A novel small RNA CoaR regulates coenzyme A biosynthesis and tolerance of *Synechocystis* sp. PCC6803 to 1-butanol possibly via promoter-directed transcriptional silencing

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**Abstract**

**Background:** Microbial small RNAs (sRNAs) have been proposed as valuable regulatory elements for optimizing cellular metabolism for industrial purposes. However, little information is currently available on functional relevance of sRNAs to biofuels tolerance in cyanobacteria.

**Results:** Here, we described the identification and functional characterization of a novel 124 nt sRNA Ncl1460 involved in tolerance to biofuel 1-butanol in *Synechocystis* sp. PCC 6803. The expression of Ncl1460 was verified by blotting assay and its length was determined through 3′ RACE. Further analysis showed that Ncl1460 was a negative regulator of slr0847 (*coaD*) and slr0848 operon responsible for coenzyme A (CoA) synthesis possibly via promoter-directed transcriptional silencing mechanisms which has been widely discovered in eukaryote; thus Ncl1460 was designated as CoaR (CoA Biosynthesis Regulatory sRNA). The possible interaction between CoaR and target genes was suggested by CoA quantification and green fluorescent protein assays. Finally, a quantitative proteomics analysis showed that CoaR regulated tolerance to 1-butanol possibly by down-regulating CoA biosynthesis, resulting in a decrease of fatty acid metabolism and energy metabolism.

**Conclusions:** As the first reported sRNA involved CoA synthesis and 1-butanol tolerance in cyanobacteria, this study provides not only novel insights in regulating mechanisms of essential pathways in cyanobacteria, but also valuable target for biofuels tolerance and productivity modifications.

**Keywords:** sRNA, *Synechocystis*, Tolerance, 1-Butanol, CoA biosynthesis, Transcriptional silencing

**Background**

Bacterial small RNAs (sRNAs) are functional non-coding RNAs with a typical length of 50–300 nt, among which trans-acting sRNAs (ncRNAs) transcribe from the intergenic regions and function at a distance to alter the expression of target RNAs, while cis-acting sRNAs (asRNAs) transcribe from the complementary strand of the known open reading frames (ORFs) and regulate translation or decay mRNA molecules through base pairing [1]. Photosynthetic cyanobacteria have been proposed as “autotrophic cell factories” for biofuels production due to its ability to utilize CO₂ and sunlight directly for growth [2]. In cyanobacteria *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), though previous studies have approximately identified 300 putative sRNAs [3], their functional characterizations still significantly lag behind. So far only several sRNAs of *Synechocystis* have been functionally revealed, including an asRNA IsrR functioning as a negative regulator of the CP43 homolog IsiA, a negative...
regulator named as_flv4 involved in response to shifts in inorganic carbon supply. PsbA2R and PsbA3R acting as positive regulators to enhance the stability of target mRNAs as well as PsrR1 controlling photosynthetic functions [4]. Recently, NsrR4 was found involved in nitrogen assimilation control [5].

1-Butanol biosynthetic pathways have been introduced into various cyanobacterial systems and its production could reach 404 mg/L in Synechococcus elongatus PCC 7942 and 37 mg/L in Synechocystis [6, 7]. However, current productivity is still much lower than the native Clostridium or even synthetic Escherichia coli systems [8, 9], partially due to the high toxicity of 1-butanol to cyanobacteria [2]. Recently, Kaczmarzyk et al. [10] over-expressed an RNA polymerase sigma factor sigB in Synechocystis and successfully enhanced the tolerance to 1-butanol in a butanol-shock experiment. Our recent efforts on studying metabolic responses of Synechocystis to various biofuels have also led to the discovery of two response regulator genes (i.e., slr1037 and sll0039) related to 1-butanol tolerance [11, 12], and several transcriptional regulators (i.e., slr0794, sll1392, sll1712, and slr1860) related to ethanol tolerance [13, 14]. These studies demonstrated the possibility of improving biofuel tolerance by engineering transcriptional regulation. Previous studies have shown the vital roles of sRNAs in responding to environmental perturbations among various microbes through transcriptional or post-transcriptional regulation [1]. In a recent study, Gaida et al. [15] showed that overexpression of three sRNAs, DsrA, RprA, and ArcZ, could increase acid tolerance of E. coli up to 8500-fold. Meanwhile, the transcriptomic analysis of cellular responses to ethanol production in Synechocystis identified several regulated functionally unknown sRNAs including ncl1740, ncl1390, and ncl1600 [16], suggesting that sRNAs could be involved in the regulation of biofuels tolerance.

In this work, we employed a RNA-seq approach to identify sRNAs related to exogenous 1-butanol tolerance in Synechocystis, which led to discover a novel sRNA Ncl1460 responsive to 1-butanol. Further analysis demonstrated that Ncl1460 was a negative regulator of coaD involving coenzyme A (CoA) biosynthetic pathway, and was accordingly designated as CoaR (CoA Biosynthesis Regulatory sRNA). The interaction between CoaR and target genes was verified by CoA quantification and GFP assays. Finally, quantitative proteomics analysis revealed the decreased CoA content caused by coaR overexpressing could down regulate fatty acid metabolism and energy metabolism thus led to a decreased 1-butanol tolerance. As the first reported sRNA involved CoA synthesis and 1-butanol tolerance in cyanobacteria, this study provides not only novel insights in regulating mechanisms of essential pathways in cyanobacteria, but also valuable target for biofuels tolerance and productivity modifications.

