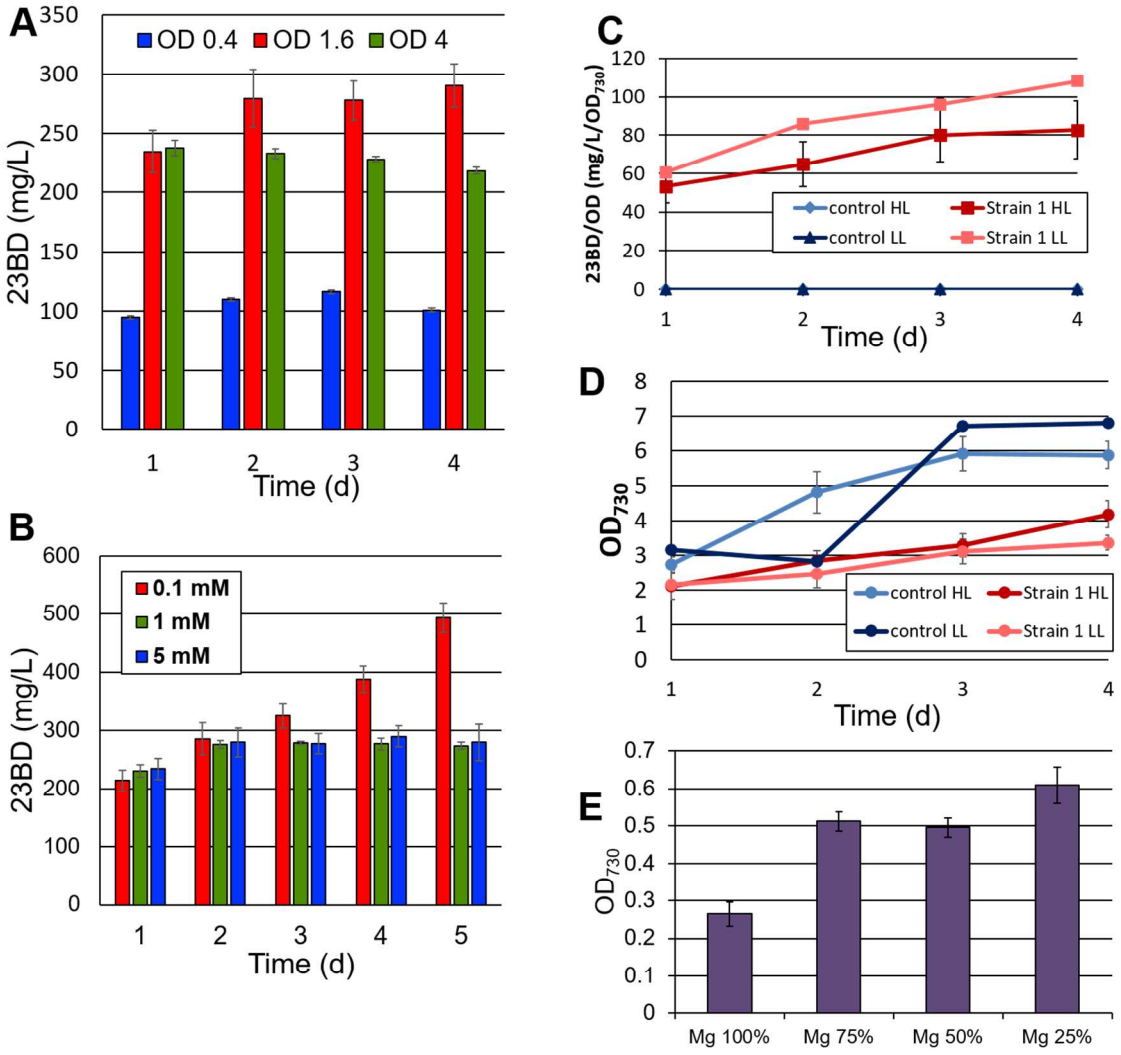


Figure 6. 23BD production conditions in 7002

The production conditions including induction timing **(A)**, IPTG concentration **(B)**, light levels (**C** & **D**), and magnesium concentration in the media (**E**) were modified. LL = low light (300 µmol photons/m^2/sec light), HL = high light (600 µmol photons/m^2/sec light). Control indicates strains lacking any pathway genes. Mg^{2+} percentage indicates percentage of the originally recommended concentration of $MgSO_4 \cdot 7H_2O$ added to the culture. Error bars indicate SD for a least 3 biological replicates.

