Proteomic analysis reveals resistance mechanism against biofuel hexane in *Synechocystis* sp. PCC 6803

Jie Liu¹,², Lei Chen¹,², Jiangxin Wang¹,², Jianjun Qiao¹,² and Weiwen Zhang¹,²*

**Abstract**

**Background:** Recent studies have demonstrated that photosynthetic cyanobacteria could be an excellent cell factory to produce renewable biofuels and chemicals due to their capability to utilize solar energy and CO₂ as the sole energy and carbon sources. Biosynthesis of carbon-neutral biofuel alkanes with good chemical and physical properties has been proposed. However, to make the process economically feasible, one major hurdle to improve the low cell tolerance to alkanes needed to be overcome.

**Results:** Towards the goal to develop robust and high-alkane-tolerant hosts, in this study, the responses of model cyanobacterial *Synechocystis* PCC 6803 to hexane, a representative of alkane, were investigated using a quantitative proteomics approach with iTRAQ - LC-MS/MS technologies. In total, 1,492 unique proteins were identified, representing about 42% of all predicted protein in the *Synechocystis* genome. Among all proteins identified, a total of 164 and 77 proteins were found up- and down-regulated, respectively. Functional annotation and KEGG pathway enrichment analyses showed that common stress responses were induced by hexane in *Synechocystis*. Notably, a large number of transporters and membrane-bound proteins, proteins against oxidative stress and proteins related to sulfur relay system and photosynthesis were induced, suggesting that they are possibly the major protection mechanisms against hexane toxicity.

**Conclusion:** The study provided the first comprehensive view of the complicated molecular mechanism employed by cyanobacterial model species, *Synechocystis* to defend against hexane stress. The study also provided a list of potential targets to engineer *Synechocystis* against hexane stress.

**Keywords:** Hexane, Tolerance, Proteomics, *Synechocystis*

**Background**

Human society has been depending on fossil fuels in the past centuries. However, fossil fuels are not infinite resources, and a sharp price increase of these natural resources in recent years has posed an urgent call for alternative ways to produce fuels and chemicals. Moreover, over-utilizing fossil fuels has also caused environmental issues such as global warming and pollution. To address these issues, photosynthetic cyanobacteria have attracted significant attention recently as a cell factory to produce renewable biofuels and chemicals due to their capability to utilize solar energy and CO₂ as the sole energy and carbon sources [1-4]. In addition, cyanobacteria have a relatively simple genetic background and are easy for genetic manipulation [5,6]. In recent studies, two approaches have been taken to utilize cyanobacteria for biofuel production: i) to isolate fatty acids from lipid-rich cyanobacterial biomass and then convert them chemically to other products, such as biodiesel [7,8]. However, lipid extraction process from cyanobacteria is very energy-intensive and has been one of the major hurdles for commercial application [9-11]; and ii) to employ genetically manipulated cyanobacteria to produce secreteable fuel products directly. So far the second approach has led to successful production of a dozen of fuel products in engineered cyanobacterial systems, including ethanol [12,13], ethylene [14], isoprene...
[15], free fatty acids [16], fatty alcohols [17], isobutyraldehyde [18], 1-butanol [19,20] and hydrogen [21,22]. Although the current productivity level by these systems is still very low, the studies clearly demonstrated the feasibility of developing sustainable production systems based on cyanobacterial cells.

Biofuels offer a diverse range of promising alternatives. Although currently ethanol constitutes 90% of all biofuels in the United States, other fuels with better chemical properties, such as bio-based alkane due to their low water solubility and high energy density [23], are also being pursued around the world [24,25]. Alkanes composed of 5 to 9 carbons, which are liquid at room temperature and among the usual suspects in gasoline, can be used as a good fuel in internal combustion engine [26], while C8–C21 alkanes are the predominant components of diesel fuel [27]. Biosynthesis of alkanes has also been reported in a diversity of microorganisms including photosynthetic cyanobacteria since later 1960s [24,27,28]; however, its production in native producing hosts has not received much attention due to their low productivity. In a recent study, the researchers isolated a biosynthesis pathway consisting of an acyl-acyl carrier protein reductase and an aldehyde decarbonylase, which together convert intermediates of fatty acid metabolism to alkanes and alkenes in cyanobacterium Synechocystis elongatus, and expressed it heterologously in Escherichia coli, leading to the production and secretion of C13 to C17 mixtures of alkanes at ~0.3 g/L after 40 h cultivation in E. coli [29]. The work for the first time demonstrated the potential to use heterologous hosts for high–efficiency alkane production. Currently efforts are ongoing in both academic and industry settings to express synthetic alkane pathways in photosynthetic cyanobacterial hosts for the production of the third-generation carbon-neutral biofuels.

As solvents, alkane products are toxic to microbes [30]. Their toxicity is inversely correlated with the log-P\textsubscript{ow} value, which is the common logarithm of the partition coefficient (P\textsubscript{ow}) for the distribution of the organic solvent between n-octanol and water phases [31,32]. A series of genes involved in alkane tolerance in E. coli have been identified and utilized as targets to improve alkane tolerance by genetic engineering, which has led to some progress in improving alkane tolerance in E. coli [33-37]. Cyanobacteria have low tolerance to alkanes; meanwhile, currently the knowledge on alkane tolerance in cyanobacteria is very limiting. To fully understand the effects of alkane on the cyanobacterial cells so that a construction of more robust alkane-producing cyanobacterial hosts can be possible, in this study, we employed a quantitative proteomics approach with isobaric tag for relative and absolute quantification (iTRAQ) technique and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to reveal the global metabolic response of the model cyanobacterium Synechocystis to the treatment of hexane, a representative alkane. The results showed that common stress responses which have been reported for other microbes under solvent/biofuel stress were induced by hexane in Synechocystis. Notably, the analysis revealed the induction of large numbers of transporters and membrane-bound proteins, proteins related to sulfur relay system, oxidative stress response and photosynthesis, suggesting that they were among the major protection mechanisms against hexane. The study provided the first comprehensive view of the complicated molecular mechanism employed by cyanobacterial model species, Synechocystis to defend against hexane stress, and also constituted an important foundation for rational engineering of more robust photosynthetic hosts for the production of the carbon-neutral biofuel alkane.

**Results and discussion**

**Hexane effect on Synechocystis sp. PCC 6803**

The growth of Synechocystis supplemented with 0, 0.7%, 0.8% and 0.9% hexane was assessed to determine an appropriate hexane concentration for proteomic studies (Figure 1). The results showed that the hexane concentration that caused an approximately 50% growth inhibition was found to be 0.8% (v/v) at both 24 h and 48 h (corresponding to middle-exponential and exponential-stationary transition phases, respectively), and was selected for the proteomics analysis in this study. The tolerance level of Synechocystis to hexane was similar to what has been reported for Aeromonas hydrophila and Pseudomonas aeruginosa [38]. Cell morphology examination showed no visible change after hexane treatment (data not shown). For proteomic analysis, two independent cultivations for both control and 0.8% hexane

![Figure 1 Effects of hexane. Growth time courses with varying concentration of hexane.](image-url)

Growth (OD\textsubscript{730})

Time (h)

0% 0.7% 0.8% 0.9%
treatment were conducted, and cells were collected by centrifugation (8,000 x g for 10 min at 4°C) at 24 h and 48 h, resulting in two biological replicates for each time point of control and hexane-treated samples (Figure 1).

