SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENVIRONMENTAL MICROBIOLOGY
PLANT BIOTECHNOLOGY

Received
29 November 2013

Accepted
3 March 2014

Published
28 March 2014

Correspondence and requests for materials should be addressed to Y.L. (yli@im.ac.cn)

Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria

Jie Zhou[1], Haifeng Zhang[1], Hengkai Meng[1], Yan Zhu[1], Guanhui Bao[1,2], Yanping Zhang[1], Yin Li[1] & Yanhe Ma[3]

[1]CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, [2]University of Chinese Academy of Sciences, Beijing, China, [3]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

Cyanobacteria are oxygenic photosynthetic prokaryotes that play important roles in the global carbon cycle. Recently, engineered cyanobacteria capable of producing various small molecules from CO_2 have been developed. However, cyanobacteria are seldom considered as factories for producing proteins, mainly because of the lack of efficient strong promoters. Here, we report the discovery and verification of a super-strong promoter P_{cpc560}, which contains two predicted promoters and 14 predicted transcription factor binding sites (TFBSs). Using P_{cpc560}, functional proteins were produced at a level of up to 15% of total soluble protein in the cyanobacterium *Synechocystis* sp. 6803, a level comparable to that produced in *Escherichia coli*. We demonstrated that the presence of multiple TFBSs in P_{cpc560} is crucial for its promoter strength. Genetically transformable cyanobacteria neither have endotoxins nor form inclusion bodies; therefore, P_{cpc560} opens the possibility to use cyanobacteria as alternative hosts for producing heterogeneous proteins from CO_2 and inorganic nutrients.

CO_2 is one of the main greenhouse gases. Cyanobacteria can convert CO_2 into organic compounds through oxygenic photosynthesis with an efficiency higher than that of terrestrial plants[1,2]. Cyanobacteria account for 20–30% of earth's photosynthetic productivity; that is, the conversion of solar energy into chemical energy[3,4]. The use of engineered cyanobacteria capable of producing fuels and other chemicals could reduce greenhouse gas emission and help to address the shortages of energy and resources[5]. As nonpathogenic aquatic photoautotrophs with a high nutritional value, some cyanobacteria are attractive hosts for producing heterologous proteins that can be used in the health, food, fodder, fertilizer, and environment sectors[6–8].

Proteins of industrial or environmental interest that have been produced in cyanobacteria include a metallothionein protein capable of absorbing heavy metals in wastewater[7], a larvicidal protein that can kill mosquito eggs in water[8], and a human CuZn superoxide dismutase that can be used in health products[6]. The human CuZn superoxide dismutase was expressed at a level of 3% of total soluble protein in the cyanobacterium *Anacystis nidulans* 6301[6]. This expression level was achieved by using a native strong promoter, P_{rbc343}, and an optimized SD sequence. Because this expression cassette was delivered by a replicative plasmid, the constant addition of antibiotics was required to maintain the expression level[6].

Several heterologous genes have been expressed in cyanobacteria in an effort to create novel biosynthetic pathways to produce chemicals. Heterologous strong promoters such as P_{trc} and P_{lac} and native promoters such as P_{cpc}, P_{rbc}, and P_{psbA2} have been used in integrated cyanobacterial expression systems. Lan et al.[9] used P_{trc} to express trans-enoyl-CoA reductase (Ter) for butanol production, and Guerrero et al.[10] used P_{lac} to express ethylene-forming enzyme (EFE) for ethylene production. Lindberg et al.[11] and Bentley et al.[12] used P_{psbA2} to drive expression of isoprene synthase (Isps) for isoprene production in cyanobacteria. Although there was successful production of the target chemicals, the expression levels of the proteins encoded by the heterologous genes were not reported[9,10]. Recently, Angermay et al.[13] experimented with various promoters (P_{rnpB}, P_{psbA2}, and P_{trc}) to drive expression of L-lactate dehydrogenase in cyanobacteria, and found that even powerful native and artificial promoters from *Synechocystis* were not strong enough to produce the enzyme in sufficient quantities.

The aim of this study was to create novel expression elements that lead to strong expression of heterologous genes in cyanobacteria. We discovered a super-strong promoter, P_{cpc560}, consisting of two promoters from the *cpcB* gene and 14 predicted transcription factor binding sites (TFBSs). Using P_{cpc560}, two heterologous genes were

expressed in the cyanobacterium *Synechocystis* sp. 6803 (hereafter, *S.* 6803) to a level comparable to that obtained using the *Escherichia coli* expression system, demonstrating the strength and efficiency of P_{cpc560}. We further showed that the presence of multiple TFBSs is crucial for the promoter strength of P_{cpc560}. The discovery of this super-strong promoter will stimulate research on using cyanobacteria as hosts to produce valuable recombinant proteins from CO_2 and inorganic nutrients.