**Results**

**Growth of Synechocystis under 1-butanol stress**

Effects of 1-butanol on Synechocystis were performed using wild-type (WT) in BG-11 media supplemented without or with 1-butanol at a concentration of 0.15, 0.20 and 0.25% (v/v), respectively (Fig. 1a). Consistent with previous study [17], the results showed that 0.20% (v/v) 1-butanol caused about 50% growth inhibition at 48 h, and this concentration was selected for the transcriptomic analysis. WT cultivated with or without 1-butanol (0.20%, v/v) were collected at 24, 48, and 72 for small RNA-seq analysis, respectively, named accordingly as C24, C48, and C72 for control as well as B24, B48, and B72 for butanol-treated samples, respectively.

**sRNomic analysis**

After adapter trimming and data filtering, a total of 9,669,974, 13,383,938, 6,919,528, 9,938,132, 6,951,722, and 8,373,222 reads were obtained in six samples (i.e., C24, C48, C72 and B24, B48, B72, respectively). Then the sRNA reference libraries of Synechocystis (including approximately 1,071 known asRNAs and 320 known ncRNAs) described previously by Mitschke et al. [3] were used to identify the sRNAs in our study. Although the effective sequencing depth may vary in different samples, the obtained reads were able to match to approximately 1000 asRNAs and 290 ncRNAs (Additional file 2: Table S1), suggesting a good coverage.

Reads per kilobase per million mapped reads (RPKM) was employed to normalize the raw reads and a principal component analysis (PCA) was used to visualize the sRNomic expression profiles. The score plot showed that 1-butanol-treated samples (i.e., B24, B48 and B72) were visibly separated from the control samples (i.e., C24, C48, and C72) (Fig. 1b), suggesting that significant responses existed after 1-butanol treatment. Comparative expression analysis of the identified sRNAs showed that a total of 98, 168, 121 asRNAs and 42, 60, 43 ncRNAs were differentially expressed at 24, 48, and 72 h after 1-butanol treatment, respectively, using a criterion of the fold change >1.5 and p values ≤0.05 (Additional file 2: Table S2). To validate the reliability, 10 sRNAs were randomly selected for a quantitative real-time RT-PCR (qRT-PCR) analysis (Primers were listed in Additional file 2: Table S3). Comparative qRT-PCR analysis between the butanol-treated and control samples showed a significant correlation with a correlation coefficient \( R^2 > 0.70 \) using the Pearson correlation analysis (Fig. 1c), suggesting the high reliability of the sRNomic analysis.
sRNA Ncl1460 involved in 1-butanol stress response

A series of differentially regulated ncRNAs were selected for constructing overexpression [sense fragments of sRNA, expressed as sRNA(+)] and suppression strains [antisense fragments of sRNA, expressed as sRNA(−)] using a replicating vector pJA2 [10] and the phenotypic difference was examined under 0.20% (v/v) 1-butanol stress (Additional file 2: Table S4; Additional file 1: Fig. S1A) [18]. Although most of the mutants constructed showed no growth difference from WT, Ncl1460(+) (i.e., Synechocystis with overexpressed ncl1460) became more sensitive to 0.20% (v/v) 1-butanol than WT (Fig. 2a). This phenotype was even more significant under high 1-butanol concentration like 0.25% (v/v) (Fig. 2b, c). Interestingly, Ncl1460(−) (i.e., Synechocystis with suppressed ncl1460) became more tolerant to 1-butanol than WT (Fig. 2a, b). The overexpression and suppression of Ncl1460 in Ncl1460(+) and Ncl1460(−) were confirmed by reverse transcriptional PCR (RT-PCR) using WT as a control (Additional file 1: Fig. S3A), which clearly showed that the transcriptional level was increased in Ncl1460(+) but decreased in Ncl1460(−), respectively.

The above phenotype indicated that ncl1460 may be a negative regulator during 1-butanol stress response. In the sRNomic data, the RKPM values for Ncl1460 were 14.52, 8.40, and 11.37 under control condition at 24, 48, and 72 h, respectively, while they respectively decreased to 5.65, 5.66, and 4.03 after 1-butanol treatment, indicating a remarkably decrease of the relative expression by 2.5-, 1.5-, and 2.9-fold (Additional file 2: Table S1). To verify whether Ncl1460(+) was sensitive specifically to 1-butanol, growth patterns were also determined under Cd2+(4.6 μM), low pH (pH = 6.1) and NaCl (4.0% w/v) stress conditions. However, no differences were observed under Cd2+, NaCl, and low pH conditions, thus we focused on 1-butanol tolerance in this study.