Overview of quantitative proteomics
A total of 167,191 spectra were obtained from the iTRAQ - LC-MS/MS proteomic analysis. After data filtering to eliminate low-scoring spectra, a total of 24,162 unique spectra that met the strict confidence criteria for identification were matched to 1,491 unique proteins, representing approximately 41.8% of the 3569 predicted proteins in the *Synechocystis* genome (Additional file 1: Table S1). In terms of protein molecular weight (MW) distribution, good coverage (averages of 30–45% of the total proteins in each MW group) was obtained for a wide MW range for proteins larger than 10 kDa (Figure 2A). In addition, most of the proteins were identified with good peptide coverage, of which ~65% of the proteins were with more than 10% of the sequence coverage, and ~44% were with 20% of the sequence coverage (Figure 2B). Among all the proteins detected, 1,181 and 1,172 were identified from the samples of 24 h and 48 h, respectively. The proteins that were identified only in control or treated samples so that ratio calculation is not available were excluded from the analysis. Based on the number of unique proteins identified in each functional category, the most frequently detected functional categories were “general function prediction only” and “signal transduction mechanisms”, representing 11.1% and 10.85% of all the proteins identified, respectively (Figure 2C). Proteins involved in signal transduction network are generally with low abundance, quick protein turnover time and membrane-bound [39], a high coverage of the group of signal proteins also suggested the methodology used in the study is with high

Figure 2 Distribution, coverage, and functional category of proteins identified in this study. A) Distribution of protein identified among different molecular weights; B) Coverage of proteins by the identified peptides; C) Functional category coverage of the proteins identified.
sensitivity and very reliable. The high percentage of functionally unknown proteins identified is not unreasonable, considering more than 40% of proteins in the _Synechocystis_ genome are still annotated as hypothetical proteins without any functional prediction. Other most frequently detected functional categories included “amino acid transportation and metabolism” (9.17%), “energy production and conversion” (7.55%), and “translational, ribosomal structure and biogenesis” (7.55%) and “cell wall/membrane/envelope biogenesis” (7.24%), suggested that the proteins in these functional categories were highly expressed and may be active during the growth and treatment conditions.

Reproducibility of the proteomic analysis was assessed in two types of comparisons (Figure 3). First we labeled and mixed two biological replicates of a given condition directly for proteomic analysis (i.e. biological replicate 1 and 2 of control at 24 h, replicate 1 and 2 of control at 48 h, biological replicate 1 and 2 of hexane treatment at 24 h, biological replicate 1 and 2 of hexane treatment at 48 h), the difference was plotted verse the percentage of the proteins identified, the results showed that approximately 60% of the proteins with difference less than delta error of 0.1, and more than 95% of the proteins with difference less than delta error of 0.5 (Figure 3-I). Second we labeled and mixed each pair of hexane-treated sample and its control for proteomic analysis, the difference between different biological pairs was plotted in Figure 3-II. The dispersion of the iTRAQ ratios of the quantified proteins (i.e. 1,181 and 1,172 for 24 h and 48 h, respectively) was found with very similar trends between four biological replicates at either 24 h (Figure 3-II-A) or 48 h (Figure 3-II-B), suggesting that the biological noise was reasonably low.

Pathway enrichment analysis of the hexane-responsive proteins

Using a cutoff of 1.5-fold change and a _p_-value less than 0.05, we determined that 140 and 148 unique proteins were differentially regulated between control and hexane treatments conditions at 24 h and 48 h, respectively (Additional file 1: Table S1). Among them, a total of 164 and 77 proteins were up-regulated and down-regulated by the hexane treatments, respectively. Forty-two up-regulated and 4 down-regulated proteins were shared between 24 h and 48 h, while more of the responsive proteins were unique for each of the time points, consistent with the expected physiological differences between middle-exponential and transition phases of the cell growth (Figure 1).

Metabolic pathway enrichment analysis was carried out for the differentially regulated proteins to determine
the affected cellular metabolism (Additional file 2: Table S2). The analysis was performed by matching the responsive proteins to the proteins annotated with KEGG Pathway database, and then comparing the frequencies of the responsive proteins in each KEGG pathway to determine statistically authenticity of the involvement of that KEGG pathway in hexane response. A series of KEGG pathways affected by the hexane treatment, with p-value less than 0.05 as cut off were identified. The results showed that at 24 h after hexane treatment, four KEGG pathways were differentially regulated by the hexane treatment: they are “Ribosome” (KO03010), “Sulfur relay system” (KO04122), “Photosynthesis” (KO00195) and “Arachidonic acid metabolism” (KO00590). All four pathways were enriched by at least two out of four biological replicates, with “Ribosome” (KO03010) enriched with good confidence in all four replicates and “Photosynthesis” (KO00195) enriched in two replicates, consistent with the relatively high expression of ribosomal proteins and photosynthetic proteins, and the sensitivity of protein biosynthesis and photosynthetic processes to stress. Most of the ribosomal proteins identified were down-regulated, suggesting an overall slowdown of the protein biosynthesis and possible slow metabolism. At 48 h, five KGG pathways, “Sulfur relay system” (KO04122), “ABC transporters” (KO02010), “Photosynthesis” (KO00195), “Steroid biosynthesis” (KO00100), and “Biosynthesis of ansamycins” (KO01051) were enriched by the differentially expressed proteins. Although these KEGG pathways were enriched in only two out of four biological replicates, the p-values were reasonably low, suggesting in general the reliability of the data analysis. The enrichment of “Ribosome” pathway (KO03010) at only 24 h was interesting, which may imply that modification of the primary metabolism, such as protein biosynthesis, may be one of the major strategies that cells used to deal with stress during middle-exponential phase. In this study, we found that several ribosomal proteins, Sll1816 (rpsM), Sll1803 (rplV) and Sr0482 (rpsP) were down-regulated under hexane treatment. Interestingly, these three genes, rpsM, rplV, and rpsP, were named as targets as they were also down-regulated in E. coli against organic solvent also indicated [35]. “Sulfur relay system” (KO04122) was differentially regulated at both 24 and 48 h, in three out of four biological replicates at 24 h, and two out of four biological replicates at 48 h, respectively (Additional file 2: Table S2). The sulfur-relay system classified in tRNA modification has been demonstrated to modify a few nucleotides of tRNA molecules, their increased expression has been reported contributing to stabilization of their structure, and was required for survival at an extremely high temperature in E. coli [40] and Thermus thermophilus [41]. In addition, ubiquitin (Ub) like proteins are signaling messengers that control many cellular functions in bacteria. It has been proposed that the Ub-protein modification evolved from prokaryotic sulfurtransfer systems [42]. Molybdenum cofactor (Moco) and thiamin are sulfur-containing cofactors whose biosynthesis includes a key sulfur transfer step that uses unique sulfur carrier proteins, MoaD and ThiS. Detailed analysis showed that upon hexane stress, two proteins in “Sulfur relay system” pathway, Slr0821 and Ssl1707 with homologies to sulfur carrier proteins MoaD and sulfur-accepting protein TusA were up-regulated, consistent with their possible roles against stress.