Results

Discovery of the super-strong promoter P_{cpc560}. Efficient expression elements are required to increase the expression level of heterologous genes. Since c-phycocyanin and ribulose-bisphosphate carboxylase/oxygenase (Rubisco) are the major soluble proteins in cyanobacteria, expression elements from genes encoding these two proteins have been used widely to construct cyanobacterial expression vectors[6,7,14–16]. For example, a metallothionein gene was expressed in the cyanobacterium *Synechococcus* sp. 7942 using the promoter from *cpcB*, the gene encoding the c-phycocyanin beta subunit[7], while a human CuZn superoxide dismutase gene was expressed in *Synechococcus* sp. 7002 using the promoter and terminator from the *rbc* operon, which encodes Rubisco[6].

RBSDesigner[17] predicted that the RBS in the *cpcB* promoter is approximately 10-times more efficient than that in the *rbc* promoter in terms of the predicted translation efficiency. Therefore, we chose the *cpcB* promoter for further investigations. The genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene (sll1577) was subjected to promoter analysis using Virtual Footprint[18]. This analysis revealed two promoters located at 135 bp and 374 bp upstream of the initiation codon of the *cpcB* gene. Scanning the genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene also revealed 14 TFBSs located between 381 bp and 556 bp from the initiation codon of the *cpcB* gene[18] (Supplementary Table S1). Negative TFBSs are usually located between 500 bp and 1,000 bp upstream of the transcription start site[19], and some mammalian proteins such as soluble adenylyl cyclase[20] are highly similar to these proteins in cyanobacteria. Therefore, we chose the genomic sequence of 560 bp upstream of the initiation codon of *cpcB* as the new promoter sequence; this sequence was designated as P_{cpc560}. P_{cpc560} contains two predicted promoters and 14 predicted TFBSs (Fig. 1).

No terminator was found in the genomic region 300 bp downstream of the stop codon of the *cpcB* gene. This may be because *cpcB* is the first gene of the cpc operon. Since the *rbc* terminator was used in a previous study that achieved high-level gene expression (yielding target protein at a level of 3% of total soluble protein) in a cyanobacterium[6], we chose the terminator of *rbcL*, the gene encoding the Rubisco large subunit (slr0009), as the terminator (T_{rbcL}).

High-level expression of crotonyl-CoA-specific trans-enoyl-CoA reductase gene in *S.* 6803. To validate the efficacy of the newly discovered promoter P_{cpc560}, we used it to drive the expressions of

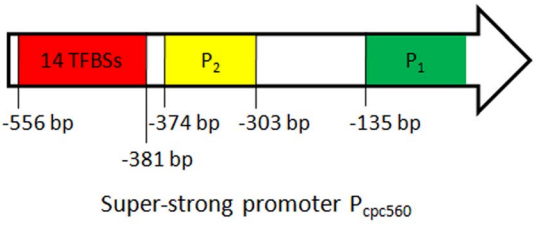

Figure 1 | Schematic structure of super-strong promoter P_{cpc560}. Green box shows first predicted promoter P_1; yellow box shows second predicted promoter P_2; red boxes show 14 predicted transcription factor binding sites (TFBSs).

two genes encoding important enzymes involved in metabolism in *S.* 6803. One enzyme was crotonyl-CoA-specific trans-enoyl-CoA reductase (Ter), a key enzyme in increasing the driving force towards butanol biosynthesis[21,22]. *ter* has been overexpressed in *Synechococcus* sp. PCC 7942 with the aim to produce butanol from CO_2[9]. A strong promoter from *E. coli*, P_{trc}, was used to express a codon-optimized *ter* in *Synechococcus* sp. PCC 7942[9]. However, the activity of Ter in the crude cell extract of *Synechococcus* sp. PCC 7942 was 0.057 ± 0.005 μmol/min/mg crude cell extract. This was 64-fold lower than that of Ter (3.7 ± 0.5 μmol/min/mg) in *E. coli*, in which the *ter* gene was expressed under the control of a medium-strength promoter, $P_{L\ lacO1}$[22]. This suggests that P_{trc} is not an efficient promoter in cyanobacteria in terms of gene expression strength. Therefore, we tested whether the newly discovered promoter P_{cpc560} could significantly increase the expression level of *ter* in cyanobacteria.