Northern blotting and 3′ RACE of Ncl1460

To verify the expression of Ncl1460, northern blotting assays was conducted using the specific probe of Ncl1460 and the results shown in Additional file 1: Fig. S3B clearly
demonstrated its presence as a sRNA. In a previous study, the 5′ end of Ncl1460 has been determined while its 3′ end reminded unclear since the longest transcript among the several candidate transcripts for ncl1460 through deep sequencing was simply selected in the previous study [19]; here, we determined the 3′ end using 3′ RACE (Additional file 1: Fig. S3C). After Sanger sequencing, the corrected length of Ncl1460 was confirmed as 124 nt, which was 55 nt shorter than previously reported (179 nt) [3].

sRNA Ncl1460 up-regulated in butanol-adapted strains
In our previous study, Wang et al. [20] achieved a 150% increase in tolerance to 1-butanol in Synechocystis after a continuous 94 passages with gradually increased 1-butanol concentration from 0.20 to 0.50% (v/v). As ncl1460 involve in 1-butanol tolerance, its expression may also be regulated during the evolutionary process. To prove the hypothesis, we applied a qRT-PCR analysis to measure the relative expression levels of ncl1460 in seven evolutionary strains enduring different concentrations of 1-butanol. The results showed that relative expression level of ncl1460 decreased clearly in all the evolutionary strains, and it was even lower in E3 and E5 which can endure 0.30 and 0.40% 1-butanol (v/v) than that in WT, respectively, (Fig. 2d), suggesting that besides coding genes, the transcriptional level of non-coding sRNAs could also change in laboratory-based adaptive strains.

sRNA Ncl1460/CoaR as a negative regulator for coenzyme A biosynthesis
ncl1460 was located between genes encoding a phytochrome-like protein (Sll0821) and a phosphopantetheine adenyllytransferase (PPAT, Slr0847) (Fig. 3a). To identify potential target genes regulated by Ncl1460, the CopraRNA software developed by Wright et al. (2014) was employed for target prediction [21] and six
cyanobacteria genomes (i.e., NC_000911, NC_008312, NC_009091, NC_009840, NC_011738, NC_007776) were taken as the sRNA homologs for inputs [22]. The result found one of the flanking genes slr0847 ranked first in the candidates (Additional file 1: Fig. S4). In *Synechocystis*, PPAT encoded by slr0847 was the only enzyme reported so far to catalyze the conversion from pantotheine 4′-phosphate to 3′-dephospho-CoA (dPCoA) in CoA biosynthesis [23]. In addition, slr0847 was found to be organized into one possible operon with slr0848 encoding a hypothetical protein which led to an enlarged cell size after deletion [24, 25]. As ncl1460 located upstream the slr0847 on the complementary strand, we hypothesized that Ncl1460 may regulate slr0847 by directly base paring. Transcriptional starting site (TSS) of slr0847 has been identified in previous study and Ncl1460 was on its upstream but not within the transcribed region of slr0847 (Fig. 3a), excluding the possibility that Ncl1460 regulated slr0847 through post-transcriptional mechanisms by base paring [19]. As the possible interaction region between ncl1460 and upstream region of slr0847 was clearly predicted by CopraRNA (Additional file 1: Fig. S5), suggesting other mechanism may exist. In eukaryotes like *Saccharomyces cerevisiae*, plants and even mammalian cells, promoter-directed transcriptional silencing mediated by siRNA, have been discovered in recent years [26]. Under this mechanism, sRNA located beyond the TSS of target genes and silenced targets through mediating the methylation of promoters [27]. To verify whether Ncl1460 could affect its potential targets slr0847 and slr0848, we examined the intracellular contents of dPCoA, CoA as well as the cell size differences between WT, Ncl1460(+) and Ncl1460(−) under both normal BG11 or BG11 medium with 0.25% (v/v) 1-butanol. Using liquid chromatography–mass spectrometry (LC/MS), the intracellular contents of dPCoA in Ncl1460(+) strain were found decreased by 14.7 and 13.5% under normal medium as well as 13.1 and 15.5% under 1-butanol stress condition, respectively. Although it is not significant, their abundances especially the abundance of dPCoA were increased by 4 and 6% in Ncl1460(−) strain compared with WT respectively under

![Fig. 3](image-url)
normal medium or 1-butanol stress condition (Fig. 3c, d).
Furthermore, flow cytometry analysis showed that the
mean size of Ncl1460(+) was enlarged than that of WT
while the size of Ncl1460(−) cells showed no significant
difference (Fig. 3b), consistent with the previous report
[25]. These results further indicated that Ncl1460 was a
negative regulator of slr0847 and slr0848 operon which
could affect the CoA biosynthesis and cell size; thus, the
sRNA Ncl1460 was renamed as CoaR (CoA Biosynthesis
Regulatory sRNA).

**GFP assays in Synechocystis**

To demonstrate the possible interaction between CoaR
and the promoter of slr0847, the whole intergenic
sequence between sll0821 and slr0847 (containing the
promoter of slr0847 named Pslr0847) was amplified and
fused to the GFP coding gene. The fused fragment was
then ligated to backbone of pXT37b (without original
promoter, Additional file 1: Fig. S1B) and introduced into
WT, CoaR(+) and CoaR(−) strains (Fig. 4a). Full segre-
gation of the transformants (i.e., WT-GFP, CoaR(+)-GFP,
and CoaR(−)-GFP) were achieved by consecutively pas-
saging and verified by PCR (Fig. 4b). Fluorescence of
WT-GFP, CoaR(+)-GFP, and CoaR(−)-GFP was meas-
ured after cultivation for 48 h using a HITACHI F-2700
fluorescence spectrophotometer. The fluorescence
intensity was normalized by optical density (OD730)
of each sample and the basal fluorescence of WT. The
results illustrated in Fig. 4c showed that the fluorescence
intensity of CoaR(+)-GFP was decreased significantly
than that of WT-GFP and CoaR(−)-GFP, demonstrating
that the function of CoaR may functioned by silencing
the promoter region of slr0847.