As one core function of cyanobacteria, “Photosynthesis” pathway (KO00195) was differentially regulated by hexane exposure at both 24 h and 48 h, in two out of four biological replicates (Additional file 2: Table S2). Early studies showed that some natural stresses, such as salt and sulfur starvation, decreased expression level of genes for phycobilisome, photosystems I and II, cytochrome b6/f, and ATP synthase, indicating overall reduced light-harvesting and photosynthetic activity upon stress [43,44]. However, detailed analysis showed that six proteins involved in photosystem I and II (Slr0172: photosystem I assembly protein; Slr0629: photosystem I subunit X; Slr1645: photosystem II 11 kD protein; Slr1194: photosystem II complex extrinsic protein precursor U; Slr1418: photosystem II oxygen-evolving complex 23 K protein PsbP homolog; and Slr1398: photosystem II reaction center protein Psb28), four ferredoxin proteins (Slr1828, Ssl0020, Slr1382, Slr1205) and two cytochrome (Sr3451: cytochrome B559 subunit alpha; and Slr0258: cytochrome C-550) were all up-regulated by hexane exposure (Table 1). Although it remains unclear how the increased expression of photosynthesis-related proteins will help combat the hexane toxicity, the results were consistent with our recent study on ethanol toxicity in Synechocystis sp. PCC 6803, where the similar proteomics analysis also demonstrated a profoundly increased expression of many proteins related to photosynthesis [45].

“Arachidonic acid metabolism” pathway (KO00590) was differentially regulated by hexane exposure at 24 h, in two out of four biological replicates (Additional file 2: Table S2). Detailed analysis showed two proteins of glutathione peroxidase (Slr1992 and Slr1171) were up-regulated and thus contributed to the enrichment of this pathway (Table 1). Peroxidases are heme-cofactored enzymes responsible for hydrogen peroxide removal, cytoplasmic glutathione peroxidase (Gpx) has been found involved in oxidative defense in many bacteria [46].

“Steroid biosynthesis” pathway (KO00100) was differentially regulated by hexane exposure at 48 h (Additional file 2: Table S2). Detailed analysis showed that sll0513 encoding a putative squalene synthase was down-
| Protein ID | 24 h | 48 h | Description |
|------------|------|------|-------------|
|            | 1.74 | 1.73 | Protein mura rpon intergenic region precursor |
| Sll1804    | 1.55 | 1.54 | 305 ribosomal protein S3 |
| Str0444    | 1.54 | 1.45 | 3-phosphoshikimate 1-carboxyvinyltransterase |
| Sll1821    | 1.80 | 1.72 | 50S ribosomal protein L13 |
| Sll1813    | 1.60 | 1.60 | 50S ribosomal protein L15 |
| Sll1821    | 1.80 | 1.71 | 50S ribosomal protein L29 |
| Sll1813    | 1.60 | 1.54 | 50S ribosomal protein L35 |
| Sll1017    | 1.93 | 1.91 | Acyl carrier protein |
| Str0242    | 1.58 | 1.66 | Ammonium/methylammonium permease |
| Str0043    | 2.56 | 2.56 | Bacterioferritin co migratory protein |
| Str0041    | 3.60 | 3.60 | Bicarbonate transport system ATP-binding protein |
| Sll0834    | 1.54 | 1.66 | Bicarbonate transporter |
| Str0436    | 1.66 | 1.66 | Carbon dioxide concentrating mechanism protein Ccmk |
| Str1853    | 1.61 | 1.61 | Carboxymuconolactone one decarboxylase |
| Str2131    | 1.66 | 1.66 | Cation or drug efflux system protein |
| Sll0672    | 1.63 | 1.63 | Cation-transporting ATPase E1-E2 ATPase |
| Sll1895    | 1.80 | 1.71 | C-di-GMP phosphodiesterase A |
| Sll039     | 1.83 | 1.78 | CheY family protein positive phototaxis protein |
| Str0757    | 1.79 | 1.68 | Cysteine clock protein KaiB |
| Sll0712    | 2.04 | 2.04 | Cysteine synthase A |
| SsSr3451   | 2.40 | 2.34 | Cytochrome B559 subunit alpha |
| Sll0258    | 1.55 | 1.55 | Cytochrome C-550 |
| Protein | Fold Change | Description                                      |
|---------|-------------|--------------------------------------------------|
| sll1712 | 1.80        | DNA binding protein HU                           |
| sll1699 | 2.55        | Extracellular solute binding                     |
| slr1330 | 1.52        | F0F1 ATP synthase subunit epsilon                |
| slr1828 | 1.59        | Ferredoxin                                       |
| sll1382 | 1.61        | Ferredoxin component                             |
| slr1205 | 1.56        | Ferrichrome-iron receptor                        |
| slr1761 | 1.52        | FKBP-type peptidyl prolyl cis-trans isomerase    |
| sll1382 | 1.61        | Gamma-glutamyltranspeptidase                     |
| slr1269 | 1.56        | Glutamyl-tRNA(Gln) amidotransferase subunit C    |
| slr1992 | 2.07        | Glutathione peroxidase                           |
| slr1171 | 1.54        | Glutathione peroxidase                           |
| slr1761 | 1.68        | Glycine decarboxylase complex H-protein           |
| sll0020 | 1.62        | Glutathione oxidase subunit GlcD                 |
| sll1382 | 1.61        | Heat shock protein GrpE                         |
| sll0033 | 2.45        | Heterocyst to vegetative cell connection protein |
| sll1171 | 1.70        | Hydrogenase component                            |
| sll1404 | 1.54        | Inosine-5-monophosphate dehydrogenase related    |
| sll1558 | 1.79        | Mannose-l-phosphate guanyltransferase             |
| sll1394 | 1.53        | Mercuric transport protein periplasmic component precursor |
| sll1558 | 1.59        | Methionine sulfoxide reductase A                 |
| sll0689 | 1.85        | Na/H+ antiporter                                 |
| sll0493 | 1.77        | Na-activated K transporter subunit KtrA          |
| sll0891 | 1.56        | N-acetylmuramoyl-L-alanine amidase                |
| sll0223 | 2.22        |                                               |
| Accession | Ratio(n/hexane) | Description |
|-----------|----------------|-------------|
| Sll1262   | 1.52           | NAD(P)H-quinone oxidoreductase subunit 2 |
| Ssl2667   | 2.92 2.82      | NAD(P)H-quinone oxidoreductase subunit N |
| Str0513   | 1.58 1.81 1.58 | NifU protein |
| Str1160   | 1.66 1.51      | Periplasmic protein, function unknown |
| Str2144   | 3.66           | Periplasmic iron-binding protein |
| Str1513   | 4.31           | Periplasmic protein, function unknown |
| Sll0319   | 1.57           | Periplasmic protein, function unknown |
| Sll0172   | 1.84 1.56      | photosystem I assembly protein |
| Sll0629   | 2.86           | photosystem I subunit X |
| Str1645   | 1.51 1.64      | Photosystem II 11kD protein |
| Sll1194   | 2.88           | Photosystem II complex extrinsic protein precursor U |
| Sll1418   | 1.51           | Photosystem II oxygen-evolving complex 23K protein |
| Sll1398   | 1.79 1.62 1.83 | Photosystem II reaction center protein Psb28 |
| Sll0617   | 2.01 1.71 1.98 | Plasma membrane protein essential for thylakoid formation |
| Sll0199   | 1.77 1.61      | Plastocyanin |
| Sll0779   | 1.83 1.88      | PleD-like-protein |
| Sll0043   | 1.72           | Positive phototaxis protein |
| Str0775   | 1.68           | Preprotein translocase subunit SecF |
| Str0042   | 1.56 1.69      | Probable porin: major outer membrane protein |
| Sll1734   | 2.19 1.98 1.55 | Protein involved in low CO₂-inducible, high affinity CO₂ uptake |
| Ssr2049   | 1.54           | Protochlorophyllide reductase 57 kD subunit |
| Sll0135   | 2.20 1.93      | Putative 5'-methylthioadenosine phosphorylase |
### Table 1 Up-regulated proteins by hexane *,**,*** (Continued)

