Biofuel Toxicity and Mechanisms of Biofuel Tolerance in Three Model Cyanobacteria

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Abstract

While recent studies have demonstrated direct photosynthetic production of biofuels via engineered cyanobacteria, biofuel yields from cyanobacteria remain at low levels. As with heterotrophic biofuel production, product toxicity is likely a limiting factor. Some mechanisms of toxicity may be similar to those studied in common heterotrophic hosts; however, the photosynthesis-dependent pathways for carbon fixation and energy production in cyanobacteria present unique targets for biofuel toxicity. This study investigates biofuel toxicity for three model cyanobacterial strains: *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7002. While cyanobacterial biofuel tolerances were generally lower than that of heterotrophic hosts, the marine strain *Synechococcus* sp. PCC 7002 showed increased tolerance to short chain alcohols, and long-chain biofuel products, such as fatty alcohols, saturated free fatty acids, alkanes, and alkenes had minimal toxicity for all three cyanobacteria. Targeted mutants were generated to explore natural mechanisms of biofuel tolerance in cyanobacteria, such as cell membrane composition, reactive oxygen species degradation, and efflux pumps. These mutants confirmed the influence of cell membrane composition on cyanobacterial tolerance to short-chain alcohols. This study provides data to guide both biofuel product and cyanobacterial host selection and further identifies potential targets for improving biofuel tolerance in cyanobacteria.

Keywords: biofuel toxicity, cyanobacterial biofuel toxicity, biofuel tolerance, cyanobacterial biofuel tolerance, cyanobacterial biofuels

1. Introduction

Microbes can be engineered to produce a variety of potential fuel products with varying carbon chain lengths and chemical properties (Figure 1). In fact, cyanobacteria have been successfully engineered to produce ethanol [1, 2], butanol [3, 4], isoprene [5, 6], squalene [7], free fatty acids [8, 9], fatty alcohols [10], and alkanes/alkenes [10, 11]. The productivities associated with microbial synthesis of these biofuels, however, is often too low to meet requirements for large-scale production. One of the major factors limiting high biofuel productivity is product toxicity. Product toxicity must therefore be an important consideration when selecting a cyanobacterial host and target biofuel product. Furthermore, mechanisms for improving cyanobacterial biofuel tolerance may be important targets for genetic manipulation to optimize biofuel production.

Attempts to engineer cyanobacteria for photosynthetic biofuel production have increased dramatically within the past decade, and as these fuel-producing cyanobacteria are characterized, an increasing number of observations of biofuel toxicity are reported. While this is not surprising, as biofuel toxicity has been a long-standing issue in heterotrophic biofuel production, additional characterization and investigation of biofuel toxicity in cyanobacteria and cyanobacterial biofuel tolerance is necessary to optimize biofuel production in these photosynthetic hosts. Reduced cell growth was observed during ethanol and isobutanol production in *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 [2, 3], and free fatty acid production resulted in reduced growth, decreased photosynthetic yield, reduced chlorophyll-a, increased reactive oxygen species, changes in membrane composition, and changes in gene expression in engineered *S. elongatus* PCC 7942 [9, 12]. Additionally, ethanol, isobutanol, and hexane production in *Synechocystis* sp. PCC 6803 led to significant

transcriptomic and proteomic responses, including changes in genes and proteins involved in stress response, transport, cell envelope modification, cell mobility, and photosynthesis [13-17]. While some reduction in cell growth is expected as biofuel production consumes carbon normally used for cell growth, these additional physiological effects indicate that biofuel production impacts the cyanobacterial host in a broad, systems-level manner.

To date, cyanobacterial biofuel tolerance has received little attention, particularly compared to the heterotrophic hosts *Escherichia coli* and *Saccharomyces cerevisiae* [18, 19]. Some earlier studies investigated the toxic effects of organic solvents on cyanobacterial growth to determine the environmental impact of pollutants and the feasibility of using cyanobacteria in aqueous-organic two-phase bioprocesses [20, 21]. However, the organic solvents tested in these studies are not ideal biofuel targets and some of the model cyanobacterial strains were not included in these toxicity tests. A more recent study investigated exogenous addition of low and medium chain alcohols, acids, aldehydes, and alkanes to two model freshwater cyanobacteria, *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 [22]. This study found the potential biofuel products to inhibit growth, with alcohols leading to the greatest inhibition. Moreover, *Synechocystis* sp. PCC 6803 was found to generally have a higher biofuel tolerance compared to *S. elongatus* PCC 7942. While this study provides important information on biofuel toxicity and cyanobacterial tolerance, it does not cover key aspects, including the toxicity and tolerance to longer chain biofuel products (C16 – C18) which are more likely to be metabolically produced in cyanobacteria, the tolerance of marine cyanobacterial strains such as the model cyanobacterium *Synechococcus* sp. PCC 7002, and the identification of potential biofuel tolerance mechanisms in cyanobacteria.

While mechanisms of biofuel toxicity for cyanobacteria have yet to be identified, mechanisms of toxicity in heterotrophic, Gram-negative bacteria such as *E. coli* have been extensively studied. The primary mechanism of toxicity for biofuels initiates through their interaction with and integration into cellular membranes [23, 24]. This biofuel-membrane interaction alters membrane fluidity and permeability, ultimately leading to inhibition of membrane-associated enzymes, disruption of the electron transport chain and oxidative phosphorylation, dissipation of the proton motive force, loss of intracellular pH homeostasis, and possibly even cell lysis [18, 24, 25]. Biofuels with long carbon chains (generally greater than C12) are typically less toxic to microbes [26], and unsaturated free fatty acids have greater toxicity than their saturated counterparts, presumably due to lipid peroxidation [25]. Additional information about biofuel toxicity and tolerance can be found in recent reviews [26-30].