**Quantitative proteomics and lipid profiles reveals possible
tolerance mechanisms**

Finally, a quantitative proteomic analysis was utilized to
identify the differential metabolic responses to 1-butanol
between the WT and CoaR(+) strains. Samples of WT
and CoaR(+) were harvested after cultivation with
(0.20%, v/v) 1-butanol for 48 h, each with two biologi-
cal replicates for proteomic analysis. After data filter-
ing, the qualified spectra were matched to 2036 proteins
(Additional file 1: Fig. S7). Comparative analysis showed
that 159 proteins were down-regulated while 166 pro-
teins up-regulated in the CoaR(+) strain using a 1.2-
fold change as cut-off (p value <0.05) compared to WT
(Additional file 2: Table S5). Proteomic data showed
that the relative abundance of Slr0847 was decreased by
20% in the CoaR(+) strain, in good agreement with the
15.5% decrease of the CoA content in the same cells.
CoA is an essential cofactor for numerous metabolic and

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**Fig. 4**  a Scheme of the GFP assays. b PCR validation of WT-GFP, CoaR(+)-GFP and CoaR(−)-GFP. Lane 1: DNA ladder; Lane 2, 3, and 4: genomic DNA of WT-GFP, CoaR(+)-GFP and CoaR(−)-GFP as templates, respectively; primers Pslr0847-F and GFP-R were applied (Additional file 2: Table S3) and the target size was 1165 bp; Lane 5, 6, and 7: genomic DNA of WT-GFP, CoaR(+)-GFP, CoaR(−)-GFP, and WT as templates, respectively; primers 0168-F and 0168-R were applied (Additional file 2: Table S3) and the target size was approximately 3600 bp for WT-GFP, CoaR(+)-GFP and CoaR(−)-GFP, 500 bp for WT; c GFP fluorescence in the WT-GFP, CoaR(+)-GFP, and CoaR(−)-GFP strains. The error bars represent the calculated standard deviation of the measurements of three biological replicates.
energy-yielding reactions, also notable for its role in fatty acids metabolism and energy metabolism [28]. Consistently, the proteomic results showed that a wide range of metabolic changes including fatty acids metabolism, energy metabolism, amino acids metabolism, signal conduction, ABC transporters, ribosome metabolism, and cofactor biosynthesis were differentially regulated in the CoaR(+) strain, probably directly or indirectly due to the decreased cellular content of CoA in CoaR(+) under 1-butanol stress (Fig. 5).

The changes of intercellular CoA abundance could directly affect fatty acids biosynthesis and amino acids metabolism, and these metabolisms have been found related to tolerance to biofuels previously [29]. Malonyl-CoA-acyl carrier protein transacylase (FabD, Slr2023) was found down-regulated in CoaR(+) strain compared to WT. Interestingly, although the total lipid content in the CoaR(+) strain was approximately the same as that in WT, C18:3n6 decreased in CoaR(+) were increased in CoaR(−), which could be resulted from the repression of *coaR* in CoaR(−), consistent with the previous study in *Haematococcus pluvialis* finding the relative abundance of C18:3n6 was increased in cells under nitrogen depletion and low-temperature conditions [33].

The energy metabolism is also directly related with CoA [28]. Four proteins (i.e., Slr0343, Sll1796, Slr3093, and Sll1484) related with energy metabolism and photosynthesis process were down-regulated in the butanol sensitive CoaR(+) strain. Slr0343 (PetD) and Sll1796

Fig. 5 Proposed regulatory networks mediated by CoaR. The different metabolisms in *Synechocystis* were illustrated as different colors (e.g., yellow for amino acid metabolism and purple for energy metabolism). The down-regulated genes were listed as green down arrows.
(PetJ) were important parts of cytochromes and played vital roles in electron transfer [34]. Ssl3093 (CpcD) is a phycobilisome small rod linker polypeptide and involved in light harvesting. Sll1484 (Ndh) is a type-2 NADH dehydrogenase serving as redox-active electron transport intermediates [35]. Up-regulation of Slr0343 was reported previously under thermo-stress condition and its increased abundance allowed for rapid electron transfer from PSII to PSI [35]. In addition, Sll1796 was found significantly up-regulated in our previous transcriptomic and proteomic studies of Synechocystis under 1-butanol stress [17, 36], and Sll3093 was found significantly up-regulated in Synechocystis upon ethanol stress [37].

Besides the pathways directly related with CoA, five ABC transporters and nine two-component signal transduction system (TCS) proteins were found down-regulated in the butanol sensitive CoaR(+) strain (Fig. 5). ABC transport proteins have been suggested as an important mechanism against biofuel toxicity [38]. Two-component systems have been proved involving regulation of multiple stress conditions [39]. Chen et al. [11] recently found that the deletion of slr1037 gene encoding a response regulator in Synechocystis led to a decreased tolerance to 1-butanol; consistently, our proteomic analysis also showed that Slr1037 was down-regulated in CoaR(+). Nevertheless, it is still unclear of the functional relationship between CoaR and Slr1037.

