Identification of alcohol stress tolerance genes of *Synechocystis* sp. PCC 6803 using adaptive laboratory evolution

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**Abstract**

**Background:** *Synechocystis* sp. PCC 6803 is an attractive organism for the production of alcohols, such as isobutanol and ethanol. However, because stress against the produced alcohol is a major barrier for industrial applications, it is highly desirable to engineer organisms with strong alcohol tolerance.

**Results:** Isobutanol-tolerant strains of *Synechocystis* sp. PCC 6803 were obtained by long-term passage culture experiments using medium containing 2 g/L isobutanol. These evolved strains grew on medium containing 5 g/L isobutanol on which the parental strain could not grow. Mutation analysis of the evolved strains revealed that they acquired resistance ability due to combinatorial malfunctions of slr1044 (*mcpA*) and slr0369 (*envD*), or slr0322 (*hik43*) and *envD*. The tolerant strains demonstrated stress resistance against isobutanol as well as a wide variety of alcohols such as ethanol, *n*-butanol, and isopentanol. As a result of introducing an ethanol-producing pathway into the evolved strain, its productivity successfully increased to 142% of the control strain.

**Conclusions:** Novel mutations were identified that improved the stress tolerance ability of various alcohols in *Synechocystis* sp. PCC 6803.

**Keywords:** *Synechocystis* sp. PCC 6803, Alcohol stress tolerance, Adaptive laboratory evolution, Isobutanol

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**Background**

Cyanobacteria are promising industrial microorganisms for bio-production because the cells can directly fix atmospheric carbon dioxide and convert it to a target compound using energy from photosynthesis. In recent years, the cyanobacterial production of alcohols, such as ethanol, *n*-butanol, and isobutanol, as an alternative to petroleum has attracted attention [1]. Isobutanol is an attractive fuel because of its higher energy density and lower hygroscopicity than ethanol. Overexpression of ribulose 1,5-bisphosphate carboxylase/oxygenase genes has led to the production of 450 mg/L isobutanol production in *Synechococcus elongatus* PCC 7942 [2]. However, its productivity is still lower than that of fermentation microorganisms such as *Escherichia coli* and *Corynebacterium glutamicum* [3, 4]. Alcohol stress is one of the causes of low productivity in cyanobacteria. In isobutanol production of *Synechocystis* sp. PCC 6803, production of biomass and isobutanol increased about 1.2 and 2.5 times, respectively, by in situ removal of the produced isobutanol using a solvent trap [5]. Because the growth reduction caused by alcohol stress occurs at a low concentration of 200 mg/L isobutanol, it is highly desirable to engineer cyanobacterial strains with strong alcohol tolerance for industrial production of isobutanol.

In a previous study, transcriptome responses of *Synechocystis* sp. PCC 6803 to 1-butanol identified candidate genes for enhancing 1-butanol tolerance, and the expression of small heat-shock protein HspA (sll1514) and hypothetical protein CccS (slr1617) successfully improved 1-butanol tolerance [6]. Furthermore, screening from the response regulator library revealed that sll0039 and slr1037 are involved in 1-butanol tolerance in...
Adaptive laboratory evolution (ALE) is a method for strain engineering to provide tolerance against a stress condition \cite{11,12}. During ALE, cells are repeatedly subcultured for prolonged periods under stress conditions leading to a reduced growth rate. This allows the selection of mutants with improved growth phenotypes under the stress environment \cite{13}. Compared to random mutagenesis approaches using mutagens such as chemicals and ultraviolet light, ALE with sequential serial passages is a relatively easy method for identifying key mutations related to the improvement because of its low mutation frequency \cite{14}. The productivity of the target compound can be enhanced by introducing the identified mutations that improve the tolerance of the strain into an engineered producer. Because a complete genome sequence of Synechocystis sp. PCC 6803 has already been determined \cite{15}, mutation positions are easily identified by genome resequencing analysis, which aligns genomic reads of the evolved strain to a reference genome \cite{16}. The mutations for improving acid stress tolerance in Synechocystis sp. PCC 6803 were obtained through ALE experiments under low pH conditions and by whole genome resequencing analysis \cite{17}.