To express *ter* in *S.* 6803, a codon-optimized *ter* gene (Supplementary Fig. S1) from *T. denticola* flanked by P_{cpc560} and T_{rbcL} expression elements was integrated into the *S.* 6803 chromosome at the *pta* (slr2132) insertion site via homologous recombination. The *pta* gene encodes phosphotransacetylase, the first enzyme in acetate synthetic pathway, and disruption of *pta* does not affect cell growth[16]. Homoplasmy and gene insertion were verified by PCR and sequencing (Fig. 2a and 2b). SDS-PAGE analysis demonstrated that *ter* was strongly overexpressed in the *S.* 6803 mutant Δpta::P_{cpc560}ter. The protein expression level was approximately 15% of total soluble protein (Fig. 2c), a surprisingly strong expression for cyanobacteria. The band excised from the position at approximately 43.7 kDa was confirmed as Ter from *T. denticola* by MALDI-TOF MS analysis (Fig. 2d).

The specific activity of Ter was determined to be 32.22 ± 1.93 μmol/min/mg crude cell extract of *S.* 6803 strain Δpta::P_{cpc560}ter (Table 1). In a previous study in which the *ter* gene was expressed in *Synechococcus* sp. PCC 7942 under the control of the P_{trc} promoter[9], the activity of Ter was 0.057 ± 0.005 μmol/min/mg crude cell extract. When *ter* was expressed in *E. coli* BW25113 under the control of the $P_{L\ lacO1}$ promoter, on a medium-copy number plasmid, the Ter activity was 3.7 ± 0.5 μmol/min/mg crude cell extract[22]. These activities of Ter reported in the literature were measured in crude lysates obtained from different organisms cultured in various different conditions. The activity of Ter determined in our study indicates that the *ter* gene was strongly expressed in *S.* 6803 strain Δpta::P_{cpc560}ter.

TFBSs are crucial for P_{cpc560} strength. The newly discovered super-strong promoter P_{cpc560} differs from other commonly used promoters in that it contains 14 predicted TFBSs located between −556 bp and −381 bp from the initiation codon of *cpcB*. To investigate whether these TFBSs contribute to *cpcB* promoter strength, *ter* was expressed under the control of P_{cpc374}, which contained only the two promoters, in *S.* 6803 Δpta::P_{cpc374} (Fig. 2a and 2b). Although RT-PCR confirmed *ter* transcription from P_{cpc374} (Supplementary Fig. S2), the level of Ter protein was too low for detection by SDS-PAGE, whereas the level of Ter protein expressed from *ter* driven by P_{cpc560} was up to 15% of total soluble protein (Fig. 2c). The specific activity of Ter in strain Δpta::P_{cpc374}ter was only 0.12 ± 0.01 μmol/min/mg crude extract (Table 1). This was 268-fold lower than that of Ter in the strain Δpta::P_{cpc560}ter (32.22 ± 1.93 μmol/min/mg), and only slightly higher that of Ter in *Synechococcus* sp. PCC 7942, in which the *ter* gene was expressed under the control of the P_{trc} promoter[9]. These data show that the newly discovered super-strong promoter P_{cpc560} is indeed much stronger than P_{cpc374} and P_{trc}, and the multiple TFBSs present in P_{cpc560} are crucial for its promoter strength.

High-level expression of D-lactate dehydrogenase in *S.*6803. To investigate whether the newly discovered super-strong promoter