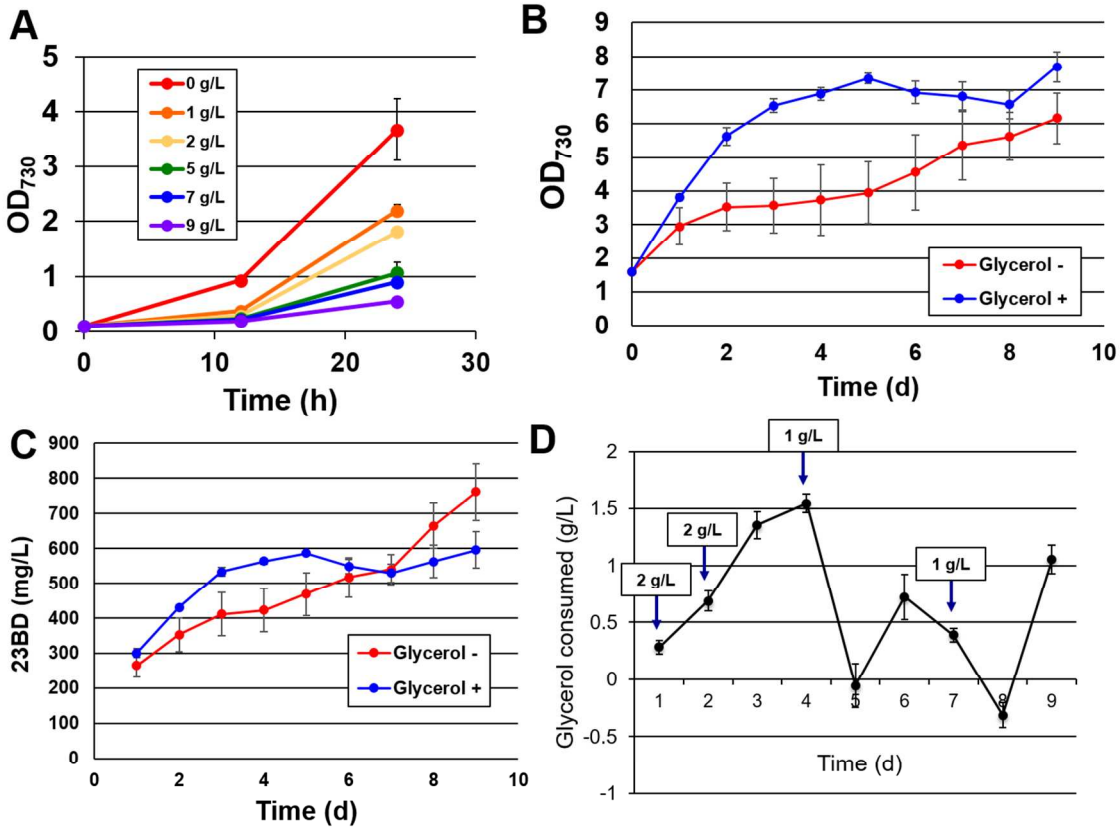


Figure 7. 23BD production with glycerol feeding

(A) Toxicity screen of a range of glycerol concentrations. Strain 1 growth (**B**) and 23BD titer **(C)** in the presence (blue) and absence (red) of added glycerol. **(D)** Concentration of glycerol consumed each day. Arrows indicate glycerol additions after the first addition of 2 g/L at time zero. Error bars indicate SD for a least 3 biological replicates.