It has been reported that cells acquiring a stress tolerance exhibit a tolerance to the same type of stress \cite{18,19}. In the present study, we aimed to obtain a tolerant strain of Synechocystis sp. PCC 6803 against various types of alcohols using ALE with medium containing isobutanol, which has strong toxicity. Mutations of evolved strains were identified by whole genome resequencing and were introduced into the genome of the parental strain to evaluate the contribution for alcohol tolerance by a reverse genetics approach. We demonstrated that the acquired resistance ability of the evolved tolerant strain enhances ethanol production.

Results

Adaptive laboratory evolution of Synechocystis sp. PCC 6803 under isobutanol stress conditions

Isobutanol-tolerant strains were obtained via long-term passage culture experiments. Prior to passage cultures, Synechocystis sp. PCC 6803 was cultured under different isobutanol concentrations (0, 1, 2, 3, and 5 g/L) to determine the appropriate concentration for ALE. The \(\text{OD}_{730}\) at 72 h decreased by one-half in medium containing 2 g/L isobutanol compared to samples under no isobutanol condition (Additional file 1: Fig. S1). Passage cultures were performed using medium containing 2 g/L isobutanol. Because various evolved strains with different mutations can be obtained in ALE experiments, four independent passage cultures were performed. The relationship between the specific growth rate and accumulated culture time during ALE experiments are shown in Additional file 1: Fig. S2. The specific growth rates obviously increased until 500 h in all four cultures. Although cell aggregation was observed until 300 h, cells eventually separated. Because no increase in the specific growth rate was observed after 1000 h, the evolved cells at 1824 h were stored at \(-80^\circ\text{C}\). Four strains, named T(1), T(2), T(3), and T(4), were isolated from the stored cultures of Nos. 1, 2, 3, and 4, respectively.

To evaluate the isobutanol tolerance of the four evolved strains obtained from the ALE experiments, the parental and the evolved strains were cultured at different isobutanol concentrations (0, 1, 2, 3, and 5 g/L). The specific growth rate of the evolved strains increased compared to that of the parental strain with more than 2 g/L isobutanol (Fig. 1). Furthermore, the evolved strain grew in the presence of 5 g/L isobutanol which completely inhibited the growth of the parental strain. Although cell aggregation was observed in the parental strain, this was not observed in the evolved strains exposed to 2 g/L isobutanol (Fig. 2 and Additional file 1: Fig. S3).

Whole genome resequencing of the evolved strains

To investigate the mutations within the evolved strains, whole genome resequencing was performed using a MiSeq sequencer through paired-end sequencing with a read length of 250 bp. Coverage depth was approximately 100–200 reads. Identified mutations of the evolved strains are summarized as a Venn diagram in Fig. 3. The
numbers of mutations in T(1), T(2), T(3), and T(4) strains were 2, 12, 2, and 2, respectively. T(1) and T(4) strains possessed mutations in mcpA, whereas T(2) and T(3) strains possessed mutations in hik43. All four evolved strains possessed mutations in envD. No mutation was identified in non-coding regions. Detailed information about the mutations is summarized in Fig. 3 and Table 1. Because the T(2) strain possessed a stop codon mutation in mutS, which encodes DNA mismatch repair protein, a higher number of mutations were accumulated during ALE than that in other evolved strains. It has been reported that mcpA and hik43 are involved in the biogenesis of pili for generating motile force [20], and envD is involved in the transport of toxin [21]. The genome resequencing results suggest that these common mutations of mcpA, hik43, and envD in the evolved strains could be related to the observed enhanced isobutanol tolerance.

Because the mutations of mcpA in T(1) and T(4) strains lead to a frame shift and a premature stop codon, the resulting putative truncated products are 75 and 30 amino acid residues, respectively, out of 869 amino acid residues. These truncated products must have no function due to their short length. The mutation of hik43 in T(2) also lead to a frame shift and a premature stop codon. As the result, the putative truncated product is only 252 amino acid residues out of 1095 amino acid residues. The mutation of envD in T(1) also lead to a frame shift and a premature stop codon. As the result, the putative truncated product is only 158 amino acid residues out of 1083 amino acid residues. On the other hands, the putative products of EnvD in T(2), T(3), and T(4) strains had only few amino acid substitutions.