| Protein ID  | Ratio 1 | Ratio 2 | Ratio 3 | Ratio 4 | Ratio 5 | Ratio 6 |
|-------------|---------|---------|---------|---------|---------|---------|
| Slr0821     | 1.55    | 1.59    | 2.34    | 2.36    | 1.74    | 2.23    |
| Sll1277     | 1.56    |         |         |         |         |         |
| Slr1198     | 1.54    | 1.52    |         |         |         |         |
| Slr1164     | 3.34    |         |         |         |         |         |
| Sll0469     |         |         |         |         |         | 1.51    |
| Sr10193     |         |         |         |         |         | 1.52    |
| Sll1440     | 1.59    |         |         |         |         |         |
| Sll1277     | 2.25    |         |         |         |         |         |
| Sr1198      | 1.74    | 1.54    | 1.52    |         |         |         |
| Sll1549     | 5.32    |         |         |         |         |         |
| Sll0575     |         |         |         |         |         |         |
| Sll0575     | 2.02    |         |         |         |         |         |
| Sr1697      | 1.68    |         |         |         |         |         |
| Sr1531      | 1.68    |         |         |         |         |         |
| Sll1440     | 1.71    |         |         |         |         |         |
| Sll0575     | 2.26    |         |         |         |         |         |
| Slr1034     | 1.74    | 1.53    |         |         |         |         |
| Sll1742     | 1.63    |         |         |         |         |         |
| Sll1483     | 2.21    |         | 2.43    |         |         |         |
| Slr0709     |         |         |         | 1.53    |         |         |
| Sll1277     |         |         |         |         | 1.59    |         |
| Sll1277     |         |         |         |         |         | 2.25    |
| Sll1440     |         |         |         |         |         | 1.52    |
| Sll1549     |         |         |         |         |         | 5.32    |
| Sll0469     |         |         |         |         |         | 1.51    |
| Sr10193     |         |         |         |         |         | 1.52    |
| Sll1440     |         |         |         |         |         | 1.71    |
| Sll0575     |         |         |         |         |         | 2.02    |
| Sr1697      |         |         |         |         |         | 1.68    |
| Sr1531      |         |         |         |         |         | 1.68    |
| Sll1440     |         |         |         |         |         | 1.71    |
| Sll0575     |         |         |         |         |         | 2.26    |
| Sr1034      |         |         |         |         |         | 1.74    |
| Sll1742     |         |         |         |         |         | 1.63    |
| Sll1483     |         |         |         |         |         | 2.21    |
| Slr0709     |         |         |         |         |         | 1.53    |
| Sll1277     |         |         |         |         |         | 1.59    |
| Sll1277     |         |         |         |         |         | 2.25    |
| Sll1440     |         |         |         |         |         | 1.52    |
| Sll1549     |         |         |         |         |         | 5.32    |
| Sll0469     |         |         |         |         |         | 1.51    |
| Sr10193     |         |         |         |         |         | 1.52    |
| Sll1440     |         |         |         |         |         | 1.71    |
| Sll0575     |         |         |         |         |         | 2.02    |
| Sr1697      |         |         |         |         |         | 1.68    |
| Sr1531      |         |         |         |         |         | 1.68    |

*Proteins with 1.5 fold change and p-value less than 0.05.

**Hypothetical proteins listed with gene ID only, full information in Additional file 1: Table S1.

***Blank represents that ratio can't be calculated for the condition.
regulated, which then contributed to the enrichment of this pathway (Table 2). Role of this enzyme in cyanobacteria upon hexane stress still needs more investigation, but it has been reported that squalene synthase (Erg9) in yeast was down regulated upon oxidative stress and ergosterol level plays a key role in adaptation to oxidative stress [47].

Membrane-bound proteins significantly induced by hexane

Pathway enrichment analysis showed that “ABC transporters” (KO02010) was differentially regulated by hexane exposure, among which four ABC transporters were matched to the KEGG pathway KO02010 at 48 h (Additional file 2: Table S2). Further analysis, however, showed that more putative transporters were up-regulated by the hexane treatment (Table 1). Induction of transporters by stress or toxic solvent has been reported in many microbes, such as acrAB-toleC in E. coli and srpABC in Pseudomonas putida which have been shown to export hexane, heptane, octane, octanol and nonane, and the enhanced expression of these genes were related to high tolerance [48,49]. Up-regulation of transporters has been reported for various stresses in many other cyanobacterial species, such as arsenate resistance in Anabaena variabilis, salinity in Synechocystis [50-53]. In Synechocystis sp. PCC 6803, we recently found that five putative transporters involved in transporting of different substrates (i.e. polar amino acid, bicarbonate, iron, Na+/K+ ) were up-regulated by ethanol exposure [45]. Upon hexane exposure, a spectrum of putative transporters, including five transporters involved in bicarbonate transporting (Slr0041, Slr0043, Slr0044, Slr1783, Slr1909), two involved in cation transporting (Slr1512, Slr1783), two involved in Na+ and K+ transporting (Slr0043, Slr0044), one involved in mercuric transporting (Slr2857), were up-regulated (Table 1). In Synechocystis sp. PCC 6803, the sfpAgene, a putative porin gene (sfpA), were induced under low CO2 stress conditions to increase the bicarbonate transporting [54]. Slr1512 was previously found essential for thylakoid formation Sll0617 (vipp1) with a similar gene, pspA, in E. coli up-regulated by organic solvents [35], a periplasmic iron-binding protein (Slr0513) [57], four periplasmic proteins with unknown function (Slr1160, Slr2144, Slr1513, Sll0319), a salt-enhanced periplasmic protein (Sll1549). Meanwhile, several membrane-bound proteins were also found down-regulated by hexane exposure (Table 2). Although the exact function of these proteins was mostly unknown, the significant changes occurred at the cell membrane level may represent an important resistance strategy against hexane toxicity.

Bacteria use a variety of secretion systems to transport proteins beyond their cell membrane in order to interact with their environment. In E. coli and other gram-negative bacteria, one of the major translocation systems is the twin arginine translocation pathway consisted of TatA, TatB and TatC to export folded proteins across the cytoplasmic membrane [58]; and another is the Sec-dependent protein translocation system whose complex molecular machine comprises a flexible transmembrane conduit coupled to a motor-like component [59]. Our proteomics analysis showed that hexane treatment induced the expression of both systems in Synechocystis sp. PCC 6803. The putative TatA protein (Slr1046) of twin arginine translocation pathway and SecF (Slr0775) of the Sec-dependent protein translocation system were both up-regulated (Table 1).

