ACS SyntheticBiology

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Engineered Platform for Bioethylene Production by a Cyanobacterium Expressing a Chimeric Complex of Plant Enzymes

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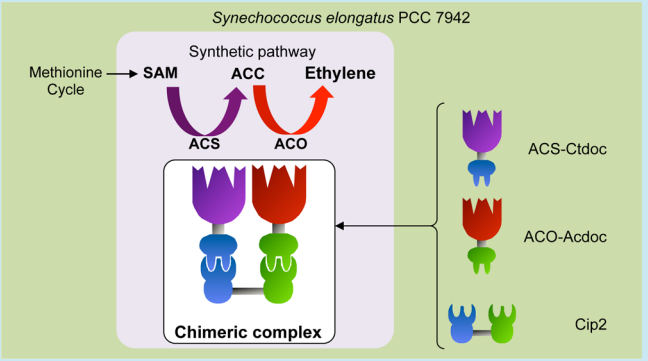
Supporting Information

ABSTRACT: Ethylene is an industrially important compound, but more sustainable production methods are desirable. Since cellulosomes increase the ability of cellulolytic enzymes by physically linking the relevant enzymes via dockerin−cohesin interactions, in this study, we genetically engineered a chimeric cellulosome-like complex of two ethylene-generating enzymes from tomato using cohesin−dockerins from the bacteria *Clostridium thermocellum* and *Acetivibrio cellulolyticus*. This complex was transformed into *Escherichia coli* to analyze kinetic parameters and enzyme complex formation and into the cyanobacterium *Synechococcus elongatus* PCC 7942, which was then grown with and without 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Only at minimal protein expression levels (without IPTG), the chimeric complex produced 3.7 times more ethylene *in vivo* than did uncomplexed enzymes. Thus, cyanobacteria can be used to sustainably generate ethylene, and the synthetic enzyme complex greatly enhanced production efficiency. Artificial synthetic enzyme complexes hold great promise for improving the production efficiency of other industrial compounds.



KEYWORDS: *ethylene, cyanobacteria, photosynthesis, cellulosomes, dockerin−cohesin interaction, synthetic enzyme complex*

Ethylene is an important chemical commodity; globally, more than 133 million tons are produced annually. Ethylene is a critical industrial raw material because it is used to produce various petrochemical products. Ethylene is currently manufactured mainly from naphtha by thermal cracking, that is, so-called *steam cracking*. In the U.S.A., 70% of the total ethylene production capacity comes from steam cracking of naphtha.[1] This steam cracking process is highly energy intensive[2] and produces large quantities of CO_2 (1.5−3.0 tons of CO_2 per ton of ethylene),[3] which is a greenhouse gas and requires petroleum, a finite resource. To reduce the rate of global warming, development of a sustainable and carbon-neutral bioethylene synthetic process is desired.

Ethylene is naturally produced by some organisms. In higher plants, such as tomato (*Solanum lycopersicum*), ethylene is produced as gaseous hormone, and its synthesis increases during several stages of plant growth and development, including seed germination, abscission, leaf and flower senescence, and fruit ripening.[4] In *S. lycopersicum*, ethylene is synthesized from *S*-adenosylmethionine (SAM) in a two-step reaction catalyzed by two key enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS, EC 4.4.1.14), and aminocyclopropane carboxylate oxidase (ACO, EC 1.14.17.4). SAM is a metabolite in the Yang cycle (methionine cycle), which recycles sulfur-containing metabolites into the amino acid methionine.[5] The cycle is a universal pathway found in organisms ranging from unicellular bacteria to plants and animals.[6−10] Some bacteria, such as *Pseudomonas syringae*, are reported to produce ethylene-forming enzyme (EFE) that catalyzes the formation of ethylene from 2-oxoglutarate, which is a member in tricarboxylic acid (TCA) cycle.[11]

Cyanobacteria are becoming increasingly attractive as cell factories for producing renewable chemicals, because they capture solar energy and CO_2 and because their relatively simple genomes facilitate genetic manipulation.[12] Direct production of valuable chemicals from CO_2 by photosynthetic organisms has been proposed.[12,13] Recently, bioethylene has been photoautotrophically generated by introducing the *EFE* gene from *P. syringae* into a cyanobacterium.[3,14] In that system, however, the recombinant cyanobacteria had a severely depressed rate of cell growth as well as a yellow-green phenotype, indicating that ethylene production caused severe metabolic stress by consuming 2-oxoglutarate and liberating the cellular carbon as ethylene.[15,16]

Anaerobic cellulolytic bacteria produce an enzyme complex called a cellulosome that increases the ability of cellulolytic enzymes to degrade biomass cellulose into soluble sugars.[17] A common feature of cellulosomes is that they consist of a large number of catalytic components arranged around a noncatalytic

Received: December 2, 2013
Published: February 17, 2014

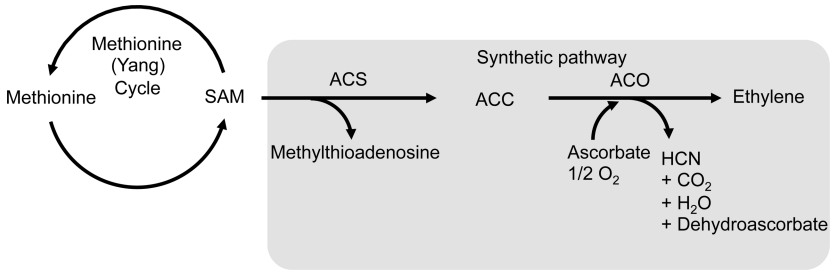


Figure 1. Pathway for ethylene production in *Synechococcus elongatus* PCC 7942. The endogenous methionine cycle recycles *S*-adenosylmethionine (SAM) from methionine in *S. elongatus* PCC 7942. Ethylene is synthesized from SAM by a synthetic pathway containing two enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), and aminocyclopropane carboxylate oxidase (ACO), derived from *Solanum lycopersicum*.