**Effect of mutations on isobutanol tolerance**

To evaluate the effect of the mutations in mcpA, hik43, and envD of the evolved strains on isobutanol tolerance, the mutations and corresponding gene deletions were introduced into the genome of the parental strain.

mcpA encodes a methyl-accepting chemotaxis protein. No growth recovery was observed by introducing the mcpA deletion in the presence of 2 g/L isobutanol (Additional file 1: Fig. S4). Cell aggregation, which was observed in the parental strain, was dispersed in the ∆mcpA strain (Fig. 2 and Additional file 1: Fig. S5). This indicates that mcpA contributes to cell aggregation under the isobutanol stress condition. The strain did not grow in the presence of 5 g/L isobutanol, as well as its parental strain. The mcpA-R1 and mcpA-R4 strains were constructed by introducing mcpA mutations derived from the T(1) and T(4) strains, respectively, into the ∆mcpA strain. Because the mcpA-R1 and mcpA-R4 strains showed a similar phenotype as the ∆mcpA strain, the mcpA mutations were considered loss-of-function mutations.

hik43 encodes a histidine kinase, which is a member of the two-component hybrid sensor and regulator. Because stop codon mutations occurred in hik43 of the
T(2) and T(3) strains (Table 1), these mutations were also considered loss-of-function mutations. The Δhik43 strain showed the same growth phenotype as the mcpA mutants in the presence of isobutanol (Fig. 4). Cell aggregation was also dispersed in the Δhik43 strain in the presence of 2 g/L isobutanol (Fig. 2), indicating that hik43 also contributes to cell aggregation under alcohol stress.

envD encodes the resistance nodulation division multidrug efflux transporter protein. Although the specific growth rate of the ΔenvD strain decreased compared to the parental strain, in the presence of 2 g/L isobutanol, the ΔenvD strain grew in the presence of 5 g/L isobutanol, which completely inhibits the growth of the parental strain (Fig. 4 and Additional file 1: Fig. S6). The specific growth rate of the ΔenvD strain was lower than that of the evolved strain. The envD-R1, envD-R2, envD-R3, and envD-R4 strains were constructed by introducing envD mutations derived from the T(1), T(2), T(3), and T(4) strains, respectively, into the ΔenvD strain. Cell aggregation was observed in all envD mutants in the presence of 2 g/L isobutanol as well as the parental strain (Fig. 2 and Additional file 1: Fig. S7). Because the envD-R1, envD-R2, envD-R3, and envD-R4 strains showed a similar phenotype to the ΔenvD strain (Additional file 1: Table 1 Details about the mutations of evolved strains a Del, Ins, and SNP indicate an deletion, insertion, and single nucleotide polymorphism, respectively b Mutation ratios indicates

| Strain | Type | Position | Reference | Mutation | Gene | Type | Mutation ratio (%) | Function |
|--------|------|----------|-----------|----------|------|------|---------------------|----------|
| T(1)   | Del  | 492349   | ATCCCTA   | -T------ | slr1044 (mcpA) | Frameshift, stop codon | 95 | Methyl-accepting chemotaxis protein |
| Ins    | 2364114 | CCG   | CCGA      | slr0369 (envD) | Frameshift, stop codon | 94 | RND multidrug efflux transporter |
| T(2)   | Ins  | 345787   | TGG       | TGGG     | sll0923 (epsB) | Frameshift, stop codon | 92 | Unknown protein |
| Ins    | 389860 | CCC   | CCCC      | sll1025  | Frameshift, stop codon | 86 | Hypothetical protein |
| Ins    | 549361 | CCG   | CGGG      | sll1636 (fbp) | Frameshift, stop codon | 84 | Ferricyochelin-binding protein |
| SNP    | 755131 | CTG   | TTG       | sll1924 (sycp2) | Leu → Leu | 99 | cAMP receptor protein |
| Ins    | 1041211 | GGG   | GGGG      | sll1233 (frdA) | Frameshift, stop codon | 94 | Succinate dehydrogenase flavoprotein subunit |
| Del    | 2270044 | GGG   | -GG       | slr0322 (hik43) | Frameshift, stop codon | 93 | Two-component hybrid sensor and regulator |
| SNP    | 2279149 | CAG   | GAG       | slr0326  | Gln → Glu | 97 | Hypothetical protein |
| SNP    | 2364333 | GTT   | GGT       | envD     | Val → Gly | 100 | RND multidrug efflux transporter |
| Del    | 2585782 | CCC   | -CC       | sll0058 (dnaK) | Frameshift, stop codon | 93 | Heat-shock protein 70, molecular chaperone |
| Del    | 3430453 | CTT   | C-T       | slr0756 (kaiA) | Frameshift, stop codon | 95 | Circadian clock protein KAI homolog |
| Ins    | 1372331 | CAA   | CAAC      | sll1165 (mutS) | Frameshift, stop codon | 80 | DNA mismatch repair protein MutS |
| T(3)   | Del  | 1766850 | ACGGCGCTTC-CAAGG | AC--------G | slr1963 (ocp) | Del (Ala, Leu, Pro) | 88 | Water-soluble carotenoid protein |
| SNP    | 2271565 | TGG   | TGA       | hik43    | Stop codon | 98 | Two-component hybrid sensor and regulator |
| Del    | 2366401 | TCGGGGCGA | -------- | envD    | Del (Ser, Gly, Arg) | 88 | RND multidrug efflux transporter |
| T(4)   | Ins  | 492262 | TAT       | mcpA     | Frameshift, stop codon | 95 | Methyl-accepting chemotaxis protein |
| SNP    | 2366659 | GTT   | TTT       | envD     | Val → Phe | 99 | RND multidrug efflux transporter |