**Discussion**

It is estimated that CoA is utilized by 9% of known enzymes and involved in over 100 reactions in cells [40]. In bacteria, CoA biosynthesis includes five enzymatic steps starting from pantothenate (Fig. 6) [41]. Given the fact that CoA is an essential cofactor in all living organisms [42], its regulation has attracted significant attentions. Early studies found that pantothenate kinase (CoaA) catalyzing the first step as well as phosphopantetheine adenlyltransferase (CoaD) in the fourth step were two key regulatory points in CoA biosynthesis process [43] (Fig. 6). Homologues of the coaD gene have been identified in a wide range of bacterial species, sharing a similar size (140–169 residues) and a high degree of sequence homology (32–52% identical) to the E. coli counterpart [44]. In addition, an early study showed that expression of coaD was regulated through a feedback of end-product CoA in Pseudomonas aeruginosa [45]. For Synechocystis, although no experimental information is available, slr0847 is annotated as coaD involved in catalyzing the penultimate step of the CoA synthesis [23]. Besides, the identity between Slr0847 and E. coli CoaD was 48% as revealed by Blast [23].

### Table 1 Lipid profiles of WT, CoaR(+), and CoaR(−)

|       | WT       | CoaR(+) | CoaR(−) | CoaR(+)/WT | CoaR(−)/WT |
|-------|----------|---------|---------|------------|------------|
| C14:1n9 | 0.85 ± 0.027 | 0.82 ± 0.056 | 0.87 ± 0.038 | 0.96 ± 0.073 | 1.02 ± 0.055 |
| C16:0 | 26.77 ± 0.087 | 23.99 ± 0.99 | 25.92 ± 0.13 | 0.9 ± 0.037 | 0.97 ± 0.006 |
| C16:1n7 | 3.69 ± 0.031 | 3.67 ± 0.20 | 3.68 ± 0.24 | 0.99 ± 0.055 | 1 ± 0.066 |
| C16:2n4 | 0.35 ± 0.004 | 0.37 ± 0.025 | 0.43 ± 0.042 | 1.05 ± 0.073 | 1.23 ± 0.12 |
| C16:4n6 | 0.53 ± 0.024 | 0.55 ± 0.017 | 0.52 ± 0.033 | 1.03 ± 0.056 | 0.97 ± 0.076 |
| C18:1n9 | 1.37 ± 0.058 | 1.32 ± 0.08 | 1.46 ± 0.056 | 0.96 ± 0.071 | 1.06 ± 0.061 |
| C18:1n7 | 0.28 ± 0.029 | 0.37 ± 0.018 | 0.45 ± 0.003 | 1.33 ± 0.15 | 1.62 ± 0.17 |
| C18:2n6 | 7.88 ± 0.069 | 7.39 ± 0.48 | 7.75 ± 0.52 | 0.94 ± 0.10 | 0.98 ± 0.11 |
| C18:3n6 | 7.26 ± 0.21 | 7.00 ± 0.52 | 7.56 ± 0.44 | 0.96 ± 0.077 | 1.04 ± 0.068 |
| Total area | 48.97 ± 1.23 | 45.47 ± 1.26 | 48.61 ± 1.16 | 0.93 ± 0.035 | 0.99 ± 0.034 |

content: each type of fatty acids was shown as the normalized peak area (area/mg) by GC. The first three columns were the mean peak area and the standard deviation of each fatty acid as well as the total area from three replicates. The last two columns represent the relative content of each fatty acid as well as the total lipid in CoaR(+) or CoaR(−) to that in WT.
expression of coaD by overexpressing coaR led to a decreased tolerance to 1-butanol while suppressing coaR resulted in higher tolerance to 1-butanol. Although the dPCoA and CoA changes were not significant (which may be due to the low abundances of intracellular dPCoA and CoA), the dPCoA and CoA contents in CoaR(−) had a slight increase, when compared with WT (Fig. 3c, d). Roles of sRNAs in biofuel stresses have been reported in other species. Venkataramanan et al. [46] recently discovered 159 sRNAs in responding to the native but toxic metabolites 1-butanol and butyrate in C. acetobutylicum. In addition, Cho et al. [47] validated 3 sRNAs (i.e., Zms2, Zms6, and Zms18) with differential expression in Z. mobilis under 5% ethanol stress. In addition, Aguilar et al. [48] found that sRNAs omrA and omrB regulating protein composition of the outer membrane were site-mutated after an adaptive laboratory evolution process of E. coli in glucose for 120 h. Here we found the transcriptional level of coaR also changed in 1-butanol adaptive evolution process, suggesting the important roles of sRNAs in biofuels response. Unlike overexpression of genes, regulation mediated by sRNA hardly imposes any metabolic burden on cells, and represents a more effective strategy for strains modification [15]. Here we identified the sRNA CoaR related to biofuel tolerance through regulating CoA biosynthesis pathways, constituting a valuable basis for further sRNA engineering in cyanobacteria in the future.

Our studies via quantitative proteomics showed that CoA was involved in the 1-butanol stress response mainly through affecting the fatty acid biosynthesis and energy metabolism. In Bacillus subtilis, fatty acid biosynthetic genes including the fab initiation genes fabHB and fabD as well as the elongation genes fabF, fabG, and
fabI were found significantly up-regulated after sorbic acid exposure [49]. Consistently, our proteomics analysis also revealed the reduction of FadD in the CoaR(+) strain after 1-butanol exposure, and the down-regulation of FadD caused by decreased CoA may affect the membrane remodeling thus contributed to the low 1-butanol tolerance. In a study of *E. coli* in responding to heat shock stress, it was shown that intracellular ATP levels could transiently increase to meet the higher consumption for protein- and DNA-repair mechanisms [50]; besides, decreased expression of amino acyl-tRNA synthetases might promote the release of amino acids that feed energy-providing pathways [51]. Consistently, we found in this study, the decreased abundance of energy metabolism related proteins (i.e., Srl0343, Sll1796, Ssl3093 and Sll1484) and ribosome metabolism related proteins (i.e., Rps17, Rpl21, Rpl31 and Rpl33), could also lead to a sensitive phenotype of CoaR(+) to 1-butanol.