In this study, we aim to advance and expand the current knowledge of cyanobacterial biofuel toxicity and tolerance to help inform the selection of cyanobacterial hosts and product targets for biofuel strain development efforts. We investigate three model cyanobacteria strains, freshwater strains *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 and a marine strain *Synechococcus* sp. PCC 7002. The inclusion of a marine cyanobacterium is important, as it will likely have different membrane composition and properties compared to the freshwater species. Moreover, cellular responses to organic solvents and biofuels are often similar to other stress responses [29]; therefore, *Synechococcus* sp. PCC 7002, with its high light, high temperature, and high salt tolerances, may also have high biofuel tolerance [31, 32]. The biofuel products investigated in this study include those readily produced from the cyanobacterial metabolism (Figure 1): ethanol, isobutanol, 1-hexadecanol, α -linolenic acid (unsaturated FFA), palmitic and stearic acids (saturated FFAs), pentadecane, and 1-heptadecene. These long-chain biofuel targets

(C15 – C18) are likely biofuel targets based on the fatty acid composition of cyanobacterial membranes, which are predominantly C16 and C18 FAs [33]. This study analyzes the growth inhibition and physiological effects of biofuel addition to cyanobacterial cultures along with a preliminary investigation into three possible mechanisms of natural biofuel tolerance in cyanobacteria: cell membrane composition, reactive oxygen species degradation, and efflux pump activity.

2. Results and Discussion

2.1 Cyanobacterial Growth Inhibition and Physiological Effects of Exogenous Biofuels

As described in the Introduction, three model cyanobacterial species were characterized for toxic or inhibitory effects upon exogenous addition of potential metabolically-produced fuels or fuel precursors (collectively referred to as ‘biofuels’ in the subsequent text) (Figure 1). For each target biofuel, the concentration of biofuel was increased until either (1) complete growth inhibition was observed 5 days after inoculation in the biofuel-spiked medium or (2) the concentration exceeded the solubility limit of the biofuel in aqueous solution. Biofuel solubility was particularly problematic for targets containing long chain hydrocarbons (1-hexadecanol, linolenic acid, palmitic acid, stearic acid, pentadecane, and 1-heptadecene). The experimental or predicted aqueous solubilities for each biofuel are listed in the Supplemental File, Table S1. Due to difficulties in obtaining accurate concentrations at the low solubility limits, the tested concentration ranges are often well above the solubility limits. Due to the volatile nature of the short-chain biofuel products, namely ethanol and isobutanol, evaporation of the biofuel is likely to occur under the cultivation conditions of 30°C. As this inherent property is difficult to control, the reader should note that the concentrations reported in this study are initial concentrations of the biofuel in the culture.

The effects of biofuel addition on cell growth are shown in Figure 2. The short-chain alcohols, ethanol and isobutanol, had inhibitory effects on the growth of all three strains within 5 days of biofuel addition. Of the three model strains, *Synechococcus* sp. PCC 7002 was most tolerant of short chain alcohols, showing some growth at ethanol concentrations as high as 0.6 M and isobutanol concentrations as high as 0.08 M (Figure 2A,B). On the other hand, *S. elongatus* PCC 7942 was the least tolerant with growth at ethanol concentrations as high as 0.2 M and isobutanol concentration as high as 0.02 M. Overall, cyanobacterial ethanol and isobutanol tolerance is slightly lower than that of *Escherichia coli*, which has native tolerances up to 1.03 M and 0.13 M, respectively [18, 34]. While these short chain alcohols proved toxic to cyanobacteria, the long-chain fatty alcohol, 1-hexadecanol, did not lead to any observable growth inhibition for all 3 cyanobacterial strains under the concentrations tested (Figure 2C). Due to the low solubility of 1-hexadecanol, concentrations higher than 0.75 μ M were not tested. While it's anticipated that higher concentrations would not generate a toxic effect due to phase separation of 1-hexadecanol, this still remains to be investigated.

The toxicity of long-chain free fatty acids (FFAs) was dependent upon the degree of saturation. Saturated FFAs, palmitic (16:0) and stearic (18:0) acids, did not have an inhibitory effect on any of the model cyanobacterial strains (Figure 2E,F); however, the polyunsaturated FFA, α -linolenic acid (18:3), reduced cell growth in all 3 cyanobacteria (Figure 2D). Interestingly, *Synechococcus* sp. PCC 7002 was the most susceptible to α -linolenic acid (LA) toxicity, with complete inhibition at 25 μ M of LA, while *Synechocystis* sp. PCC 6803 was the most tolerant, showing no significant effect on growth at 25 μ M of LA. Growth of both *Synechocystis* sp. PCC 6803 and *S. elongatus* PCC 7942 was completely inhibited at 50 μ M of LA. These results suggest that different mechanisms contribute to short-chain alcohol and

unsaturated FFA tolerances in cyanobacteria. Furthermore, this illustrates that biofuel toxicity may be observed at concentrations exceeding the solubility limit of the biofuel, as growth inhibition of *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 is only observed at concentrations (10 and 50 μ M, Figure 2D) exceeding the predicted solubility limit for LA (0.444 μ M, Supplemental File - Table S1).

The long-chain alkane, pentadecane (C15), and long-chain alkene, 1-heptadecene (C17:1), showed no significant inhibitory effect on *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, even at concentrations exceeding the solubility limits by 10 to 11 orders of magnitude (Figure 2G,H). *S. elongatus* PCC 7942, however, had a 92% reduction in growth at pentadecane concentrations exceeding 50 mM (Figure 2G) and a 15% reduction in growth at 100 mM of 1-heptadecene (Figure 2H). These results are in agreement with previous toxicity studies using undecane (C11), which showed growth inhibition of *S. elongatus* PCC 7942 at 51 and 96 mM and only slight inhibition of *Synechocystis* sp. PCC 6803 at the same concentrations [22]. Again, these results confirm that toxicity effects may be observed at concentrations exceeding the solubility limit.