a Del, Ins, and SNP indicate an deletion, insertion, and single nucleotide polymorphism, respectively
b Mutation ratios indicates
Fig. S6), the envD mutations were also considered loss-of-function mutations.

The specific growth rates of the constructed strains with single mutations of mcpA, hik43, and envD were lower than that of the evolved strains obtained from ALE experiments (Fig. 4). Because all four evolved strains possess combinatorial mutations of mcpA and envD, or that of hik43 and envD (Fig. 3), it was assumed that the evolved strains acquired the isobutanol tolerance ability through the synergistic effect of these mutations. Since these mcpA, hik43, and envD mutations are considered loss-of-function mutations, the isobutanol tolerance of double deletion mutants (ΔmcpA/ΔenvD and Δhik43/ΔenvD strains) were evaluated. As expected, the ΔmcpA/ΔenvD and Δhik43/ΔenvD strains showed identical growth with the evolved strain under 0, 2, and 5 g/L isobutanol concentrations (Fig. 4). Cell aggregation dispersed in ΔmcpA/ΔenvD and Δhik43/ΔenvD strains in the presence of 2 g/L isobutanol (Fig. 2). These findings suggest that the evolved strains acquire isobutanol tolerance through the loss of function of mcpA and envD, or that of hik43 and envD.

Evaluation of stress tolerance against other alcohols of the evolved strains

Cells acquiring stress tolerance exhibit a tolerance to the same type of stress [18, 19]. Tolerance of the evolved strains against other alcohols such as ethanol, n-butanol, and isopentanol were evaluated (Fig. 5). The mutations important for alcohol tolerance were combinatorial malfunctions of envD and mcpA, or that of envD and hik43. Since both T(1) and T(4) strains have functional deficits in mcpA and envD, and both T(2) and T(4) strains have functional deficits in hik43 and envD (Fig. 3), alcohol tolerances were evaluated for representative T(1) and T(2) strains. The specific growth rate of the evolved strains increased compared to the parental strain in the presence of 16 g/L ethanol, which contains less carbon atoms than isobutanol. In the presence of 1 and 3 g/L n-butanol, which has the same number of carbon atoms of isobutanol with a different main chain, the specific growth rate of the evolved strains increased compared to the parental
strain. In the presence of 0.8 and 1 g/L isopentanol, which contains more carbon atoms than isobutanol with the same main chain, the specific growth rate of the evolved strains also increased compared to the parental strain (Fig. 5). These findings suggest that the evolved strains obtained by ALE under isobutanol stress exhibited tolerance against isobutanol as well as ethanol, n-butanol, and isopentanol.

Ethanol production using the evolved strain
The evolved strains showed tolerance against not only isobutanol but also ethanol (Fig. 5). The effect of alcohol tolerance on ethanol production was investigated. Ethanol-producing strains of the parental and T(1) strains, called SE and SE-T(1) strains, were constructed by introducing heterologous genes for pdc and adhII from Zymomonas mobilis. Culture profiles of SE and SE-T(1) strains are shown in Fig. 6. Although the specific growth rate of the SE-T(1) strain was slightly lower than that of the SE strain, enhanced ethanol production was observed in the SE-T(1) strain. The ethanol productivity at 72 h of SE and SE-T(1) strains were 10.4 ± 0.4 and 14.8 ± 0.2 mg L⁻¹ OD⁻¹, respectively.