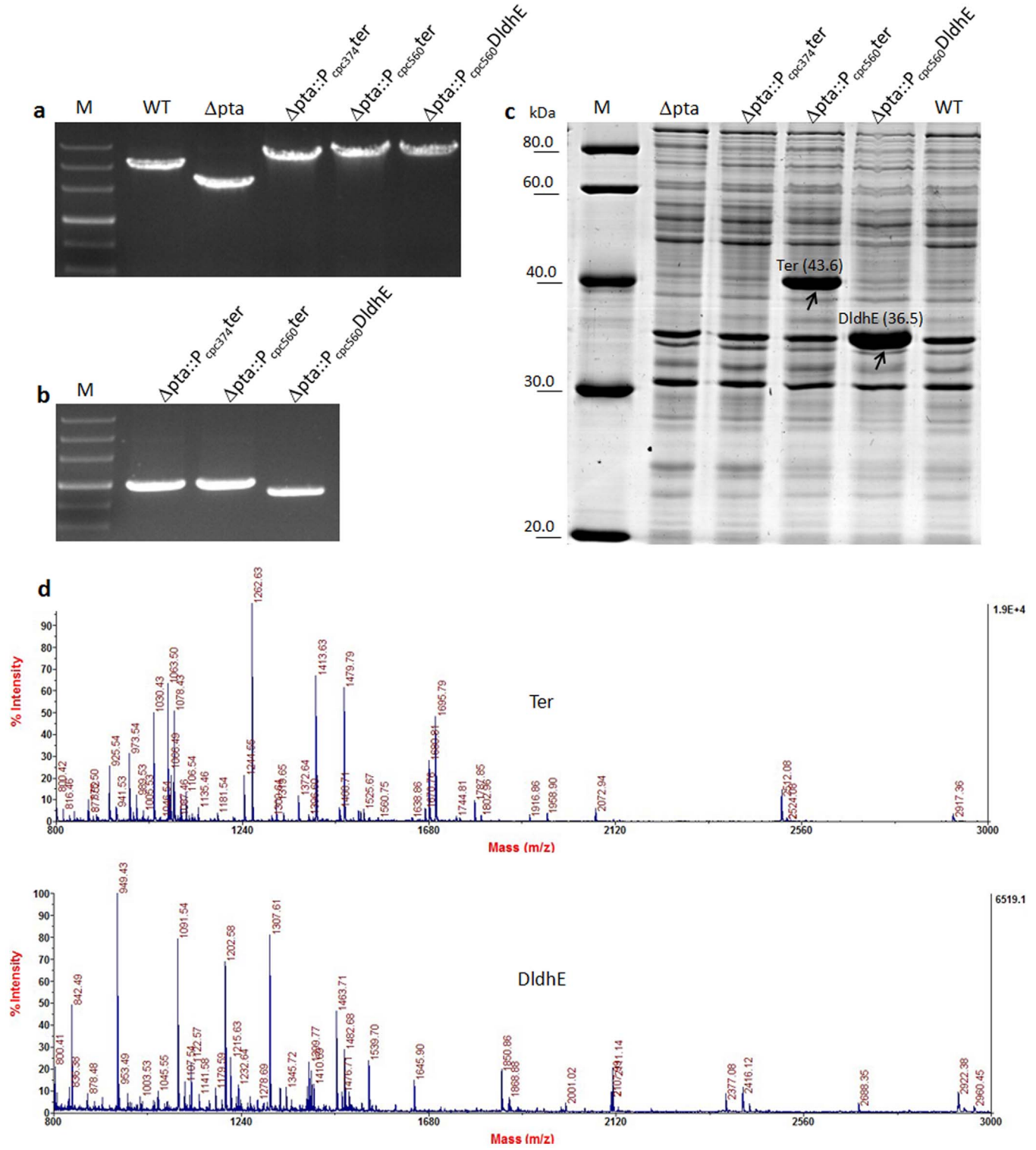

Figure 2 | Heterologous gene insertion into genome of *S.* 6803 at *pta* site, and expression of *ter* and *DldhE* in *S.* 6803. (a) For all mutants, complete segregation was demonstrated by whole-cell PCR with primers from recombinant cassette at *pta* site and a primer from 100-bp outside of recombinant cassette. (b) Whole-cell PCR with primers for each heterologous gene verified insertion of each heterologous gene into genome of corresponding strain. M: DNA marker III (top to bottom; 4.5, 3, 2, 1.2, 0.8, 0.5 kb). (c) Detection of heterologous gene expression in corresponding strain by 12% SDS-PAGE analysis. Each marker protein band contains 2 μg protein; 20 μg protein was loaded into each lane. Crude extracts from each strain were analyzed. Expression levels of Ter or DldhE under control of strong promoter P_{cpc560} were approximately 15% of total soluble protein, whereas Ter expressed under control of P_{cpc374} in Δpta::P_{cpc374}ter strain was undetectable on gel. (d) Peptide mass fingerprint of bands at approximately 43.6 and 36.5 kDa. 43.6-kDa band was identified as Ter from *T. denticola*, 36.5-kDa band was identified as DldhE from *E. coli* K12. Mascot score greater than 53 (default MASCOT threshold for such searches) was accepted as significant (p value < 0.05). All strains (WT, Δpta, Δpta::P_{cpc374}ter, Δpta::P_{cpc560}ter and Δpta::P_{cpc560}DldhE) were continuously passaged more than 10 times without antibiotics.

Table 1 | Enzymatic activities of Ter and DldhE in *S.* 6803 strains

Strain	Enzyme activity	
	Ter	DldhE
WT	n.d.	0.01 ± 0.005
Δpta	n.d.	0.01 ± 0.008
Δpta::P_{cpc374}ter	0.12 ± 0.01	-
Δpta::P_{cpc560}ter	32.22 ± 1.93	-
Δpta::P_{cpc560}DldhE	-	51.92 ± 2.31

n.d. not detected. Specific activity: μmol/min/mg crude cell extract from *S.* 6803 strains. Ter, trans-2-enoyl-CoA reductase (*T. denticola*); DldhE, D-lactate dehydrogenase (*E. coli* K12). Data are means of three independent measurements (±SD).