In addition to biofuel-induced growth inhibition, photosynthetic yield measurements were also taken as an indication of the overall cell health of the cyanobacteria (Figure 3) [35]. In general, the photosynthetic yield measurement correlated with growth inhibition, with reduced photosynthetic yields accompanying reduced growth. One exception was the photosynthetic yield of *Synechococcus* sp. PCC 7002 in response to 1-heptadecene (Figure 3H). The growth of *Synechococcus* sp. PCC 7002 was unaffected by all concentrations of 1-heptadecene tested; however, photosynthetic yield was significantly decreased in *Synechococcus* sp. PCC 7002 cultures with the addition of 50 and 100 mM of 1-heptadecene. The reduced photosynthetic yield

measurements indicate a reduced efficiency of electron transport through photosystem II. As photosynthesis drives carbon fixation and energy production, it is surprising that these cultures do not also show reduced growth. It is possible that later time points would show growth inhibition in these cultures.

2.2 Cyanobacterial Ethanol Tolerances of Exponential Phase Cultures

During microbial biofuel production, biofuel accumulation generally correlates with cell growth, reaching high levels only in the late exponential or stationary phases [9]. Therefore, it is important to not only test growth inhibition but to also investigate the tolerance of exponential phase cultures to biofuel addition. To this end, ethanol was added to cyanobacterial cultures during the mid-exponential growth phase ($OD_{730} \sim 0.3$) at various concentrations, with higher ethanol concentrations for *Synechococcus* sp. PCC 7002 due to its improved tolerance. Compared to the control, with no ethanol addition, all three strains showed growth inhibition (Figure 4A,D,G). Again, the photosynthetic yield measurements correlated with the growth measurements (Figure 4B,E,H), but the photosynthetic yield measurements showed a faster response, with F_v'/F_m' measurements near zero within 10 hours after ethanol addition. There is one notable exception, however; the photosynthetic yield measurements for *Synechocystis* sp. PCC 6803 with 0.5 M ethanol remained nearly as high as the no ethanol control after a slight dip in the first few hours following ethanol addition (Figure 4E). Interestingly, this high photosynthetic yield did not correlate with improved growth (Figure 4D), suggesting that the excess electrons are dissipated or utilized for a function other than cell growth. This result cautions against the sole reliance on photosynthetic yield measurements to determine cell health of cyanobacterial cultures.

As described in the Introduction, one of the main mechanisms for biofuel toxicity is reported to be the disruption of the cell membrane. To determine whether ethanol addition disrupts the cell membrane, the membrane impermeable fluorescent dye, SYTOX, was used to measure cell membrane permeability (Figure 4C,F,I). All three cyanobacterial strains showed increased membrane permeability with the addition of ethanol, with the percentage of membrane-permeable cells increasing with increasing ethanol concentrations. However, ethanol concentrations as high as 0.5 M do not have a significant effect on membrane permeability in the three cyanobacteria despite the significant reduction in cell growth and photosynthetic yield (with the exception of *Synechocystis* sp. PCC 6803) at this ethanol concentration. Unexpectedly, *Synechocystis* sp. PCC 6803 had a lower percentage of membrane-permeable cells (a maximum of 51%, Figure 4F – green diamonds) compared to the more ethanol resistant *Synechococcus* sp. PCC 7002 (99% at the same time point for the same ethanol concentration, Figure 4I – blue triangles). Overall, these results suggest that membrane permeability is increased by ethanol addition, but that the level of permeability does not directly correlate with the inhibition of growth or reduction in photosynthetic yield.

2.3 Cell Membrane Composition Influences Cyanobacterial Biofuel Tolerance

In addition to permeabilizing the membrane, biofuels are also predicted to intercalate within cellular membranes, affecting membrane fluidity and the activity membrane-associated enzymes. In cyanobacteria, membrane-associated enzymes are not only responsible for the transport and export of nutrients and metabolites, they also generate cellular energy and reducing equivalents via photosynthesis in the intracellular thylakoid membranes. Based on this possible mechanism of toxicity, the chemical composition of the cell and thylakoid membranes may play an important role in cyanobacterial biofuel tolerance. Both *S. elongatus* PCC 7942 and

Synechocystis sp. PCC 6803 are freshwater strains which may be expected to have similar membrane compositions, while the marine strain *Synechococcus* sp. PCC 7002 may have a unique membrane composition to allow for its higher salt tolerance.

To investigate changes in the chemical composition of membranes among the three strains, lipids were extracted from late exponential phase cultures and analyzed using ESI-MS. Significant differences in the degree of saturation of membrane lipids were measured between the three cyanobacteria (Table 1). *S. elongatus* PCC 7942 had predominantly monounsaturated lipid molecules (74.3%) while *Synechocystis* sp. PCC 6803 had a high level of tri-unsaturated lipids (63.1%) and *Synechococcus* sp. PCC 7002 had a distribution of mono (21.2%), di (27.3%) and tri (39.7%) –unsaturated lipids. Previous studies suggest that microbial salt tolerance can be improved by decreasing membrane viscosity [36]. Therefore, the lower degree of saturation of membrane lipids in *Synechococcus* sp. PCC 7002 may contribute to both the salt and short-chain alcohol tolerances of this strain compared to *S. elongatus* PCC 7942, yet this does not explain the lower salt and short-chain alcohol tolerances of *Synechocystis* sp. PCC 6803 in comparison to *Synechococcus* sp. PCC 7002. The lipid analysis presented in Table 1 is a whole cell analysis, including both cell and thylakoid lipids. In this analysis, the thylakoid lipids accounted for 94.7 – 99.6% of the total signal, with the cell membrane lipids comprising only 0.4 – 5.3% of the signal. As the thylakoids membranes are intracellular, their composition may differ from the composition of the inner and outer cell membranes. While the signals for phosphatidylglycerol (PG), a major component of the cell membrane, are too low and variable to draw statistically significant conclusions, *S. elongatus* PCC 7942 had primarily monounsaturated signal while *Synechococcus* sp. PCC 7002 had mono and di-unsaturated signals, and PG signals from *Synechocystis* sp. PCC 6803 were too low for detectable signal above 1% of the total. Therefore,

although thylakoid and cell membrane biosynthesis utilize the same pathway for acyl-ACP provision, chemical variations in fatty acid composition between these membranes may explain why *Synechocystis* sp. PCC 6803 does not have higher ethanol tolerance.