Finally, although we proposed a possible mechanism for CoaR based on our study, we cannot exclude other possible mechanisms as the direct interaction mechanism between *ncl1460* and *slr0847* has not been verified experimentally. Nevertheless, this mode of mechanism has been well studied and demonstrated in eukaryotes like yeast, plants and mammalian cells [26]. In addition, previous studies found that almost all relevant regulatory genes can only affect tolerance at a small degree individually in *Synechocystis* suggesting that the tolerance may be regulated by multiple regulatory genes simultaneously [11–14], which is also consistent with the excellent results of enhancing acid tolerance significantly by overexpressing three sRNAs (DsrA, RprA, and ArcZ) in *E. coli* [15]. We believe with more of the tolerance-relevant sRNAs identified in the future, together with this finding, a better understanding of the tolerance regulation in *Synechocystis* will be eventually achieved.

**Conclusions**

This study found a novel sRNA CoaR regulating CoA synthesis as well as the tolerance of *Synechocystis* to 1-butanol. Overall, the study provided novel insight to the regulation of CoA biosynthesis in cyanobacteria, and also indicated that sRNAs could be useful targets in improving biofuel tolerance.

**Methods**

**Bacterial growth conditions**

For *Synechocystis*, WT and mutants were grown on BG11 agar plate or in medium (pH 7.5) under a light intensity of approximately 50 μmol photons m⁻² s⁻¹ in an illuminating incubator or shaking incubator at 130 rpm at 30 °C [17]. Medium for different mutants in this study was supplemented with appropriate antibiotic(s) to maintain plasmids with sRNAs or genes fused to genome (i.e., 10 μg/mL kanamycin, 10 μg/mL spectinomycin or a combination). For *E. coli*, DH 5α were grown on LB agar plate or in LB medium with appropriate antibiotic(s) to maintain plasmids (i.e., 100 μg/mL ampicillin) at 37 °C using incubator or shaking incubator at 200 rpm, respectively.

**SRNome sequencing and data analysis**

Small RNA-seq transcriptome sequencing was carried out as described previously [17]: (1) Samples composition: WT grew under BG11 medium with or without 0.20% (v/v) 1-butanol were collected for the transcriptome analysis at 24, 48, and 72 h, respectively; (2) RNA preparation and cDNA synthesis: approximately 10 mg of cell pellets was frozen by liquid nitrogen immediately after centrifugation. Total RNA extraction of 6 samples was achieved through a mirNeasy Mini Kit (Qiagen, Valencia, CA) following the protocols, and 500 ng total RNA was subjected to cDNA synthesis using a NuGEN Ovation® Prokaryotic RNA-seq System (NuGEN, San Carlos, CA); (3) RNA sequencing: RNA 2 × 100 bp paired-end sequencing was performed using Illumina’s Solexa Genome Analyzer II using the standard protocol. The image deconvolution and calculation of quality value were performed using Goat module (Firecrest v.1.4.0 and Bustard v.1.4.0 programs) of Illumina pipeline v.1.4. Sequenced reads were generated by base calling using the Illumina standard pipeline.

Genome sequence and sRNA annotation information of *Synechocystis* were downloaded from NCBI and Mitschke et al. [3]. sRNA sequence reads were pre-processed using NGS QC Toolkit (Version: 2.3) to remove low-quality bases and adapter sequences. Reads after QC were aligned to the *Synechocystis* genome using Burrows-Wheeler Alignment tool software version 0.7.10 with perfect match parameters. Raw counts of reads that uniquely mapped to each sRNA region were calculated by HTseq (Version: 0.6.1). Then read counts were normalized to the aligned RPKM to obtain the relative expression levels.

**qRT-PCR analysis**

The RNA extraction method was the same as described above. cDNAs were synthesized using SuperScript® VILO™ cDNA Synthesis Kit following manufacturer’s protocol (Invitrogen, Carlsbad, CA) and 1 μL of each dilution was used as template for following qRT-PCR reaction. The qPCR reaction was carried out in 10 μL reactions containing 5 μL of UltraSYBR Mixture (CW Biotech, Beijing, China), 3 μL dH₂O, 1 μL dilute template cDNA, and 1 μL of each PCR primer, employing the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) [36]. Three technical replicates were
performed for each sample. Data analysis was carried out using the StepOnePlus analytical software (Applied Biosystems, Foster City, CA) and the $2^{-\Delta\Delta CT}$ method [52]. 16S rRNA was selected as a reference gene, and the data were presented as ratios of the amount of normalized transcript in the treatment to that from the WT control.

**RT-PCR analysis**

The RNA extraction method was the same as described above. cDNAs were synthesized using specific reverse primer of Ncl1460 named qRT-ncl1460-R (Additional file 2: Table S3) and SuperScript® VILO™ cDNA Synthesis Kit following manufacturer’s protocol (Invitrogen, Carlsbad, CA). Then 1 μL cDNA was used as template for RT-PCR using primer qRT-ncl1460-F and qRT-ncl1460-R.