P_{cpc560} also drives strong expressions of other genes, we used it to control expression of *Dldh*, which encodes D-lactate dehydrogenase. The *Dldh* gene from *E. coli* K12 (designated as *DldhE*) flanked by P_{cpc560} and T_{rbcL} was integrated into the chromosome at the *pta* insertion site via homologous recombination. Homoplasmy and *DldhE* gene insertion were verified by PCR and sequencing (Fig. 2a and 2b). SDS-PAGE analysis demonstrated that *DldhE* was strongly overexpressed in the *S.* 6803 mutant Δpta::P_{cpc560}DldhE; the protein expression level was approximately 15% of total soluble proteins (Fig. 2c). MALDI-TOF MS analysis confirmed that the band excised from the position at approximately 36.5 kDa was Dldh from *E. coli* (Fig. 2d). The specific activity was determined to be 51.92 ± 2.31 μmol/min/mg crude extract in strain *S.* 6803 Δpta::P_{cpc560}DldhE (Table 1). In a previous study in which the *DldhE* gene was expressed under the control of $P_{LtetO-1}$ on a low-copy number plasmid[23], the activity of DldhE in *E. coli K12* MG1655 was 0.96 ± 0.06 μmol/min/mg crude cell extract. This result confirmed that the *DldhE* gene was strongly expressed in *S.* 6803 under the control of P_{cpc560}.

We also investigated the genetic stability of the mutant containing the strong integrative expression cassette. The mutants Δpta::P_{cpc560}ter and Δpta::P_{cpc560}DldhE were continuously passaged 10 times in the absence of antibiotics. The expression levels of both *ter* and *DldhE* did not change during this procedure, demonstrating the stability of this expression system.

Discussion

In this study, we discovered and verified a super-strong promoter P_{cpc560} for efficient and strong expression of heterologous genes in cyanobacteria. The newly discovered super-strong promoter P_{cpc560} consists of two predicted promoters from the *cpcB* gene and 14 predicted TFBSs. Using P_{cpc560}, two heterologous genes were expressed in the cyanobacterium *S.* 6803 to levels of up to 15% of total soluble protein. Despite the fact that only a single copy of the target gene was inserted into the chromosome of *S.* 6803, the expression level of the target gene driven by P_{cpc560} was comparable to that which can be achieved in an *E. coli* expression system using low- or medium-copy number plasmids. This demonstrates that P_{cpc560} has potential applications for efficient production of recombinant proteins in cyanobacteria.

It is generally accepted that the genomic sequence 200–300 bp upstream of the initiation codon of a gene that is constitutively expressed at high levels can be used as a promoter sequence. Previous studies have shown that the thymine at 259 bp upstream of the initiation codon of *cpcB* is crucial for *cpc* promoter activity[24]. Recently, a genomic map of the transcriptional start sites (TSS) of *S.* 6803 showed that the *cpcB* gene is one of the genes with a long distance between its TSS and start codon[25]. Further examination revealed that most of the *S.* 6803 genes with a long distance between the TSS and start codon are responsive to environmental factors[25]. Since the *cpcB* gene is a light-and redox-responsive gene[26,27], it is plausible that the length of the *cpcB* promoter is related to its responsiveness to environmental factors. P_{cpc560} is unusually long in that it contains not only two predicted promoters (at 135 bp and 374 bp), but also 14 predicted TFBSs located between 381 bp and 556 bp upstream of the initiation codon of *cpcB*. P_{cpc560} differs from P_{cpc374} in that it has an extra 186 bp DNA fragment containing the 14 predicted TFBSs. The large-scale difference in the expression levels of *ter* under the control of P_{cpc560} and P_{cpc374} demonstrated that the extra 186 bp DNA fragment in P_{cpc560} may contain positive TFBSs and that the presence of these multiple TFBSs is crucial for its promoter strength.

Transcription factors are proteins that play roles in virtually every aspect of the transcription process[28]. In prokaryotes, RNA polymerase recognizes and binds to the promoter region and initiates transcription, and a sigma factor is required for RNA polymerase to bind to the promoter. In eukaryotes, three types of eukaryotic RNA polymerases all require transcription factors to bind to the promoter sequence before transcription can be initiated[28]. Therefore, compared with eukaryotic promoters such as the yeast promoter[29], the promoter of *E. coli* is rather short (approximately 30–50 bp) and TFBSs are usually not required. For instance, when we scanned the sequences of *E. coli* strong promoters (including P_{trc}, P_{lac}, and T7) that have been used to drive gene expressions in cyanobacteria previously, we found only one or two TFBSs in each of the promoters. In this study, we found 14 predicted TFBSs located between 381 bp and 556 bp upstream of the initiation codon of *cpcB*, and verified that the extra 186 bp DNA fragment containing multiple predicted TFBSs is crucial for P_{cpc560} promoter strength in cyanobacteria. This novel discovery raises the possibility that the lack of TFBSs in *E. coli* strong promoters may explain why they perform poorly in driving gene expression in cyanobacteria. Thus, we propose that native positive TFBSs should be considered when designing promoters to drive gene expression in cyanobacteria.