Comparative genomics using the three model cyanobacterial strains was used to identify desaturases that may contribute to the observed differences in the degree of membrane saturation (Table 2). The number of desaturase genes in the genome generally correlates with the measured degree of saturation ($7942 < 6803 < 7002$). Both *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 have a significantly greater number of desaturases within their genomes compared to *S. elongatus* PCC 7942. *Synechococcus* sp. PCC 7002, the most ethanol tolerant of all 3 strains, has two desaturases unique to its genome, *desE* (SYNPCC7002_A2833) and *desF* (SYNPCC7002_A1989). These two desaturase genes along with *desB* (SYNPCC7002_A0159), a desaturase shown to be important for low temperature tolerance [37], were targeted for gene knockout in *Synechococcus* sp. PCC 7002 to assess their role in ethanol tolerance. No significant change in ethanol tolerance was observed for the *desE* and *desF* mutants (7002 Δ 2833 and 7002 Δ 1989 in Figure 5A); however, ethanol tolerance was reduced in the *desB* mutant (7002 Δ 0159) at a concentration of 0.5 M ethanol. This suggests that the degree of saturation of membrane lipids may play a role in short-chain alcohol tolerance. It is unlikely that this is the sole mechanism of short-chain alcohol tolerance, as *desB* gene knockout did not lead to a drastic reduction in ethanol tolerance and *Synechocystis* sp. PCC 6803 also contains *desB* but has lower short-chain alcohol tolerance.

2.3 Reactive Oxygen Species and Biofuel Toxicity

Generally, microbes under stress conditions have been shown to have high levels of reactive oxygen species (ROS). The ROS themselves can be toxic to the cell, leading to lipid

peroxidation as well as protein and DNA damage [38]. It is likely that biofuel stress also leads to ROS generation, which may contribute to the observed toxicity. In fact, the reduction of ROS accumulation by overexpression of sigma factor SigB improved butanol tolerance in *Synechocystis* sp. PCC 6803 [39]. Our attempts to measure ROS levels in *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 using a fluorescent ROS stain (2,7-dichloro fluorescein diacetate - H₂DCFDA) in response to ethanol addition were largely unsuccessful due to high variability; however, the addition of ethanol to cultures of *Synechococcus* sp. PCC 7002 resulted in statistically significant increases in the percentage ROS-positive cells (up to $60 \pm 33\%$) compared to the control with no ethanol addition ($1.4 \pm 0.63\%$). Furthermore, our previous work investigating FFA production in engineered strains of *S. elongatus* PCC 7942 showed elevated ROS levels in the FFA-producing strains relative to the wild type (cite). This data indicates that ROS generation may be a contributing factor in biofuel toxicity.

Comparative genomics analysis of ROS-degrading proteins did not reveal any significant genomic differences between the three cyanobacterial strains (Table 2). All three strains have approximately the same number of ROS-degrading protein genes and only a few of these genes are unique to a particular species. To investigate whether ROS-degrading proteins contribute to biofuel tolerance, *S. elongatus* PCC 7942 was engineered to overexpress four ROS-degrading proteins: glutathione peroxidase (Synpcc7942_1214), catalase/peroxidase HPI (Synpcc7942_1656), glutathione peroxidase (Synpcc7942_0437), and superoxide dismutase (Synpcc7942_0801). *S. elongatus* PCC 7942 was selected as the host for ROS-degrading protein overexpression, as this strain was the most sensitive to ethanol. One of the target genes (Synpcc7942_0437) does not have a close homolog in the other two cyanobacterial strains (Table 2), while the other three targets have predicted homologs. The ethanol tolerances of all

four ROS-degrading protein overexpression mutants of *S. elongatus* PCC 7942 were very similar to that of the control strain (Figure 5B), suggesting that while ROS levels may be elevated in response to biofuel addition, they are not a significant contributing factor to biofuel toxicity.

2.4 Do Efflux Pumps Contribute to Cyanobacterial Biofuel Tolerance?

Previous studies have shown that efflux pump proteins can play a pivotal role in biofuel tolerance for *E. coli* [28]. Genomic analysis of predicted efflux pump protein genes in the three model cyanobacteria indicates that cyanobacteria possess numerous efflux pump proteins, yet the distribution of efflux pump proteins among the three strains is quite similar in both number and predicted function (Table 2). As a preliminary investigation of the role of efflux pump proteins in ethanol tolerance, several efflux pump protein genes were targeted for gene knockout in *Synechococcus* sp. PCC 7002: a multidrug efflux transporter (SYNPCC7002_A0719), an outer membrane efflux protein (SYNPCC7002_A0585), and a hydrophobe/amphiphile efflux-1 (HAE1) family protein (SYNPCC7002_A1013). SYNPCC7002_A1013 is a homolog of the AcrB protein of *E. coli*, which is a component of the TolC-AcrAB efflux pump involved in solvent tolerance and FFA export [28, 40]. The other two targets, SYNPCC7002_A0719 and SYNPCC7002_A0585, have homologs in *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803, but these homologs have low amino acid sequence identity with the *Synechococcus* sp. PCC 7002 targets (37.48-55.03%) and are annotated as hypothetical proteins. Hence, these two efflux pump genes in *Synechococcus* sp. PCC 7002 may contribute to its improved ethanol tolerance relative to the other cyanobacteria. All three efflux pump gene mutants (7002 Δ 0719, 7002 Δ 0585, and 7002 Δ 1013) showed ethanol tolerances similar to the wild type (Figure 5C). While this preliminary investigation did not identify an efflux pump protein involved in biofuel

tolerance, further investigation is necessary to rule out the contribution of efflux pump proteins to cyanobacterial biofuel tolerance.