Regulatory systems regulated by hexane

Two-component system (TCS) is an important signal transduction mechanism employed by prokaryotes to survive the complex and volatile environments [60], and has been involved in various stress responses in cyanobacteria [61,62]. In our previous work, two response regulators (Slr1783, Slr1909) were found up-regulated by ethanol exposure; however, these two response regulators were not differentially regulated by hexane under our experimental conditions [45]; meanwhile, four different regulatory proteins were up-regulated, and two regulatory proteins were down-regulated by hexane exposure (Table 1, 2). Interestingly, up-regulated Slr1697 of a serine/threonine kinase and up-regulated Slr0043 of a positive phototaxis hybrid histidine kinase (homologous to chemotaxis protein CheA) were both required for cell motility and chemotaxis [63,64]. Chemotaxis and flagellar motility are essential mechanisms by which bacteria use to adapt to and survive in environments suffered with various natural stresses [65], our results showed that the Synechocystis cells may adapt the similar mechanism in dealing with the stress caused by hexane. In addition, the fact that a different set of regulatory proteins were involved in hexane stress from those in ethanol stress implicated that variable resistance mechanisms were
| Protein ID | 24h Hexane-24h-r1 vs Control-24h-r1 | 24h Hexane-24h-r2 vs Control-24h-r2 | 48h Hexane-48h-r1 vs Control-48h-r1 | 48h Hexane-48h-r2 vs Control-48h-r2 |
|------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| Sll1605    | 0.60                                 |                                      |                                      |                                      |
| Sll1101    |                                      |                                      |                                      | 0.59                                 |
| Sll1816    | 0.60                                 | 0.37                                 | 0.64                                 | 0.49                                 |
| Sir0628    |                                      |                                      | 0.38                                 | 0.35                                 |
| Ssr0482    | 0.52                                 | 0.48                                 |                                      | 0.66                                 |
| Sst3437    | 0.49                                 |                                      |                                      | 0.63                                 |
| Sst1399    |                                      |                                      |                                      | 0.52                                 |
| Sll1804    |                                      | 0.61                                 |                                      | 0.54                                 |
| Sir0469    | 0.47                                 | 0.41                                 | 0.59                                 | 0.47                                 |
| Sll1812    |                                      | 0.62                                 |                                      |                                      |
| Sll1097    | 0.54                                 |                                      |                                      |                                      |
| Sll1809    | 0.57                                 | 0.48                                 | 0.48                                 | 0.45                                 |
| Sll1822    | 0.47                                 | 0.32                                 | 0.63                                 | 0.48                                 |
| Sir1140    |                                      |                                      | 0.65                                 | 0.65                                 |
| Sll1821    | 0.42                                 | 0.35                                 | 0.57                                 | 0.48                                 |
| Sll1813    | 0.53                                 | 0.41                                 | 0.64                                 | 0.55                                 |
| Sll1819    | 0.45                                 | 0.32                                 | 0.46                                 | 0.32                                 |
| Sll1811    |                                      | 0.59                                 |                                      |                                      |
| Sll1802    |                                      |                                      |                                      | 0.59                                 |
| Sir1678    |                                      |                                      | 0.67                                 | 0.61                                 |
| Sll1803    | 0.53                                 | 0.64                                 | 0.52                                 | 0.65                                 |
| Sll1807    |                                      | 0.48                                 |                                      |                                      |
| Ssr2799    | 0.00                                 |                                      | 0.58                                 |                                      |
| Ssr1604    | 0.50                                 | 0.37                                 | 0.55                                 |                                      |
| Sll1810    |                                      | 0.35                                 | 0.43                                 | 0.57                                 |
| Sir1927    |                                      |                                      | 0.63                                 |                                      |
| Sir1694    |                                      | 0.63                                 |                                      |                                      |
| Str0083    |                                      | 0.55                                 | 0.39                                 |                                      |

**Description**

- (3R)-hydroxymyristoyl-ACP dehydratase
- 30S ribosomal protein S10
- 30S ribosomal protein S13
- 30S ribosomal protein S14
- 30S ribosomal protein S16
- 30S ribosomal protein S17
- 30S ribosomal protein S18
- 30S ribosomal protein S3
- 30S ribosomal protein S4
- 30S ribosomal protein S5
- 30S ribosomal protein S7
- 30S ribosomal protein S8
- 30S ribosomal protein S9
- 3-amino-5-hydroxybenzoic acid synthase
- 50S ribosomal protein L13
- 50S ribosomal protein L15
- 50S ribosomal protein L17
- 50S ribosomal protein L18
- 50S ribosomal protein L2
- 50S ribosomal protein L21
- 50S ribosomal protein L22
- 50S ribosomal protein L24
- 50S ribosomal protein L27
- 50S ribosomal protein L28
- 50S ribosomal protein L6
- ABC transporter
- Activator photopigment and puc expression
- ATP-dependent RNA helicase DeaD
|   |   |   |   |   |
|---|---|---|---|---|
| Slr0834 |   |   |   |   |
| Slr1223 | 0.60 |   |   |   |
| Slr1839 | 0.66 | 0.58 |   |   |
| Slr1292 |   |   |   |   |
| Slr1641 | 0.64 |   |   |   |
| Slr1258 |   |   | 0.58 |   |
| Slr1327 | 0.57 | 0.51 |   |   |
| Slr0513 |   |   | 0.65 |   |
| Slr0020 |   |   |   |   |
| Slr0567 |   |   | 0.65 |   |
| Slr0248 | 0.00 | 0.00 |   |   |
| Slr1370 |   |   | 0.62 |   |
| Slr0207 |   |   |   | 0.59 |
| Slr0288 |   |   | 0.66 | 0.64 |
| Slr1019 | 0.57 | 0.63 |   |   |
| Slr1123 | 0.62 |   |   |   |
| Slr0764 |   |   | 0.66 |   |
| Slr2099 |   |   |   | 0.57 |
| Slr0546 |   |   | 0.61 |   |
| Slr1214 |   |   | 0.66 | 0.63 |
| Slr0223 |   |   | 0.63 |   |
| Slr0844 |   |   | 0.64 |   |
| Slr077 | 0.55 |   |   |   |

Table 2: Down-regulated proteins by hexane *,**,*** (Continued)