Discussion
In this study, evolved strains of Synechocystis sp. PCC 6803 were obtained using the ALE method in medium containing 2 g/L isobutanol, to engineer a strain with strong tolerance against various alcohols (Fig. 5). Genome resequencing analysis demonstrated that the evolved strains possess mutations in mcpA and envD, or hik43 and envD (Fig. 3). Reverse genetics approaches revealed that the mutations of mcpA, hik43, and envD were loss-of-function mutations, and the evolved strains acquired resistance by combinatorial malfunctions of envD and mcpA, or that of envD and hik43 (Fig. 4).

In the ΔmcpA and Δhik43 strains, since no cell aggregation was observed in the presence of 2 g/L isobutanol, mcpA and hik43 genes are likely related to cell flocculation. It has been reported that deletion of mcpA or hik43 causes a loss of thick pili, which is essential for phototactic motility in Synechocystis sp. PCC 6803 [20]. Although the thick pili play a role in uptake of extracellular DNA in nature, these pili do not only extracellular DNA but also the surrounding cells under isobutanol-induced stress. Cell aggregation of Synechocystis sp. PCC 6803 was also observed under n-butanol stress [22]. Transcriptome analysis demonstrated the up-regulation of genes involved in chemotaxis [6]. The relationship between cell flocculation size and ethanol tolerance has been investigated in yeast. Yeast has improved ethanol tolerance as flocculation size increases. On the other hand, in this study, alcohol tolerance was improved by making free of flocculation, so the tolerance was acquired by a mechanism different from yeast [23, 24].

The amino acid sequence of a protein encoding envD is similar to that of AcrB and AcrD of E. coli. Pairwise alignments show that both proteins share 38% amino acid sequence identity. Amino acid sequence similarities of the EnvD to the E. coli AcrB and AcrD are 58 and 59%, respectively. E. coli AcrB and AcrF have been categorized into a same orthologous protein cluster of EnvD in Cyanobase for the protein homology database featuring cyanobacteria and plastids [25]. The AcrB is a component of the AcrAB-TolC transporter, which can export a broad variety of compounds with little chemical similarity [26]. It has been reported that the deletion of AcrAB-TolC contributed to enhance tolerance against solvents such as styrene, 1-octanol, and isobutanol [27]. Isobutanol stress causes leakage of quinone, an electron acceptor in the E. coli respiratory chain, owing to membrane damage [28]. Because AcrAB-TolC can also export quinone compounds [29] and is highly induced by isobutanol stress [28], the deletion of AcrAB-TolC increased isobutanol tolerance with reducing the quinone [18]. Although, to our knowledge, there are no studies on the molecular function of EnvD in Synechocystis sp. PCC 6803, its amino acid sequence similarity with AcrB would also be expected to involve quinone export. The envD deletion strain could acquire isobutanol tolerance with alleviation of quinone sink under the isobutanol stress condition. Despite the fact that the envD deletion makes it possible for the strain to grow in the presence of 5 g/L isobutanol, the specific growth rate of the envD deletion strain decreased compared to the parental strain in the presence of 2 g/L isobutanol. Because deletion of membrane and membrane-associated proteins, such as transporters, would affect membrane lipids [27], the envD deletion negatively affected the cell growth indirectly in the presence of 2 g/L isobutanol.
Since the ALE experiments were conducted using 2 g/L isobutanol, the mutants of envD should not be selected because of its negative effect for the tolerance to this isobutanol level. Synergistic effects for isobutanol tolerance induced by the combinatorial gene deletions of envD and mcpA, or that of envD and hik43 are shown in Fig. 4. The findings indicate that first the mutations in mcpA or hik43 were introduced, followed by the mutations in envD, since non-aggregated cells by mcpA or hik43 mutation are easy to subculture in the passaged cultures. Although the mechanisms of the synergistic effect for isobutanol tolerance by these two genes are still unclear, photon supply to each cell increases through interference with cell aggregation. Additionally, the metabolic activities could be enhanced through alleviation of quinone sink with envD mutations. It is difficult to rationally predict such synergistic effects for tolerance by multiple gene deletions. The ALE method has become a powerful tool for strain improvement to obtain stress tolerance.