3. Conclusions

For cyanobacterial biofuel production, there are many decisions which can affect biofuel productivity, such as selection of the fuel target and host strain selection. Biofuel targets have variable toxic effects on the host, and the host strain may have variable tolerance to each biofuel. The results of this study provide some guidance for these critical decisions. Long-chain alcohols, saturated FFAs, and alkanes/alkenes were found to have minimal effects on cyanobacterial growth and physiology. Therefore, long-chain alcohols and alkanes/alkenes may be good biofuel targets from a toxicity perspective. As the degree of saturation cannot be controlled for FFAs, it's likely that FFA production will have toxic effects on the cyanobacterial host. Indeed, this was observed previously during FFA production in *S. elongatus* PCC 7942 [9]. If toxic biofuel products are desirable targets for production, judicious host selection can help to minimize the detrimental impact on productivity. Based on the growth inhibition studies conducted in this work, *Synechococcus* sp. PCC 7002 should be a good host for short-chain alcohol production while *Synechocystis* sp. PCC 6803 is a good option for FFA production. Both *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 should be good hosts for alkane production due to their higher tolerances compared to *S. elongatus* PCC 7942. By selecting biofuel targets with low toxicity and cyanobacterial hosts with high biofuel tolerance, biofuel productivities should improve.

It is essential to interpret the results and conclusions of this study within the confines of the experimental parameters. One important caveat in the interpretation and application of the results in this study is that only extracellular biofuel toxicity was tested, while cyanobacterial

biofuel production will occur intracellularly. This distinction can have significant implications. For instance, a cyanobacterium may show high tolerance of an extracellular biofuel due to cell membrane properties which do not allow the biofuel to enter into the cell. When produced intracellularly, the same biofuel may have highly toxic effects. This was illustrated with FFA production in *E. coli*, whereby exogenous biofuel addition had minimal toxic effects and intracellular FFA production led to significant reductions in cell growth [41]. Additionally, ethanol production at the level of approximately 12.5 mM led to nearly a 60% reduction in cell growth of *Synechocystis* sp. PCC 6803 [2], while exogenous ethanol addition would require an ethanol concentration of greater than 200 mM according to the growth inhibition results of this study. A second caveat is that while biofuel toxicity can significantly impact productivity, it is certainly not the only factor to consider when selecting the biofuel target and cyanobacterial host. Cell growth, carbon flux through the biofuel pathway, energetic requirements, and cofactor requirements will also affect cyanobacterial biofuel productivities. These factors, therefore, should also be analyzed when optimizing productivity.

In addition to providing guidance regarding biofuel target and cyanobacterial host selection, this work investigated several possible mechanisms of cyanobacterial biofuel tolerance, including cell membrane composition, ROS generation, and efflux pump proteins. The lower ethanol tolerance of the *desB* mutant of *Synechococcus* sp. PCC 7002 confirms that cell membrane composition plays a role in short-chain alcohol tolerance. However, the ethanol tolerance of the *desB* mutant was not greatly reduced, showing complete growth inhibition at an ethanol concentration of 0.5 M, compared to 0.6 M for the wild type. Therefore, other molecular mechanisms likely contribute to cyanobacterial biofuel tolerance. Additional investigation into these mechanisms will not only increase our understanding of biofuel tolerance, it will also

identify potential metabolic engineering targets and strategies for improving cyanobacterial host tolerance. This study, along with the previous investigation into cyanobacterial biofuel tolerance [22], provide a good basis for further exploration of this important factor in the development of cyanobacterial biofuel production.

4. Materials and Methods

4.1 Materials

Chemicals used in this study were purchased from Acros Organics ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), BD (agar), MP Biomedicals (ferric ammonium citrate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Fisher Scientific (Na_2CO_3 , agarose, tris-acetate-EDTA, NaCl, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, NaNO_3 , tris base, H_3BO_3 , sodium thiosulfate pentahydrate, and kanamycin monosulfate), Gold Technology (IPTB) and Sigma-Aldrich (Na_2EDTA , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, KH_2PO_4 , vitamin B12, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, ZnCl_2 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and spectinomycin dihydrochloride pentahydrate). Primers were synthesized by Integrated DNA Technologies (IDT). DNA purifications were performed using the Plasmid Miniprep, DNA Clean & Concentrator, and Gel DNA Recovery kits from Zymo Research. Restriction enzymes, DNA polymerases, and ligases were purchased from New England Biolabs. Suppliers of all other materials used in this study are described below.

4.2 Bacterial Strains and Growth Conditions

Three model strains of cyanobacteria were analyzed for biofuel tolerance, including two freshwater strains, *Synechococcus elongatus* PCC 7942 (ATCC 33912) and *Synechocystis* sp. PCC 6803 (kindly provided by Jerilyn Timlin, Sandia National Laboratories), and one marine strain, *Synechococcus* sp. PCC 7002 (ATCC 27264). All strains used in this study are listed in

Table S2 (Supplemental File). The freshwater strains were cultured in BG-11 medium, and the marine strain was cultured in A+ medium. Cultures were grown at 30°C in a low temperature Shel Lab incubator with a VWR orbital shaker at 150 rpm under a continuous light intensity of approximately 40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ from plant & aquarium fluorescent lights. Cultures were maintained on agar plates and inoculated into test tubes containing 4 mL of liquid media.

4.3 Construction of Mutants

The ROS-overexpression mutants were constructed using *S. elongatus* PCC 7942 with genome integration of the plasmid at neutral site II (NSII). Plasmids for genome integration and the overexpression of 4 ROS-degrading proteins (pSA_0437, pSA_0801, pSA_1214, and pSA_1656) were constructed previously [12]. The plasmids were transformed as described in [42] and screened using BG-11/agar plates supplemented with 50 $\mu\text{g/mL}$ of kanamycin monosulfate and 1 mM of sodium thiosulfate. Successful transformation was confirmed by PCR amplification of NSII (primers: NSII5F and NSII3R, Table A6, [12]) and PCR amplification of the 5' homologous region of NSII along with the target gene (primers: NSII5F and 0437R/0801R/1214R/1656R, Table A6, [12]). A control strain, 7942a, was constructed with genome integration of the empty vector, pSA [43], into NSII.