* Bicarbonate transporter
** Bidirectional hydrogenase complex protein HoxU
*** Carbon dioxide concentrating mechanism protein CcmK
**** CheY family protein
***** ClpB protein
****** Deoxycytidine triphosphatase deaminase
******* F0F1 ATP synthase subunit gamma
******** Fransyl-diphosphatase farnesytraferas
********* Ferredoxin
********** Ferric uptake regulation protein
*********** Flavodoxin FldA
************ GDP-mannose pyrophosphorylase
************* Glucose-1-phosphate thymidylyltransferase
************** Glutamate–ammonia ligase
*************** Glyoxalase II
**************** Guanylate kinase
***************** High-affinity branched-chain amino acid transport
****************** Hybrid sensory kinase
******************* Indole-3-glycerol-phosphate synthase
********************* Magnesium-protoporphyrin IX monomethyl ester cyclase
******************** NAD(P)H-quinone oxidoreductase subunit 2
********************** NAD(P)H-quinone oxidoreductase subunit F
*********************** NifS protein
| Slr2005 | 0.61 | Periplasmic protein, function unknown |
| Slr0064 | 0.65 | Putative polar amino acid transport system |
| Slr1410 | 0.61 | Periplasmic WD-repeat protein |
| Slr1615 | 0.57 | Perynosamine synthetase |
| Sll0226 | 0.51 | 0.56 | Photosystem I assembly protein Ycf4 |
| Slr1894 | 0.60 | Probable DNA-binding stress protein |
| Sll0772 | | 0.57 | 0.57 | Probable porin; major outer membrane protein |
| Slr1861 | 0.59 | Probable sigma regulatory factor |
| Slr0677 | | 0.64 | Perative biopolymer transport protein exbB-like 2 |
| Slr1531 | 0.66 | Signal recognition particle protein |
| Slr1139 | | 0.63 | 0.66 | Thioredoxin |
| Sll0615 | | 0.61 | Transmembrane protein FT 27 |
| Sll0518, Sll0596, Sll0783, Sll0872, Sll1118, Slr1304, Slr0007, Slr0147, Slr0168, Slr0723, Slr1260, Slr1363, Slr1385, Ssr2254 | | | Hypothetical proteins |

*Proteins with 1.5 fold change and p-value less than 0.05.
**Hypothetical proteins listed with gene ID only, full information in Additional file 1: Table S1.
***Blank represents that ratio can't be calculated for the condition.
used by the cells for the biofuel products of different chemical properties [45].

Common stress response
Early studies have defined a set of common stress responses that cells will typically initiate upon stresses from organic solvent or biofuel products, including down-regulation of ribosomal proteins, induction of heat shock proteins to aid with proper protein folding, induction of oxidative stress response, and modification of cell membrane [66-68]. Our proteomic analysis showed that the initiation of these common stress response programs were also seen in the hexane-treated Synechocystis cells, although the protein species used for each of the function could vary between microbial species (Table 1, 2). For example, a heat shock protein (GrpE, Sll0057) was induced by hexane in the Synechocystis cells, while in E. coli treated with one of the hydrophobic organic solvents, n-hexane or cyclooctane, a 28 kDa phage-shock protein PspA was induced [69]. While in the ethanol-treated Synechocystis cells, two heat shock proteins, GroES (Slr2075) and GrpE (Sll0057) were up-regulated [45]. Oxidative stress responses have been reported for cells under treatment of organic solvents as they induced production of highly reactive oxygen species (ROS). Without exception, ROS formation has been suggested playing a important role in n-hexane induced alterations in cell proliferation and membrane integrity [70]. In addition to two responsive glutathione peroxidase discussed above, our proteomic analysis showed that a bacterioferitin comigratory protein (Slr0242), a rehydrin (Sll1198) and a methionine sulfoxide reductase A (Sll1394) were also induced in response to the oxidative stress caused by hexane in Synechocystis (Table 1). Studies of solvent-tolerant microbes found that cells can modify fatty acid composition or other accessory molecules of their cell membrane to block the entry of solvents [66,67]. Up-regulation of Slr1761, a peptidylprolyl cis-trans isomerase which catalyses the cis-trans isomerization of proline imidic peptide bonds in both prokaryotic and eukaryotic cell envelope upon stress, was found in hexane-treated cells, similar up-regulation of Slr1761 by ethanol was also observed previously Synechocystis [45].

Conclusions
Alkane compounds of varying carbon lengths have been proposed as a good alternative to gasoline. However, one of the major hurdles needed to be overcome is that alkanes typically exhibit toxicity to microbes, especially cyanobacterial cells. Towards this end, we employed a global-based quantitative proteomics approach with iTRAQ - LC-MS/MS technologies to reveal the responses of Synechocystis to hexane, a representative of alkane. Using a cutoff of 1.5-fold change and a p-value less than 0.05, a total of 164 up-regulated and 77 down-regulated proteins were determined. Function annotation and KEGG pathway enrichment analyses showed that common stress responses which have been reported for other microbes under organic solvent/biofuel stress were also induced in Synechocystis [66,67]. Notably, the analysis revealed the induction of a large number of transporters and membrane-bound proteins, proteins related to sulfur relay system, oxidative stress and photosynthesis, suggesting that they were among the major protection mechanisms against hexane. In this study hexane is added exogenously, however, it is expected that the general responses uncovered by the study will be similar to that caused by hexane generated intracellularly, although intracellular hexane may be more toxic at lower concentration. Nevertheless, the study provided the first comprehensive view of the complicated molecular mechanism employed by cyanobacterial model species, Synechocystis to defend against hexane stress. Moreover, the proteomic analysis identified a list of potential target genes/proteins which can be engineered to generate stress-resistant hosts for high efficiency production of hexane [71,72].

Methods
Bacterial growth condition and hexane treatment
Synechocystis sp. PCC 6803 was obtained from American Type Culture Collection (ATCC) and grown in BG11 medium (pH 7.5) under a light intensity of approximately 50 μmol photons m⁻² s⁻¹ in an illuminating incubator operated at 130 rpm and at 30°C (HNY-211B Illuminating Shaker, Honour, China) [73-75]. Cell density was measured on a UV-1750 spectrophotometer (Shimadzu, Japan). For growth and hexane treatment, the 10 mL fresh cells of OD₇₃₀ 0.5 were collected by centrifugation at 8000 x g for 10 min at 4°C, and then were inoculated into 50 mL BG11 liquid medium in a 250-mL flask. Hexane of varying concentration was added at the beginning of cultivation. 1 mL of culture samples were collected and measured with a spectrophotometer at OD₇₃₀ every 12 h. Morphology of Synechocystis under control and treatment conditions was observed using a BX43 fluorescence microscope (Olympus, Japan). Growth experiments were repeated at least three times to confirm the growth patterns. Cells for proteomics analysis were collected by centrifugation at 8,000 x g for 10 min at 4°C.

Protein preparation and digestion
For each sample, 10 mg of cells were frozen by liquid nitrogen immediately after centrifugation and washed with
phosphate buffer (pH 7.2). The cells were broken with sonication cracking at low temperature. Cell pellets were then resuspended in a lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris–HCl), with 1 mM PMSF and 2 mM Ethylenediaminetetraacetic acid (EDTA) (final concentration). After 5 min of vigorously vortex, dithiothreitol (DTT) was also added to a final concentration of 10 mM. After mix, the sample were centrifuged for 20 min at 20,000 x g, and the supernatant was mixed well with ice-cold acetone (1:4, v/v) with 30 mM DTT. After repeating this step twice, supernatants were combined and precipitated at ~20°C overnight, and stored at ~80°C prior to sample if not for immediate use. For digestion, protein pellet from previous step was resuspended in digestion buffer (100 mM triethylammonium bicarbonate TEAB, 0.05% w/v sodium dodecyl sulfate, SDS) to a final concentration of 1 mg/mL (total protein measured by bicinchoninic acid assay (Sigma, St. Louis, MO)). Equal aliquots (500 μg) from each lysate were then digested with trypsin overnight at 37°C (Sigma; 1:40 w/w added at 0 and 2 h) and lyophilized.