**Northern blotting**

The non-radioactive northern blot method to detect small RNAs was performed following Kim et al. (2010)’s work [53]. Briefly, 10 μg total RNA was extracted from WT for blotting assays. Oligonucleotides probe of CoaR (5′-GTTAAGATATAGAGCAATTTAGCTGA-3′) was added digoxin-label using Roche DIG Oligonucleotide Tailing Kit II (F. Hoffmann-La Roche Ltd, Switzerland) for hybridization. Anti-digoxigenin-AP and CSPD (F. Hoffmann-La Roche Ltd, Switzerland) were used to detection.

**3′ Race**

Total RNA of WT was extracted as described above. Then RNA was added with a poly(A) tail using NEB E. coli poly(A) polymerase (New England Biolabs Inc., MA, USA). After that, a specific primer containing oligo dT (5′-CACACAGGAACAGCTATGACCATGTTTTTTTTTTTTTTTTT-3′) and SuperScript® VILO™ cDNA Synthesis Kit were used to synthesize cDNA. At last, forward primer (5′-CCTTTCCCAT-AAGTCTAATTGCTAGAGG-3′) and reverse primer (5′-CACACAGGAACAGCTATGACCATGTTTTTTTTTTTTTTT-3′) were used to amplify Ncl1460 containing 3′ end. PCR product was purified and ligated into pTZ57R/T using InstA-clone PCR Cloning Kit (Thermo Fisher scientific Inc., MA, USA) for sequencing analysis.

**qRT-PCR for adaptive laboratory evolutionary strains**

In this study, seven evolved strains from previous studies [20], i.e., E1, E2, E3, E4, E5, E6, and E7 which could, respectively, endure 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50% (v/v) 1-butanol, were selected and cultured in normal BG11 medium along with WT and all the samples were harvested at 48 h for sRNA extraction and qRT-PCR analysis.

**Mutant strains construction**

*Escherichia coli* DH5α strain was used for vectors construction and enrichment. Primers used in this study were listed in Additional file 2: Table S2. All the plasmids and mutants constructed in this study were concluded in Additional file 2: Table S6. For overexpression and suppression of sRNAs, fragments of sRNAs and related reverse complementary chain were amplified from the *Synechocystis* genome with specific primers that introduced XbaI and BamHI restriction sites and ligated to a broad host replicating vector pAI2 with anti-kanamycin cassette (kindly provided by Prof. Paul Hudson of KTH Royal Institute of Technology of Sweden) [10, 54]. *Synechocystis* was transformed by electroporation (~10 ng plasmid DNA) using GenePulser Xcell (Bio-rad, Hercules, CA) and grown photoautotrophically on agar plate adding 10 μg/mL kanamycin. The transformants were validated by colony PCR.

For GFP reporting assays in *Synechocystis*, genome sequence between *sll0821* and *srbo847* containing the promoter region of *srbo847* was amplified through PCR and fused to GFP sequence, then the fused fragment was introduced with *KpnI* and *EcoRI* restriction site and ligated to pXT37b (digested with *KpnI* and *EcoRI* restriction enzymes to remove the promoter region; this vector was kindly provided by Dr. Xuefeng Lu of Qingdao Institute of Bioenergy and Bioprocess Technology of Chinese Academy of Sciences) [55]. The constructed vector was introduced into the CoaR(+), CoaR(−) and WT strains through electro- transformation and grown photo-autotrophically on agar plate containing 10 μg/mL spectinomycin with or without 10 μg/mL kanamycin, respectively. The transformants were validated by colony PCR and purified through passages on plates with increased antibiotics.

**Growth patterns and fluorescence measurement**

For measurements of growth patterns, 5 mL fresh cells at OD$_{630}$ of 0.2 were collected by centrifugation at 3000×g and 4 °C and were then inoculated into 25 mL of BG11 liquid medium in a 100-mL flask. 1-butanol treatment was carried out by adding 0.20% (v/v) 1-butanol (Merck, USA) to the medium at beginning of the cultivation, each with three replicates and repeated at least three times. Cell density was measured on a UV-1750 spectrophotometer (Shimadzu, Japan) at OD$_{730}$ or on an ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT, USA) at OD$_{630}$. Culture samples (1 mL or 200 μL, respectively) were taken and measured at both OD$_{730}$ and OD$_{630}$ every 12 h.

For GFP reporting assays, *Synechocystis* strains were harvested by centrifugation (4 °C, 7800×g) and re-suspended with ddH$_2$O after 72 h. The final OD of the re-suspensions was measured on UV-1750
2 mL of hexane and 0.75 mL of distilled water were added, and the flask was stirred at 70 °C for 1 h. Then using the equation described by Liu et al. [32].

The standard dPCoA and CoA were purchased from Sigma-Aldrich (St Louis, USA). The mass spectra of two substances were determined using LC/MS. The standard curve of dPCoA and CoA was plotted through measuring the peak area of a series of dilutions of the respective standard, each dilution with 3 replicates using LC/MS (Additional file 1: Fig. S6). The extraction of total metabolites from *Synechocystis* and LC-MS analysis was conducted using the protocol described before [20]. A total of 36 samples, including WT, the CoaR(+) and CoaR(−) strains grown in normal BG11 medium or BG11 medium with 0.25% (v/v) 1-butanol were harvested at 60 h, each with six biological replicates. The quantitation of dPCoA and CoA of each sample were measured through standard curve, respectively.