The desaturase and efflux pump knockout mutants were constructed using *Synechococcus* sp. PCC 7002 and the corresponding knockout plasmids. The knockout plasmids were constructed by cloning ~ 500 bp homologous regions upstream and downstream of the 5' and 3' ends of the target gene sequence and integrating them into pSK, containing a kanamycin resistance cassette (Supplemental File, Table S5). These plasmids were linearized using *SpeI* digestion and transformed into *Synechococcus* sp. PCC 7002 as described in [42]. Transformed colonies were re-streaked a minimum of 3 times on medium A+ agar plates containing 50 $\mu\text{g/mL}$

kanamycin monosulfate and 1 mM sodium thiosulfate to obtain homozygous strains. Gene knockouts were confirmed via colony PCR of the transformants using the 5' forward and 3' reverse primers in Table S5 (Supplemental File).

4.4 Biofuel Growth Inhibition and Toxicity Studies

To assess biofuel growth inhibition, the 3 cyanobacterial strains were cultivated in 16 x 100 mm test tubes with 4 mL of medium. When culture densities reached an OD₇₃₀ of 1.0, 20 µL of culture were inoculated into a test tube containing 4 mL of medium with varying biofuel concentrations. The short chain alcohols, absolute ethanol (Fisher) and isobutanol (2-methyl-1-propanol, Fisher), were added directly to the culture medium to achieve the final biofuel concentrations. The long-chain alcohol, 1-hexadecanol (Ultra Scientific), was heated in a water bath at 50°C until melted, and the liquid 1-hexadecanol was added to the culture medium and vortexed to produce a 100 µM stock solution. This stock solution was further diluted with medium to reach the final biofuel concentrations. For the saturated FFAs, 500 µM stock solutions of palmitic acid and stearic acid were made by dissolving solid sodium palmitate and sodium stearate (Acros Organics) in the culture medium; these stock solutions were further diluted with medium to reach the final biofuel concentrations. For the polyunsaturated FFA, linolenic acid, a 200 µM stock solution of α-linolenic acid (Acros Organics) in medium was prepared fresh to prevent oxidative degradation and immediately diluted to achieve the specified biofuel concentrations. The long-chain alkane (n-pentadecane, Acros organics) and alkene (1-heptadecene, Acros Organics) were added directly to the medium to reach the final biofuel concentrations. For engineered strains containing inducible gene overexpression (7942_0437, 7942_0801, 7942_1214, and 7942_1656), IPTG was added at a concentration of 1 mM prior to inoculation. Cultures were grown for 5 days under constant light in slanted test tube racks as

described above. On day 5, cell concentration (OD_{730}) and photosynthetic yield (F_v'/F_m') were measured. The OD_{730} value for each culture was normalized using the OD_{730} of the control culture with no biofuel addition. A minimum of three biological replicates were analyzed for each biofuel concentration; data is reported as the average of these measurements with error bars representing the standard deviation. For data with high standard deviation, additional biological replicates were performed, up to a total of 5 replicates.

To determine biofuel toxicity, the 3 cyanobacterial strains were cultivated in 500 mL baffled Erlenmeyer flasks with 110 mL of media. When culture densities reached an OD_{730} of 1.0, the culture was aliquoted into four 125 mL Erlenmeyer flasks, with 25 mL of culture in each flask. Absolute ethanol (Fisher) or α -linolenic acid (Acros Organics) was added directly to these cultures to achieve the specified biofuel concentration. The cultures were grown under the conditions described previously, and each culture was sampled at 0 h (before the addition of biofuel), 4 h, 8h, 24 h, and 48 h. For each sample, the cell concentration (OD_{730}), photosynthetic yield (F_v'/F_m'), level of ROS, and cell membrane permeability were analyzed. A minimum of three biological replicates were analyzed for each data point; data is reported as the average of these measurements with error bars representing the standard deviation.

4.5 Spectroscopic Measurements

Cell concentration was determined using the optical density (OD) at 730 nm. Optical densities were measured using a LambdaBio spectrophotometer from PerkinElmer. OD_{730} readings were normalized to the control OD_{730} , having no biofuel addition.

To measure photosynthetic yield (F_v'/F_m'), a Waltz MINI-PAM photosynthesis yield analyzer was used by pressing the probe flat against the glass surface of the culture and

averaging the readings of three technical replicates for each data point. The cultures were not dark-adapted prior to this measurement.

4.6 Cell Membrane Permeability and ROS Measurements

The membrane-impermeable dye, SYTOX Green Nucleic Acid Stain (Invitrogen/Life Technologies), was used to assess cell membrane permeability upon biofuel addition in the toxicity studies. A 30 μ M stock solution of SYTOX was prepared using dimethyl sulfoxide (DMSO, Sigma). For SYTOX staining, 0.5 mL of culture was extracted from the 25 mL culture and placed into a 1.5 mL microcentrifuge tube. The SYTOX stock solution was added to the sample to obtain a final concentration of 300 nM. Samples were inverted after SYTOX addition and incubated in the dark at room temperature for 20 min. Samples were then diluted 20 – 100x before running on an Accuri C6 flow cytometer. The flow cytometer was used to analyze the cell population, using a 20,000 event limit, slow fluidics speed, and threshold of 40,000 for FSC-H. Cells with increased fluorescence emission at 533 nm were determined to have positive SYTOX staining, indicating enhanced cell membrane permeability.

The ROS-reactive dye, 2,7-dichloro fluorescein diacetate (H_2DCFDA , Sigma-Aldrich), was used to assess intracellular ROS levels. A stock solution of 10.2 mM H_2DCFDA was prepared using DMSO. For ROS staining, 0.5 mL of culture was extracted and placed into a 1.5 mL microcentrifuge tube. The H_2DCFDA stock solution was added to the sample to achieve a final concentration of 102 μ M. DMSO (20 μ L) was also added to permeabilize the cells and allow for H_2DCFDA uptake. Samples were inverted after H_2DCFDA addition and incubated under growth conditions for 1 h. Samples were then diluted 20 – 100x and analyzed using flow cytometry with the aforementioned settings. Cells with increased fluorescence emission at 533 nm were determined to be positive for elevated ROS levels.