**iTRAQ labeling**
The iTRAQ labeling of peptide samples derived from control and hexane treatment conditions were performed using iTRAQ reagent Multiplex kit (Applied Biosystems, Foster City, CA) according to manufacturer’s protocol. For each time point (i.e. 24 h or 48 h), four samples (two biological replicates for control and two biological replicates for hexane-treated samples) were iTRAQ labeled. The peptides labeled with respective isobaric tags, incubated for 2 h and vacuum centrifuged to dryness. The labeled control and hexane treatment replicate samples were 1:1 pooled, and generating four combinations of samples for each time point, which were reconstituted in Buffer A (10 mM KH₂PO₄, 25% acetonitrile, pH 2.85). The iTRAQ labeled peptides were fractionated using PolySULFOETHYL ATM SCX column (200 x 4.6 mm, 5 μm particle size, 200 Å pore size) by HPLC system (Shimadzu, JAPAN) at flow rate 1.0 ml/min. The 50 min HPLC gradient consisted of 100% buffer A (10 mM KH₂PO₄, 25% acetonitrile, pH 2.85) for 5 min; 0-20% buffer B (10 mM KH₂PO₄, 25% ACN, 500 mM KCL, pH 3.0) for 15 min; 20-40% buffer B for 10 min; 40-100% buffer B for 5 min followed by 100% buffer A for 10 min. The chromatograms were recorded at 218 nm. The collected fractions were desalted with Sep-Pak™ Vac C18 cartridges (Waters, Milford, Massachusetts), concentrated to dryness using vacuum centrifuge and reconstituted in 0.1% formic acid for LC-MS/MS analysis.

**LC-MS/MS proteomic analysis**
The mass spectroscopy analysis was performed using an AB SCIEX TripleTOF™ 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA), coupled with online micro flow HPLC system (Shimadzu, JAPAN) as described before [76,77]. The peptides were separated using nanobored C18 column with a picofrit nanospray tip (75 μm ID x 15 cm, 5 μm particles) (New Objectives, Wubrun, MA). The separation was performed at a constant flow rate of 20 μl min⁻¹, with a splitter to get an effective flow rate of 0.2 μl min⁻¹. The mass spectrometer data acquired in the positive ion mode, with a selected mass range of 300–2000 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. The three most abundant peptides above a 5 count threshold were selected for MS/MS and dynamically excluded for 30 s with ±30 mDa mass tolerance. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

**Proteomic data analysis**
The data acquisition was performed with Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). Protein identification and quantification were performed using Mascot 2.3.02 (Matrix Science, London, United Kingdom) [78]. For iTRAQ quantification, the peptide for quantification and quantification were performed using Mascot software (Applied Biosystems/MDS SCIEX). Protein identification and quantification were performed using Mascot 2.3.02 (Matrix Science, London, United Kingdom) [78]. For iTRAQ quantification, the peptide for quantification and quantification were performed using Mascot software (Applied Biosystems/MDS SCIEX). Protein identification and quantification were performed using Mascot software (Applied Biosystems/MDS SCIEX). Protein identification and quantification were performed using Mascot software (Applied Biosystems/MDS SCIEX).

\[
P = \sum_{i=0}^{n-1} \frac{M}{i} \cdot \frac{N-M}{n-i} / \binom{N}{n}
\]

where \(N\) is the number of all proteins with KEGG pathway annotation information, \(n\) is the number of the differentially regulated proteins with KEGG pathway annotation information, \(M\) is the number of proteins with a given KEGG pathway annotation, and \(m\) is the number of the differentially regulated proteins with a given KEGG pathway annotation. The KEGG pathways with \(p\)-value less than
0.05 were considered as enriched KEGG pathways by the hexane responsive proteins.

Additional files

Additional file 1: Table S1. Full list of identified proteins and their ratio across conditions *

Additional file 2: Table S2. KEGG pathway enrichment analysis of the differentially regulated proteins *.

Abbreviations
iTRAQ: Isobaric tag for relative and absolute quantitation; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; KEGG: Kyoto Encyclopedia of Genes and Genomes; TCS: Two-component system.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LC, JW, and WZ conceived of the study. LC, LW, JL, JQ and WZ drafted the manuscript. JL, LC, JW, and WZ carried out cultivation, protein isolation and proteomics analysis. LC, JW and WZ finish the statistical analysis for proteomics data. “All authors read and approved the final manuscript.”

Acknowledgements
The research was supported by grants from National Basic Research Program of China (National “973” program, project No. 2011CB608003 and No. 2012CB721101). The authors would also like to thank Tianjin University and the “985 Project” of Ministry of Education for their generous supports in establishing the research laboratory.

Received: 28 July 2012 Accepted: 30 August 2012
Published: 7 September 2012

References

1. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 1979, 111:1–61.

2. Ducat D, Sachdeva G, Silver P: Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proc Natl Acad Sci USA 2011, 108:3941–3946.

3. Robertson DE, Jacobson SA, Morgan F, Berry D, Church GM, Alleyan NB: A new dawn for industrial photosynthesis. Photosynth Res 2011, 107:269–277.

4. Qutanta N, Van der Kooy F, Van de Rhee MD, Voshol GP, Verpoorte R: Genetic tools for cyanobacteria. Biotechnol Biofuels 2011, 4:171–1733.

5. Koksharova O, Wolk C: Genetic tools for cyanobacteria. Appl Microbiol Biotechnol 2011, 91:471–490.

6. Hess WR: Cyanobacterial genomics for ecology and biotechnology. Curr Opin Microbiol 2011, 14:608–614.

7. Rittmann BE: Opportunities for renewable bioenergy using microorganisms. Biotechnol Bioeng 2008, 100:203–212.

8. Angernikay SA, Hellingswerf KJ, Lindblad P, de Mattos MJ: Energy biotechnology with cyanobacteria. Curr Opin Biotechnol 2008, 20:257–263.

9. Sheng J, Vannella R, Rittmann BE: Evaluation of cell-disruption effects of pulsed-electric-field treatment of Synechocystis PCC 6803. Environ Sci Technol 2011, 45:3795–3802.

10. Sheng J, Vannella R, Rittmann BE: Evaluation of methods to extract and quantify lipids from Synechocystis PCC 6803. Bioresour Technol 2011, 102:1697–1703.

11. Liu X, Curtiss R 3rd: Nickel-inducible lysis system in Synechocystis sp. PCC 6803. Proc Natl Acad Sci USA 2009, 106:1550–21554.

12. Deng MD, Coleman JR: Ethanol synthesis by genetic engineering in cyanobacteria. Appl Environ Microbiol 1999, 65:523–528.

13. Dexter J, Fu P: Metabolic engineering of cyanobacteria for ethanol production. Energy Environmental Sci 2009, 2857–864.

14. Takahama K, Matsuoka M, Nagahama K, Ogawa T: Construction and a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbA1 locus. J Biotechnol 2003, 93:302–305.

15. Lindberg P, Park S, Melis A: Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. Metab Eng 2010, 12:70–79.

16. Liu X, Fallon S, Sheng J, Curtiss R: CO2-Limitation-inducible green recovery of fatty acids from cyanobacterial biomass. Proc Natl Acad Sci USA 2011, 108:6905–6908.

17. Tan X, Yao L, Gao Q, Wang W, Qi F, Lu X: Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. Metab Eng 2011, 13:169–176.