The expression of small heat-shock protein HspA (sll1514) and hypothetical protein CccS (slr1617) has improved 1-butanol tolerance [6]. Furthermore, it is known that a response regulator (Slr1037) is involved in butanol tolerance of Synechocystis sp. PCC 6803 [7], and co-overexpression of slr1037 and sll0039 improves butanol tolerance by 133% [9]. An overexpression of SigB is also known to improve 1-butanol tolerance in Synechocystis sp. PCC 6803 [10]. By understanding the mechanism of the alcohol tolerance acquisition by the combinatorial malfunctions of envD and mcpA, or that of envD and hik43, it may help to further enhance the alcohol tolerance in coordination with these previous findings.

The SE-T(2) strain, in which ethanol-producing genes were introduced, shows enhanced ethanol production compared to the control strain. This indicates that the ethanol tolerance ability is effective for enhancing the ethanol production rate. Since ethanol affected the growth of the parental strain at concentrations of up to 16 g/L (Fig. 5), a higher ethanol concentration was required to inhibit growth of the SE-T(2) strain (28.3 mg/L at 48 h). If ethanol excretion is a rate-limiting step, the ethanol concentration in the cells becomes much higher than that of the extracellular culture broth. Therefore, the ethanol-producing cells would be exposed to a stress condition in which the concentration of ethanol is higher than the produced ethanol concentration. Furthermore, the transporter malfunction may indirectly contribute to ethanol production. Because the mechanism is unknown, detailed metabolic analysis is necessary to clarify this.

In this study, we chose ethanol, which has been widely studied in cyanobacterial bio-production as a target alcohol. Since the evolved strains showed tolerance abilities against various alcohols (Fig. 5), the strains have the potential to enhance the production rate of a wide variety of alcohols. Since the mutation for enhancing alcohol tolerance was obtained by ALE experiments using isobutanol, it is expected to contribute in isobutanol production, especially. Isobutanol showing more strong toxicity may clearly demonstrate the effect of the mutations.

Conclusions
Alcohol stress tolerant strains of Synechocystis sp. PCC 6803 were obtained via the ALE method using medium containing isobutanol, which induces strong toxicity. The evolved strains showed increased growth rates in more than 2 g/L isobutanol, and could grow in the presence of 5 g/L isobutanol, which completely inhibits the growth of the parental strain. Genome resequencing and reverse genetics analyses revealed that the evolved strains acquired their resistive capacity owing to combinatorial malfunctions of envD and mcpA, or that of envD and hik43. Furthermore, the evolved strains also demonstrated tolerance against a wide variety of alcohols, such as ethanol, n-butanol, and isopentanol. The ethanol productivity successfully increased to 142% of the control strain by introducing an ethanol-producing pathway into the evolved strain.

Methods
Strain and culture condition
The Synechocystis sp. PCC 6803 glucose-tolerant strain served as the parent strain. All strains used in this study are listed in Table 1. Cultures were performed autotrophically using 100-ml Erlenmeyer flasks with 20 mL BG11 medium [30] at 34 °C, with rotary agitation at 150 rpm (BR-43FL shaker; TAITEC Co., Ltd., Japan) under continuous illumination (40 μmol m^{-2} s^{-1}) by white light-emitting diodes (LC-LED450 W, TAITEC Co., Ltd., Japan). The BG11 medium was supplemented with 50 mM NaHCO₃ and the required antibiotics.

Analytical methods
Cell growth was measured as optical density at 730 nm (OD₇₃₀) using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). The ethanol concentration of the culture was measured by gas chromatography with a hydrogen flame ionization detector (7890A; Agilent Technologies, CA, USA) and a Stabilwax column (0.32 mm internal diameter, 60 m length, and 1 μm thickness; Restek, PA, USA). The analytical conditions are described elsewhere [31]. A light optical microscope (Imager M2, Carl Zeiss, Jena, Germany) with AxioVision Release 4.8 was used to examine the flocculation of the cells.
Tolerance test of alcohols
Cells were inoculated at OD_{730} of 0.05 for pre-culture, and then incubated in the above conditions. After 3 days, the pre-cultivated cells were harvested by centrifugation, and then inoculated into the fresh BG11 medium at OD_{730} of 0.03. To test the alcohol tolerance, an appropriate amount of 99% (w/w) isobutanol, 99.5% (w/w) ethanol, 99% (w/w) n-butanol, or 98% (w/w) isopentanol was supplemented into the medium. A silicon cap was used in the culture to prevent evaporation of alcohols from the culture broth. The specific growth rate was calculated from OD_{730} up to 0–48 h for each culture.