**Cell size measurement using flow cytometry**

Flow cytometric analysis was performed on a Calibur fluorescence-activated cell sorting (FACS) cytomter (Becton Dickinson) to reveal cell size differences among WT, CoaR(+) and CoaR(−) strains with the similar settings described previously [13]. Cells were harvested at 48 h and data analysis was conducted using the CellQuest software, version 3.1 (Becton Dickinson). The results were repeated by at least three times.

**Lipid profile measurement**

Total lipids from approximate 5-mg dry cells of the WT, CoaR(+), and CoaR(−) strains (each with 3 biological replicates) were extracted using 2 mL chloroform/methanol (v/v, 2/1) ultrasonic treatment for 10 min and centrifugation at 4000 rpm for 5 min. The collected supernatants were dried under nitrogen flow and then at 60 °C until the weight of samples remained constant. The samples were weighed and moved to 10-mL flasks. Then, 5 mL 2.0% H2SO4-methanol (v/v, H2SO4/methanol) was added, and the flask was stirred at 70 °C for 1 h. Then 2 mL of hexane and 0.75 mL of distilled water were added to the flask and mixed and the upper hexane layer contained the fatty acid methyl esters (FAMEs). The hexane layer was transferred to a new vial and mixed with the internal standard C17-ME for analysis by gas chromatography (GC). FAME analyses were carried out by an Agilent 6890 GC instrument and FAME yield was calculated using the equation described by Liu et al. [32].

**Quantitative proteomics and data analysis**

Samples composed of the WT and the mutant CoaR(+) were harvested after cultivation for 48 h under 0.20% (v/v) 1-butanol stress, each with two replicates for quantitative isobaric tags for relative and absolute quantification (iTRAQ) liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomic analysis. Protein preparation and digestion, iTRAQ labeling, LC-MS/MS proteomic analysis, and proteomic data analysis were the same as before [36]. Briefly, cell samples for proteomics analysis were collected by centrifugation at 8000×g for 10 min at 4 °C and immediately frozen in liquid nitrogen. After proteins preparation, the iTRAQ labeling of peptide samples was performed using iTRAQ Reagent 8-plex Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Then 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). The MS data were processed using Proteome Discoverer software (Version 1.2.0.208) (Thermo Scientific) with default parameters to generating peak list. Genome sequence and annotation information of *Synechocystis* were downloaded from NCBI and the Comprehensive Microbial Resource (CMR) of TIGR (http://www.tigr.org/CMR) [56]. Proteins with 1.2-fold change between the CoaR(+) strain and control WT samples and p-value of statistical evaluation <0.05 were determined as differentially expressed proteins.

**Additional files**

**Additional file 1:** Fig. S1. Mutant constructions using different vectors. A) Overexpression of sRNAs using pJA2. B) Suppression of sRNAs using pJA2. C) Gene overexpression using pX3T37b. Fig. S2 A) Growth phenotypes of WT, Ncl1460(+), and Ncl1460(−) under BG11 media with 4.6 μM CdSO4. B) Growth phenotypes of WT, Ncl1460(+), and Ncl1460(−) under BG11 media with 4% (w/v) NaCl. Fig. S3 A) Northern blotting detection of Ncl1460. B) 3’ RACE results of Ncl1460. C) RT-PCR results of Ncl1460 in WT, Ncl1460(+), and Ncl1460(−). Fig. S4 Predicted functional regions of Ncl1460 using CopraRNA. Fig. S5 Predicted interaction regions of Ncl1460 on its target genes using CopraRNA. Fig. S6 Standard curves for measurements of CoA A) and dPCoA B). Fig. S7 Description of the proteomics data. A) Basic information statistics including the total spectrum, unique spectrum, peptides, unique peptides, and identified proteins. B) Peptide length distribution. C) Distribution of protein’s sequences coverage. The different colors represent different coverages, and the number in each color represents the identified proteins.

**Additional file 2:** Table S1. Mapping results and the expression profiles of all identified sRNAs. C represents Synechocystis cultured in normal BG11 while B represents medium with butanol. The RPKM value was reads per kilobase per million mapped reads. The fold change of each ncRNAs was shown as R/B. Table S2 sRNAs with regulated expression at 24 h; 48 h, and 72 h. Table S3 Primers used in this study. Table S4 Constructed sRNA mutants in this study. Table S5 Regulated proteins by CoaR. Table S6 plasmids and strains constructed in this study.
Abbreviations
dPCoA, 3′-dephospho-CoA; LC/MS, liquid chromatography–mass spectrometry; qRT-PCR, quantitative real time polymerase chain reaction; RT-PCR, reverse transcriptional polymerase chain reaction; 3′ RACE, 3′ rapid amplification of cDNA-end; RPKM, reads per kilobase per million mapped reads; sRNA, small RNA; sRNAome, small RNA transcriptome.

Authors’ contributions
TS performed the experiments and wrote the manuscript; TS, GP, JW, LC and WZ analyzed the data; LC and WZ designed and revised this paper. 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
All data generated or analyzed during this study are included in this published article and its additional files.

Additional information
The raw small RNA sequence data of Synechocystis sp. PCC6803 are deposited in the SRA database of NCBI with accession numbers SRP073279.

Consent for publication
All authors agree to publication.

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