The newly discovered super-strong promoter P_{cpc560} will be useful for further research on the production of useful substances using transgenic cyanobacteria and the very cheap substrate CO_2. The principle of considering TFBS in cyanobacterial promoter design will also contribute to designing strong and controllable promoters to drive the expressions of genes involved in new biosynthetic pathways from CO_2 in cyanobacteria.

Methods

Materials. We purchased restriction endonucleases and other nucleases for plasmid construction from New England Biolabs (Beverly, MA, USA). All chemicals for enzymatic analyses were purchased from Sigma-Aldrich (St Louis, MO, USA).

Construction of expression vectors. The plasmids used and constructed in this study are listed in Supplementary Table S2. Primers are listed in Table S3. General strategies for constructing expression vectors are shown in Supplementary Fig. S3.

The sequences of P_{cpc560} and the T_{rbcL} terminator were obtained from *S.* 6803 genomic DNA by PCR. Codon-optimized *ter* from *Treponema denticola* was ordered from Sangon Biotech (Shanghai, China). *DldhE* was obtained by PCR using genomic DNA of *E. coli* K12 as the template. Fusion PCR[30] was used to ligate DNA fragments, including P_{cpc560}-ter-T_{rbcL} and P_{cpc560}-DldhE-T_{rbcL}. To construct the expression vectors pSM2-P_{cpc560}ter and pSM2-P_{cpc560}DldhE, the fusion PCR products P_{cpc560}-ter-T_{rbcL} and P_{cpc560}-DldhE-T_{rbcL} were digested with XhoI and ligated into the XhoI site between two homologous arms of vector pSM2[16]. The *ter* gene flanked by P_{cpc374} and T_{rbcL} was obtained by PCR from the constructed plasmid pSM2-P_{cpc560}ter and was inserted into the XhoI site of plasmid pSM2[16].

Strains. The bacterial strains used in these experiments are listed in Supplementary Table S2. We used *E. coli* DH5α to propagate constructs, and *S.* 6803 wild-type as the starting strain. All mutant strains were constructed by transforming *S.* 6803 wild-type with the plasmids listed in Supplementary Table S2, followed by subsequent selection until homoplasmy was achieved.

Transformations were performed as previously described[16]. All constructed strains are listed in Supplementary Table S2. Briefly, strain Δpta was constructed by integration of the plasmid pSM2[16] at the *pta* locus of wild-type *S.* 6803 via double crossover homologous recombination.

Transforming the wild-type strain of *S.* 6803 with the plasmids pSM2-P_{cpc560}ter and pSM2-P_{cpc374}ter generated strains Δpta::P_{cpc560}ter and Δpta::P_{cpc374}ter, respectively. In these strains, a part of the *pta* gene was replaced with the kanamycin resistance cassette and the P_{cpc560}- or P_{cpc374}-driven expression cassette of the

codon-optimized gene *ter* from *T. denticola* encoding crotonyl-CoA specific trans-enoyl-CoA reductase.

Transforming the wild-type strain of *S.* 6803 with plasmid pSM2-P_{cpc560}DldhE generated strain Δpta::P_{cpc560}DldhE. In this strain, a part of *pta* was replaced with the kanamycin resistance cassette and the P_{cpc560}-driven expression cassette of the D-lactate dehydrogenase gene *DldhE* from *E. coli* K12.

Culture conditions. Wild-type and mutant lines of *S.* 6803 were grown in BG11 medium buffered with 10 mM HEPES (pH 8.0) at 30°C at an illumination intensity of 100 μmol photons/s/m^2 as described elsewhere[31]. Kanamycin was added to the medium at a final concentration of 10 μg/ml when necessary. To maintain strains on agar plates, the BG11 medium was supplemented with 1.5% (w/v) agar.

RT-PCR. RT-PCR was performed as previously described[16], with modifications. Total RNA was isolated from *S.* 6803 wild-type (WT), Δpta, Δpta::P_{cpc374}ter and Δpta::P_{cpc560}ter cells using Redzol reagent (Qiagen, Beijing, China). Residual DNA in RNA preparations was eliminated by digestion with RNAse-free DNAse and reverse transcription reactions were performed using a Reverse Transcription kit (Qiagen). Reverse transcription products were amplified by PCR and analyzed by electrophoresis on 1.2% (w/v) agarose gels. The *ter* transcript was amplified using the forward primer 5′-CAAGCCTTGTACCGCAAA-3′ and the reverse primer 5′-CGATCAAAGCGTTCCACT-3′. Transcript levels of *rnpB* were analyzed as a positive control[32].