4.7 Lipid Analysis

Each of the 3 model cyanobacterial strains was cultivated in a 500 mL baffled Erlenmeyer flask with 100 mL of medium. After 5 days of continuous cultivation, 50 mL of culture was used for lipid extraction. Cellular lipids were extracted using a chloroform-methanol extraction [9] and dried under a stream of nitrogen. The dried samples were solubilized and analyzed using electrospray ionization – mass spectrometry (ESI-MS) at the Kansas Lipidomics Research Center. For each strain, three biological replicates were analyzed, and the data is an average of these replicates $\pm$ the standard deviation of these measurements.

4.8 Comparative Genomics

The genome sequences of the 3 cyanobacterial species, *S. elongatus* PCC 7942, *Synechococcus* sp. PCC 7002, and *Synechocystis* sp. PCC 6803, were analyzed to identify genes associated with 3 potential mechanisms of biofuel tolerance: 1) the degree of membrane saturation, 2) ROS-degrading proteins, and 3) efflux pump proteins. The genomes were mined manually for predicted genes with functional annotations related to these potential mechanisms. As the level of functional annotation may differ among these three genomes, a comparative genome analysis was performed using the Microbial Genome Database (MBGD) for Comparative Analysis [44] to identify homologs among the three cyanobacterial species. The results of these analyses are summarized in Table 2, with homologous genes listed in the same row.

5. Abbreviations

2-AL	2-acetolactate
2,3-DHIV	2,3-dihydroxy-isovalerate
2-KIV	2-ketoisovalerate

489	2-OG	2-oxoglutarate
490	3-PGA	3-phosphoglycerate
491	AA	acetaldehyde
492	ACP	acyl carrier protein
493	acyl-A	acyl-aldehyde
494	CL	cardiolipin
495	CoA	Coenzyme A
496	DMSO	dimethyl sulfoxide
497	ESI-MS	electrospray ionization – mass spectrometry
498	EtOH	ethanol
499	F6P	fructose-6-phosphate
500	FA	fatty acid
501	FFA	free fatty acid
502	Glc	glucose
503	H ₂ DCFDA	2,7-dichloro fluorescein diacetate
504	IBA	isobutyraldehyde
505	IBOH	isobutanol
506	IPTG	isopropyl β -D-1-thiogalactopyranoside
507	LA	α -linolenic acid
508	LPS	lipopolysaccharides
509	MBGD	microbial genome database
510	OAA	oxaloacetate
511	OD	optical density

512	P _{ow}	partition coefficient in n-octanol and water
513	PE	phosphatidylethanolamine
514	PEP	phosphoenolpyruvate
515	PG	phosphatidylglycerol
516	PPP	pentose phosphate pathway
517	PYR	pyruvate
518	ROS	reactive oxygen species
519	RuBP	ribulose-1,5-bisphosphate
520	TCA	tricarboxylic acid
521	Val	valine

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9. Figure Captions

Figure 1. Simplified schematic of cyanobacterial metabolism. Potential biofuel targets are highlighted by dashed boxes; dashed arrows indicate non-native enzymatic steps. Abbreviations - 2-AL: 2-acetolactate; 2,3-DHIV: 2,3-dihydroxy-isovalerate; 2-KIV: 2-ketoisovalerate; 2-OG: 2-oxoglutarate; 3-PGA: 3-phosphoglycerate; AA: acetaldehyde; ACP: acyl carrier protein; acyl-A: acyl-aldehyde; CoA: Coenzyme A; EtOH: ethanol; F6P: fructose-6-phosphate; FFA: free fatty acid; Glc: glucose; IBA: isobutyraldehyde; IBOH: isobutanol; OAA: oxaloacetate; PEP: phosphoenolpyruvate; PPP: pentose phosphate pathway; PYR: pyruvate; RuBP: ribulose-1,5-bisphosphate; TCA: tricarboxylic acid; Val: valine.

Figure 2. Relative growth inhibition of three model cyanobacteria exposed to various concentrations of biofuel products. Optical densities are normalized to an $OD_{730} = 1.0$ for the control cultures, with no biofuel addition. Data are an average of at least 3 biological replicates; measurements with high variation between replicates were independently repeated up to 5 times. Error bars represent the standard deviation of biological replicates. Abbreviations – 7942: *S. elongatus* PCC 7942; 6803: *Synechocystis* sp. PCC 6803; 7002: *Synechococcus* sp. PCC 7002.

Figure 3. Photosynthetic yields (F_v'/F_m') of three model cyanobacteria exposed to various concentrations of biofuel products. Each measurement is the average of 3 technical replicate measurements, and data are an average of at least 3 biological replicates. Error bars represent the standard deviation of the biological replicates, as this was more significant than the technical replicate variation. Abbreviations – 7942: *S. elongatus* PCC 7942; 6803: *Synechocystis* sp. PCC 6803; 7002: *Synechococcus* sp. PCC 7002.

Figure 4. Physiological effects of ethanol toxicity in cyanobacterial cultures during the exponential growth phase. Physiological measurements include normalized growth (A, D, and G), photosynthetic yields (F_v'/F_m') (B, E, and H), and percentage of membrane-permeable cells (C, F, and I) for cultures of *S. elongatus* PCC 7942 (A, B, and C), *Synechocystis* sp. PCC 6803 (D, E, and F), and *Synechococcus* sp. PCC 7002 (G, H, and I). Note: Ethanol concentrations are higher for *Synechococcus* sp. PCC 7002 cultures (G, H, and I) due to its higher ethanol tolerance. Data are an average of at least 3 biological replicates with error bars representing the standard deviation.

Figure 5. Relative growth inhibition of genetically modified cyanobacteria exposed to various concentrations of ethanol. Optical densities are normalized to an $OD_{730} = 1.0$ for the control cultures, with no biofuel addition. Cyanobacterial mutants include desaturase knockout mutants of *Synechococcus* sp. PCC 7002 (A), ROS-degrading protein overexpression mutants of *S. elongatus* PCC 7942 (B), and efflux pump mutants of *Synechococcus* sp. PCC 7002 (C). Data are an average of at least 3 biological replicates with error bars representing the standard deviation.