Adaptive laboratory evolution
Sequential serial passages were performed using the parental strain and BG11 medium with 2 g/L isobutanol under the previously mentioned conditions. Cells were inoculated into fresh medium every 3.5 days at OD_{730} of 0.03 to maintain the growth phase in four parallel cultures. A silicon cap was used to prevent the evaporation of isobutanol. The specific growth rate was calculated from OD_{730} during the exponential growth phase (0–48 h).

Whole genome sequencing
The genomic DNAs of the parental and its evolved strains were extracted using a genome extraction kit (Qiagen, CA, USA). The DNA samples were sequenced using a MiSeq sequencer (Illumina) with the MiSeq Reagent kit v2 (Illumina) generating 150 bp paired-end reads. The sequenced reads were mapped to the genome sequence of the *Synechocystis* sp. PCC 6803 GT-I strain (NCBI Reference Sequence No.: NC_017038.1) [16] using the Bowtie 2 software ver. 2.2.3 [32]. The SNPs of the parental strain to the reference genome were identified using Bowtie 2 software ver. 2.2.3 [32]. The SNPs of the evolved strains were also checked using the Integrative Genomics Viewer [33]. The SNPs of the evolved strains identified by whole genome sequencing were confirmed by Sanger sequencing (Eurofins) using DNA fragments, including the mutation site amplified by PCR with the primer pair listed in Additional file 1: Table S1.

Genomic manipulations
Strains used in this study are listed in Table 2. Single gene deletion strains of ΔmcpA, Δhik43, and ΔenvD were constructed using *Synechocystis* sp. PCC 6803 glucose-tolerant strain as the parental strain by replacing each corresponding gene with a kanamycin-resistance gene. To construct strains in which mutations were identified after ALE, the kanamycin-resistance gene in the corresponding gene deletion strain was replaced with the mutated gene and a streptomycin-resistance gene. Double gene deletion strains of ΔmcpA/ΔenvD and Δhik43/ΔenvD were constructed using the ΔenvD strain by replacing mcpA or hik43 with a streptomycin-resistance gene. Complete segregation of the target site by the antibiotic resistance gene was confirmed by the disparity in the sizes of the PCR products generated using the primers that bound outside the upstream and downstream regions of target site for homologous recombination. Detailed procedures for strain construction, including plasmid and primer information, are described in an additional document.

### Table 2 Strains used in this study

| Strains | Description | References |
|---------|-------------|------------|
| Parent  | *Synechocystis* sp. PCC 6803 GT-I | [31] |
| T(1)    | Obtained from ALE in No. 1 This work | |
| T(2)    | Obtained from ALE in No. 2 This work | |
| T(3)    | Obtained from ALE in No. 3 This work | |
| T(4)    | Obtained from ALE in No. 4 This work | |
| ΔmcpA   | mcpA::Kmr | The work |
| Δhik43  | hik43::Kmr | The work |
| ΔenvD   | envD::Kmr | The work |
| mcpA-R1 | mcpA::mcpA (1) Smr | This work |
| mcpA-R4 | mcpA::mcpA (2) Smr | This work |
| envD-R1 | envD::envD (1) Smr | This work |
| envD-R2 | envD::envD (2) Smr | This work |
| envD-R3 | envD::envD (3) Smr | This work |
| envD-R4 | envD::envD (4) Smr | This work |
| ΔenvD/ΔmcpA | envD::Kmr, mcpA::Smr | The work |
| ΔenvD/Δhik43 | envD::Kmr, hik43::Smr | The work |
| SE      | ndhB::P_{pdc adhII} | This work |
| SE-T(1) | T(1)::P_{pdc adhII} | This work |

**Authors’ contributions**
All authors contributed to the planning of this study. TM and KY performed experiments. TM, YT, and HS drafted the manuscript. All authors read and approved the final manuscript.

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

Availability of supporting data
The whole genome sequence data of T(1), T(2), T(3), and T(4) strains are deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) under the Accession Number DRA006219.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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