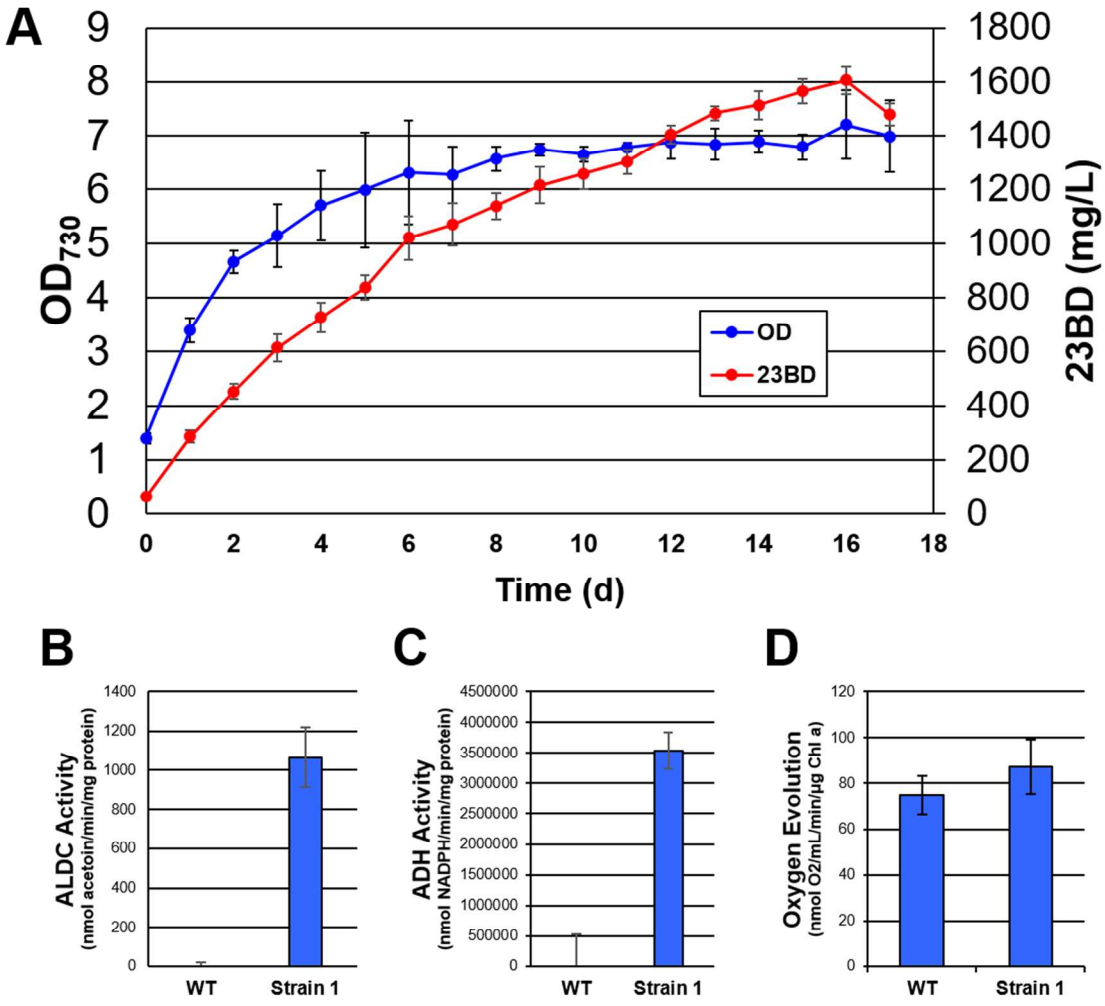
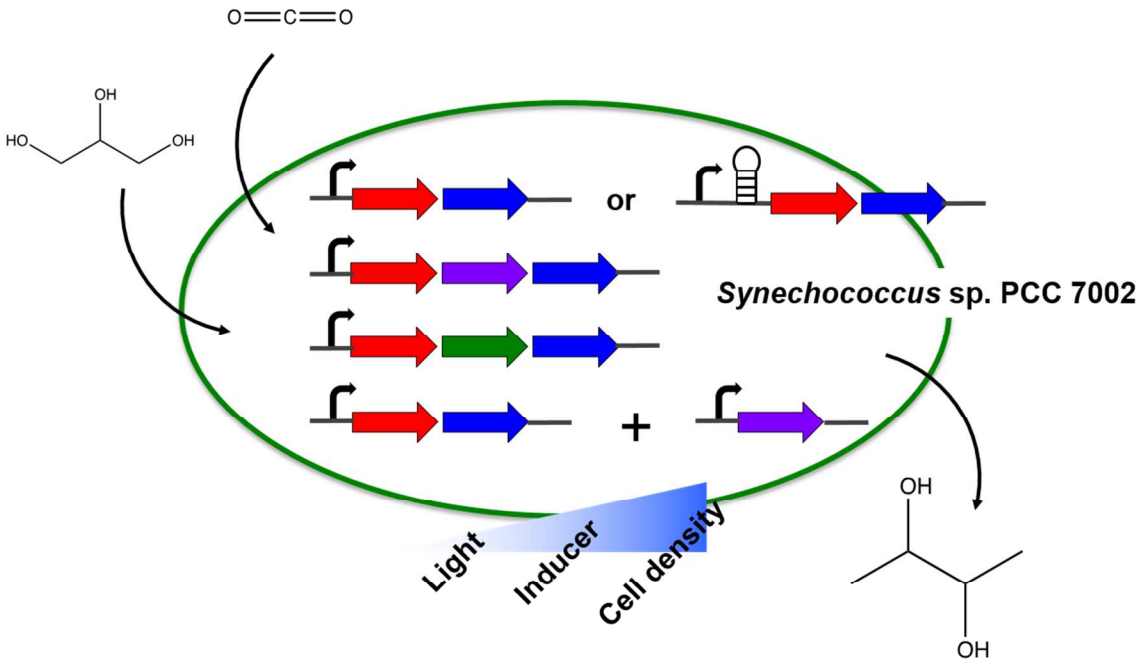


Figure 8. Long-term 23BD production

Long-term 23BD production (red) and growth (blue) of Strain 1 was measured over 16 days **(A)**. The enzyme activity in Strain 1 as compared to wild type 7002 (WT) of ALDC **(B)** and ADH **(C)** was measured as well as photosynthetic efficiency **(D)** in the form of oxygen evolution normalized by chlorophyll A content. Error bars indicate SD for a least 3 biological replicates.



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