10. Tables – see attached files

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Table 1

Table 1. Degree of saturation of fatty acids in lipids of 3 model cyanobacteria: *S. elongatus* PCC 7942, *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7002. Composition determined using ESI-MS. Data are averages of 3 biological replicates, reported with the standard deviation.

Strain	Number of Double Bonds in Lipid Molecules (% of total signal)						
	0	1	2	3	4	5	6
7942	2.07 ± 0.185	74.3 ± 3.65	15.1 ± 1.26	5.49 ± 9.15	2.87 ± 0.792	0.054 ± 0.034	0.128 ± 0.016
6803	1.48 ± 0.234	9.83 ± 0.913	15.7 ± 1.79	63.1 ± 5.16	7.92 ± 0.914	1.45 ± 0.123	0.534 ± 0.323
7002	0.945 ± 0.327	21.2 ± 2.79	27.3 ± 1.63	39.7 ± 8.89	9.63 ± 0.623	1.11 ± 0.924	0.060 ± 0.039

Table 2

Table 2. Comparative genomics for 3 model cyanobacterial strains. Genes which may contribute to cyanobacterial biofuel tolerance are analyzed, including desaturases, ROS-degrading proteins, and efflux pump proteins. Homologous genes are listed in each row.

<i>Synechococcus elongatus</i> PCC 7942		<i>Synechocystis</i> sp. PCC 6803		<i>Synechococcus</i> sp. PCC 7002	
Locus Tag	Description	Locus Tag	Description	Locus Tag	Description
<i>Desaturases</i>					
Synpcc7942_2561	delta-9 acyl-phospholipid desaturase	sll0541	acyl-CoA desaturase (DesC)	SYNPCC7002_A2198	delta-9 acyl-lipid desaturase (DesC)
		slr1350	fatty acid desaturase, homology to sll1441 (DesA)	SYNPCC7002_A2756	homology to SYNPCC70025_A0159 (DesA)
		sll1441	delta 15 desaturase, homology to slr1350 (DesB)	SYNPCC7002_A0159	omega-3 acyl-lipid desaturase, homology to SYNPCC7002_A2756 (DesB)
		sll0262	delta-6 desaturase (DesD)		
				SYNPCC7002_A1989	syn-2, delta 9 acyl-lipid fatty acid desaturase (DesF)
				SYNPCC7002_A2833	fatty acid desaturase (DesE)
<i>ROS-Degrading Proteins</i>					
Synpcc7942_0801	superoxide dismutase	slr1516	superoxide dismutase (SodB)	SYNPCC7002_A0242	Mn-superoxide dismutase (SodB)
Synpcc7942_1214	glutathione peroxidase	slr1171	glutathione peroxidase	SYNPCC7002_A0117	glutathione peroxidase (Gpo)
Synpcc7942_1656	catalase/oxidase HPI	sll1987	catalase HPI (KatG)	SYNPCC7002_A2422	catalase/oxidase HPI (KatG)
Synpcc7942_1937	peptide methionine sulfoxide reductase	sll1394	methionine sulfoxide reductase A (MsrA)	SYNPCC7002_A0215	methionine sulfoxide reductase A (MsrA)
Synpcc7942_2190	methionine sulfoxide	sll1680	methionine	SYNPCC7002_A0672	methionine-R-sulfoxide

	reductase B		sulfoxide reductase B (MsrB)		reductase (MsrB)
Synpcc7942_0437	glutathione peroxidase				
Synpcc7942_B2620	putative catalase				
		slr1992	glutathione peroxidase	SYNPCC7002_A0970	glutathione peroxidase
		slr1795	methionine sulfoxide reductase A (MsrA)		
<i>Efflux Pump Proteins</i>					
Synpcc7942_1869	cation efflux system protein	sll0142	cation or drug efflux system protein	SYNPCC7002_A0587	cation efflux system protein (CzcA)
Synpcc7942_1938	multidrug-efflux transporter	slr0944	hypothetical protein	SYNPCC7002_A0589	arsenite efflux pump ACR3
Synpcc7942_2032	multidrug-efflux transporter quinolone resistance protein (NorA)	sll1154	quinolone resistance protein (NorA)	SYNPCC7002_A0087	major facilitator transporter
Synpcc7942_2369	hydrophobe/amphiphile efflux-1 HAE1	slr2131	cation or drug efflux system protein	SYNPCC7002_A1013	hydrophobe/amphiphile efflux-1 (HAE1) family protein
Synpcc7942_2368	secretion protein (HlyD)	sll0180	hypothetical protein	SYNPCC7002_A1574	RND family efflux transporter MFP subunit
Synpcc7942_1989	cation diffusion facilitator family transporter	sll1263	hypothetical protein	SYNPCC7002_A2463	cation efflux system protein
Synpcc7942_0553	secretion protein (HlyD)	slr1207	hypothetical protein	SYNPCC7002_A2552	RND family efflux transporter MFP subunit
Synpcc7942_1761	hypothetical protein	slr1270	hypothetical protein	SYNPCC7002_A0585	Outer membrane efflux protein
Synpcc7942_1870	secretion protein (HlyD)	sll0141	hypothetical protein	SYNPCC7002_A0591	RND family efflux transporter MFP subunit

Synpcc7942_0985	hypothetical protein	slr1935	hypothetical protein	SYNPCC7002_A0719	multidrug efflux transporter
Synpcc7942_1699	MATE efflux family protein	slr0896	Probable multidrug resistance protein (NorM)		
Synpcc7942_0792	multidrug efflux MFS transporter				
		slr0369	cation or drug efflux system protein		
		slr0794	cation or drug efflux system protein		
		slr0454	cation or drug efflux system protein		
				SYNPCC7002_A1483	RND family efflux transporter MFP subunit
				SYNPCC7002_A1723	HlyD family secretion protein, homology to SYNPCC7002_A1483
				SYNPCC7002_A2628	AcrB/AcrD/AcrF family protein

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5