18. Lan E, Liu JC: ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. Proc Natl Acad Sci USA 2012, 109:6018–6022.

19. Lan E, Liu JC: Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. Metab Eng 2011, 13:353–363.

20. Atsumi S, Higashide W, Liao JC: Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat Biotechnol 2009, 27:1177–1180.

21. McNeely K, Xu Y, Bennette N, Bryant D, Dimmick G: Redirecting redundant flux into hydrogen production via metabolic engineering of fermentative carbon metabolism in a cyanobacterium. Appl Environ Microbiol 2010, 76:5032–5038.

22. Lee HS, Vermaas WF, Rittmann BE: Biological hydrogen production: prospects and challenges. Trends Biotechnol 2010, 28:262–271.

23. Rottig A, Wenning L, Broker D, Steinbuchel A: Fatty acid alkylation esters: perspectives for production of alternative biofuels. Appl Microbiol Biotechnol 2010, 85:1713–1723.

24. Jang YS, Park JM, Choi S, Choi YJ, Seung DY, Cho JH, Lee SY: Engineering of microorganisms for the production of biofuels and perspectives based on systems metabolic engineering approaches. Biotechnol Adv 2012, 30:891–1000.

25. Zhang F, Rodriguez S, Kealing JD: Metabolic engineering of microbial pathways for advanced biofuels production. Curr Opin Biotechnol 2011, 22:775–783.

26. Peralta-Yahya PP, Kealing JD: Advanced biofuel production in microbes. J Biotechnol 2010, 154:147–162.

27. Winters K, Parker PL, Van Baalen C: Hydrocarbons of blue-green algae: geochemical significance. Science 1969, 163:467–468.

28. Dembinsky VM, Srebnik M: Variability of hydrocarbon and fatty acid components in cultures of the filamentous cyanobacterium Syntomonos sp, isolated from microbial community ‘black cover’ of limestone wall in Jerusalem. Biochemistry (Mosk) 2002, 67:1276–1282.

29. Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB: Microbial biosynthesis of alkanes. Science 2010, 329:559–562.

30. Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Tenen W, Segura A: Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 2002, 56:743–768.

31. Convir H, Anderson M: The effect of intramolecular hydrophobic bonding on partition coefficients. J Org Chem 1967, 32:2583–2586.

32. Harnisch M, Mokkeli H, Schulze G: Relationship between log PD, shake-flask values and capacity factors derived from reversed-phase high-performance liquid chromatography for n-alkylbenzenes and some OECD reference substance. J Chromatogr 1983, 282:135–132.

33. Aono R, Negishi T, Nakajima H: Cloning of organic solvent tolerance gene ostA that determines n-hexane tolerance level in Escherichia coli. Appl Environ Microbiol 1994, 60:4624–4626.

34. White DG, Goldman JD, Demple B, Levy SB: Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in Escherichia coli. J Bacteriol 1997, 179:612–616.

35. Hayashi S, Aono R, Hanai T, Mori H, Kobayashi T, Hara H: Analysis of organic solvent tolerance in Escherichia coli using gene expression profiles from DNA microarray. J Biotechnol 2003, 95:379–383.

36. Jude F, Arpin C, Brachet-Castang C, Capdevy M, Caumette P, Quentin C: TbtARBM, a multidrug efflux pump associated with tributyltin resistance in Pseudomonas putida. FEMS Microbiol Lett 2004, 232:14.

37. Doukyu N, Ishikawa K, Watanabe R, Ogino H: Improvement in organic solvent tolerance by double disruptions of provB and marR genes in Escherichia coli. J Appl Microbiol 2012, 112:464–474.

38. Stanciu MW: Effect of organic solvents on solvent-tolerant Aeromonas hydrophila IBFPo8 and pseudomonas aeruginosa IBFPo10. Indian J Biotechnol 2011, 10:352–361.
39. Vennum GN, Aristidou AA: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

40. Murata M, Fujimoto H, Nishimura K, Chario K, Tanaka M, Murata N: Identical Hik-Re systems are involved in perception and transduction of salt signals and hyposmotic signals but regulate the expression of individual genes to different extents in Synechocystis. J Bacteriol 2005, 187:2531–2538.

41. Murata M, Fujimoto H, Nishimura K, Chario K: Characterization of an alcohol dehydrogenase from the cyanobacterium Synechocystis sp. strain PCC 6803 that responds to environmental stress conditions via the Hik34–Hik1 two-component system. J Bacteriol 2009, 191:4383–4391.

42. Karam A, Yousif S, Yuasa T, Geng X, Ikeuchi M: Biochemical and functional characterization of an eukaryotic-type protein kinase, SppB, in the cyanobacterium Synechocystis sp. PCC 6803. Curr Microbiol 2003, 46:296–301.

43. Karam A, Yuasa T, Okawa K, Geng X, Ikeuchi M: An eukaryotic-type protein kinase, SpkA, is required for normal motility of the unicellular cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 2001, 183:1505–1510.

44. Zhang Z, Pendse ND, Phillips KN, Cotner JB, Khodursky A: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

45. Tafforeau L, Le Blastier S, Bamps S, Dewez M, Vandenhaute J, Hermand D: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

46. Matsuda N, Kobayashi H, Katoh H, Ogawa T, Futatsugi L, Nakamura T, Bakker EP, Uozumi N: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

47. Shigii N, Sakaguchi Y, Asai S, Suzuki T, Watanabe K: Common thiolation mechanism in the biosynthesis of RNA thiolide and sulphur-containing cofactors. EMBO J 2008, 7:3267–3278.

48. Sun J, Daniel R, Wagner-Döbler I, Zeng AP: A autoinducer-2 is a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. BMC Evol Biol 2004, 4:46–46.

49. Allahverdjei SJ, Murata N: Salt stress inhibits photosystems II and I in cyanobacteria. Photosynth Res 2008, 98:529–539.

50. Zhang Z, Pendse ND, Phillips KN, Cotner JB, Khodursky A: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

51. Verma SK, Singh HN: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

52. Nomura M, Ishitani M, Takabe T, Rai AK, Takabe T: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

53. Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kosaka T, Oshima T, Ogasawara N, Yamada M: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

54. Vemuri GN, Aristidou AA: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

55. Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kosaka T, Oshima T, Ogasawara N, Yamada M: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

56. Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kosaka T, Oshima T, Ogasawara N, Yamada M: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

57. Katoh A, Sonoda M, Katoh H, Ogawa T: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.

58. Barrett CM, Freudl R, Robinson C: Twin arginine translocation (Tat)-dependent export in the apparent absence of TatABC or TatA complexes using modified Escherichia coli TatA subunits that substitute for TatB. J Biol Chem 2007, 282:26196–26201.

59. Oliver DB: SecA protein: autoregulated ATPase catalysing preprotein insertion and translocation across the Escherichia coli inner membrane. Mol Microbiol 1993, 13:159–165.

60. Stock AM, Robinson VL, Goudreau PH: Two-component signal transduction. Annu Rev Biochem 2000, 69:183–215.

61. Shoumura KY, Pathoonrangsan K, Kanesaki Y, Los DA, Zinchenko WV, Tanticharoen M, Suzuki I, Murata N: Metabolic engineering in the -omics era: elucidating and modulating regulatory networks. Microbiol Mol Biol Rev 2005, 69:197–216.