Preparation of cell extract and gene expression level analysis. Cell crude extracts were prepared as described elsewhere[33], with some modifications. We collected 10-ml samples of cultures in mid-exponential growth phase, and harvested cells by centrifugation at 14,000 g and 4°C for 2 min. Cell pellets were washed twice and then resuspended in prechilled buffer from the *in vitro* assay described below. Cells were disrupted by bead beating and cell debris and beads were removed by centrifugation at 14,000 g and 4°C for 30 min. Total protein concentrations of the crude extract were determined using the Bradford method with bovine serum albumin as the standard. To measure protein expression levels, crude extract was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The target protein bands were quantified using VisionWorksLS software, using the absolute quantity of the marker proteins as references. Each marker protein band contained 2 μg protein, and samples with 20 μg protein in total were loaded into each lane. The abundance of proteins in the Ter and DldhE target bands was about 1.5-fold that of the marker band, as determined by VisionWorksLS analysis. Therefore, there was approximately 3 μg Ter or DldhE on the gel. From this value, we calculated that the expression level of Ter or DldhE was approximately 15% of total soluble protein. The target protein bands were excised and subjected to in-gel-digestion and MALDI-TOF MS analysis as described in our previous report[34,35].

Trans-2-enoyl-CoA reductase (Ter) assay. Ter activity was assayed by monitoring the decrease in absorbance at 340 nm, which corresponded to consumption of NADH as previously described[9,36], with slight modifications. Briefly, the reaction mixture (total volume, 200 μl) contained 100 mM potassium phosphate buffer (pH 6.2), 200 μM crotonyl-CoA (Sigma), 400 μM NADH, and 0.3 μg crude extract protein from strain Δpta::P_{cpc560}ter or 7 μg crude extract protein from strains *S.* 6803, Δpta, and Δpta::P_{cpc374}ter. After preincubation at 30°C for 5 min, the reaction was initiated by adding the substrate. The activity was determined by monitoring the decrease in absorbance at 340 nm using a microtiter plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 30°C.

D-lactate dehydrogenase (Dldh) assay. Dldh activity was determined by monitoring the decrease of absorbance at 340 nm as previously reported[9,37]. The 200 μl reaction mixture contained 50 mM sodium phosphate (pH 6.5), 300 μM NADH, 2.5 mM $MgCl_2$, 3 μg crude extract protein, and 30 mM sodium pyruvate. After preincubation at 30°C for 5 min, the reaction was initiated by adding the substrate sodium pyruvate.

1. Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M. & Posewitz, M. C. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr Opin Biotechnol* **19**, 235–240 (2008).
2. Melis, A. Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. *Plant Sci* **177**, 272–280 (2009).
3. Waterbury, J. B., Watson, S. W., Guillard, R. R. L. & Brand, L. E. Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. *Nature* **277**, 293–294 (1979).
4. Pisciotta, J. M., Zou, Y. & Baskakov, I. V. Light-dependent electrogenic activity of cyanobacteria. *PLoS ONE* **5**, e10821 (2010).
5. Ducat, D. C., Way, J. C. & Silver, P. A. Engineering cyanobacteria to generate high-value products. *Trends Biotechnol* **29**, 95–103 (2011).
6. Takeshima, Y., Takatsugu, N., Sugiura, M. & Hagiwara, H. High-level expression of human superoxide dismutase in the cyanobacterium *Anacystis nidulans* 6301. *Proc Natl Acad Sci U S A* **91**, 9685–9689 (1994).
7. Sode, K., Yamamoto, Y. & Hatano, N. Construction of a marine cyanobacterial strain with increased heavy metal ion tolerance by introducing exogenic metallothionein gene. *J Mar Biotechnol* **6**, 174–177 (1998).
8. Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S. & Zaritsky, A. Mosquito larvicidal activity of transgenic *Anabaena* PCC 7120 expressing toxin genes from *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol Lett* **227**, 189–195 (2003).
9. Lan, E. I. & Liao, J. C. Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab Eng* **13**, 353–363 (2011).
10. Guerrero, F., Carbonell, V., Cossu, M., Correddu, D. & Jones, P. R. Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp. PCC 6803. *PLoS ONE* **7**, e50470 (2012).
11. Lindberg, P., Park, S. & Melis, A. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. *Metab Eng* **12**, 70–79 (2010).
12. Bentley, F. K., Zurbriggen, A. & Melis, A. Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene. *Molecular Plant* **7**, 71–86 (2013).
13. Angermayr, S. A. & Hellingwerf, K. J. On the use of metabolic control analysis in the optimization of cyanobacterial biosolar cell factories. *J Phys Chem B* **117**, 11169–11175 (2013).
14. Liu, X., Sheng, J. & Curtiss, R. Fatty acid production in genetically modified cyanobacteria. *Proc Natl Acad Sci U S A* **108**, 6899–6904 (2011).
15. Tan, X. *et al.* Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. *Metab Eng* **13**, 169–176 (2011).
16. Zhou, J., Zhang, H., Zhang, Y., Li, Y. & Ma, Y. Designing and creating a modularized synthetic pathway in cyanobacterium *Synechocystis* enables production of acetone from carbon dioxide. *Metab Eng* **14**, 394–400 (2012).
17. Na, D. & Lee, D. RBSDesigner: software for designing synthetic ribosome binding sites that yields a desired level of protein expression. *Bioinformatics* **26**, 2633–2634 (2010).
18. Münch, R. *et al.* Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**, 4187–4189 (2005).
19. Cooper, S. J., Trinklein, N. D., Anton, E. D., Nguyen, L. & Myers, R. M. Comprehensive analysis of transcriptional promoter structure and function in 1% of the human genome. *Genome Res* **16**, 1–10 (2006).
20. Chen, Y. *et al.* Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**, 625–628 (2000).
21. Bond-Watts, B. B., Bellerose, R. J. & Chang, M. C. Y. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. *Nat Chem Biol* **7**, 222–227 (2011).
22. Shen, C. R. *et al.* Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl Environ Microbiol* **77**, 2905–2915 (2011).
23. Mazumdar, S., Clomburg, J. M. & Gonzalez, R. *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. *Appl Environ Microbiol* **76**, 4327–4336 (2010).
24. Imashimizu, M. *et al.* Thymine at —5 Is crucial for cpc promoter activity of *Synechocystis* sp. strain PCC 6714. *J Bacteriol* **185**, 6477–6480 (2003).
25. Mitschke, J. *et al.* An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803. *Proc Natl Acad Sci U S A* **108**, 2124–2129 (2011).
26. Hihara, Y., Sonoike, K., Kanehisa, M. & Ikeuchi, M. DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **185**, 1719–1725 (2003).
27. Gill, R. T. *et al.* Genome-wide dynamic transcriptional profiling of the light-to-dark transition in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **184**, 3671–3681 (2002).
28. Karp, G. *Cell Biology* 163–168 (Wiley, Singarpore, 2013).
29. Vogl, T., Ruth, C., Pitzer, J., Kickenweiz, T. & Glieder, A. Synthetic core promoters for *Pichia pastoris*. *ACS Synth Biol* **doi/abs/10.1021/sb400091p** (2013).
30. Wang, H. L., Postier, B. L. & Burnap, R. L. Polymerase chain reaction-based mutageneses identify key transporters belonging to multigene families involved in Na^+ and pH homeostasis of *Synechocystis* sp. PCC 6803. *Mol Microbiol* **44**, 1493–1506 (2002).
31. Castenholz, R. W. Culturing methods for cyanobacteria. *Method Enzymol* **167**, 68–93 (1988).
32. Zhou, J., Zhou, J., Yang, H., Yan, C. & Huang, F. Characterization of a sodium-regulated glutaminase from cyanobacterium *Synechocystis* sp. PCC 6803. *Sci. China Ser. C-Life Sci.* **51**, 1066–1075 (2008).
33. Angermayr, S. A., Paszota, M. & Hellingwerf, K. J. Engineering a cyanobacterial cell factory for production of lactic acid. *Appl Environ Microbiol* **78**, 7098–7106 (2012).
34. Mao, S. *et al.* Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. *J Proteome Res* **9**, 3046–3061 (2010).
35. Zhu, L., Dong, H., Zhang, Y. & Li, Y. Engineering the robustness of *Clostridium acetobutylicum* by introducing glutathione biosynthetic capability. *Metab Eng* **13**, 426–434 (2011).
36. Tucci, S. & Martin, W. A novel prokaryotic trans-2-enoyl-CoA reductase from the spirochete *Treponema denticola*. *FEBS Letters* **581**, 1561–1566 (2007).
37. Romero, S., Merino, E., Bolívar, F., Gosset, G. & Martinez, A. Metabolic engineering of *Bacillus subtilis* for ethanol production: lactate dehydrogenase plays a key role in fermentative metabolism. *Appl Environ Microbiol* **73**, 5190–5198 (2007).

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (973, 2011CBA00800), the National Natural Science Foundation of China (30970103), and

Beijing Natural Science Foundation (5102026) to Jie Zhou. We thank Shutao Sun for assistance with MALDI-TOF MS analysis, and Zhen Cai for assistance with enzyme assays.

Author contributions

J.Z. designed and performed the research. H.Z., H.M. and Y.Z. assisted with the experiments. Y.Z. and G.B. performed expression elements analyses. Y.L. and Y.M. designed the project and supervised the research. J.Z. and Y.L. wrote the manuscript. All authors analyzed data and discussed the results.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhou, J. *et al.* Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. *Sci. Rep.* **4**, 4500; DOI:10.1038/srep04500 (2014).