scaffoldin protein (Sca). A typical Sca contains a carbohydrate-binding module (CBM) and multiple copies of cohesin domains. Each cohesin interacts persistently with a dockerin domain on an enzyme subunit, thereby incorporating the enzymes into the complex.[17−21] The cohesin−dockerin interaction is essential to form a cellulosome, is Ca^{2+}-dependent, and possesses high specificity as well as high affinity.[22]

Cellulases in the form of cellulosomes can hydrolyze cellulose faster than their noncomplexed cellulase mixtures, especially with recalcitrant celluloses. Bayer and his co-workers first proposed the construction of synthetic enzyme complexes by using dockerins and cohesins from cellulosome components.[23] A synthetic enzyme complex that places the enzymes in close proximity to each other can influence the degree of substrate channeling.[24] Using this approach, numerous designer cellulosomes have been constructed to facilitate enzymatic hydrolysis of solid polysaccharides, such as cellulose and pretreated lignocellulosic biomass.[25−28]

In this work, we aimed to produce ethylene directly from CO_2 using an engineered cyanobacterium, *Synechococcus elongatus* PCC 7942. Both ACS and ACO derived from *S. lycopersicum* were introduced into the cyanobacteria (Figure 1). In this way, ethylene could be produced from endogenous SAM without directly consuming cellular carbon sources. Furthermore, we hypothesized that efficient enzymatic activity of ethylene production could be achieved by complexing two ethylene biosynthesis enzymes. To do so, we constructed a chimeric enzyme complex using the cohesin−dockerin interaction from two cellulolytic anaerobic bacteria, *Clostridium thermocellum* and *Acetivibrio cellulolyticus*. The chimeric enzyme complex comprised three proteins: (i) a chimeric scaffoldin possessing two cohesins of divergent specificity and (ii) two enzymes, each bearing a dockerin that was complementary to one of the divergent cohesins (Figure 2A). Then, these three components were expressed in *S. elongatus* PCC 7942, and ethylene production by the resultant ternary enzyme complex was observed. Here, we report the introduction of a novel heterologous ethylene production pathway into *S. elongatus* PCC 7942.

RESULTS AND DISCUSSION

Preparation of Recombinant Proteins for Chimeric Enzyme Complex Components. To produce ethylene from SAM, two noncellulosomal enzymes, ACS and ACO, derived from *S. lycopersicum* were used. A COOH terminus deletion mutant of ACS was constructed by deleting 36 amino acids and used as the ACS gene, while the full-length open reading frame (ORF) was used for ACO. To convert these enzymes into chimeric enzyme proteins, dockerins with different binding specificities were fused to each enzyme, yielding ACS-Ctdoc and ACO-Acdoc, respectively. The ACS-Ctdoc and ACO-Acdoc constructs were made by adding dockerins from *C. thermocellum* Cel48S and *A. cellulolyticus* ScaB to the C-termini of ACS and ACO, respectively. ACS-Ctdoc and ACO-Acdoc were obtained using the overlap-extension polymerase chain reaction (PCR) method.[29] Then, each gene encoding a chimeric enzyme was separately cloned into the expression vector pET28a to produce recombinant proteins in *Escherichia coli*.

Chimeric scaffoldin (Cip2) consists of two cohesins with different binding specificities, allowing the selective binding of two different dockerin-containing enzymes. The Cip2 was prepared to contain the cohesin module 1 from *C. thermocellum* CipA at its N-terminus followed by cohesin module 3 from *A. cellulolyticus* ScaC. Three expression vectors encoding the chimeric enzymes and scaffoldin proteins designed for this study are presented schematically in Figure 2B.

Escherichia coli strain BL21(DE3)RIL was transformed with the expression plasmids pET-ACS-Ctdoc, pET-ACO-Acdoc, or pET-Cip2, and the recombinant proteins were expressed independently. The expressed proteins were purified and subjected to SDS-PAGE analysis. As shown in Figure 3, three purified recombinant proteins, ACS-Ctdoc (62.5 kDa), ACO-Acdoc (48.5 kDa), and Cip2 (35.8 kDa), each produced a single major band whose mobilities were consistent with the expected molecular masses. Cip2 was found to be very stable upon storage for several days at 4 °C, whereas low levels of spontaneous cleavage between the catalytic domain and dockerin were detectable in both ACS-Ctdoc and ACO-Acdoc.

Kinetic Parameters of Two Chimeric Enzymes, ACS-Ctdoc and ACO-Acdoc. Because the structures of ACS and ACO were modified by attaching dockerins or, in ACS, by deleting the C-terminus of the catalytic domain, the kinetic parameters of the enzymes might also have changed. To assess this possibility, we assayed the enzymatic activities of the purified ACS-Ctdoc and ACO-Acdoc. The K_m and V_{max} values were obtained by analyzing the substrate saturation kinetics and the best fit of the data points.

A previous study showed that deletion of 46−52 amino acids from the COOH terminus of ACS resulted in an enzyme with nine times higher affinity for the substrate SAM than the wild-type enzyme.[30] Thus, we constructed a 36-aa C-terminus deletion mutant of ACS-Ctdoc and assessed its kinetic parameters. ACS-Ctdoc had a K_m value of 181 μM for SAM and a V_{max} of 21.6 μmol/h·mg at 30 °C and pH 7.5. The K_m value was higher and the V_{max} value was lower than those of the wild-type ACS (K_m 22 μM, V_{max} 96 μmol/h·mg). Both values

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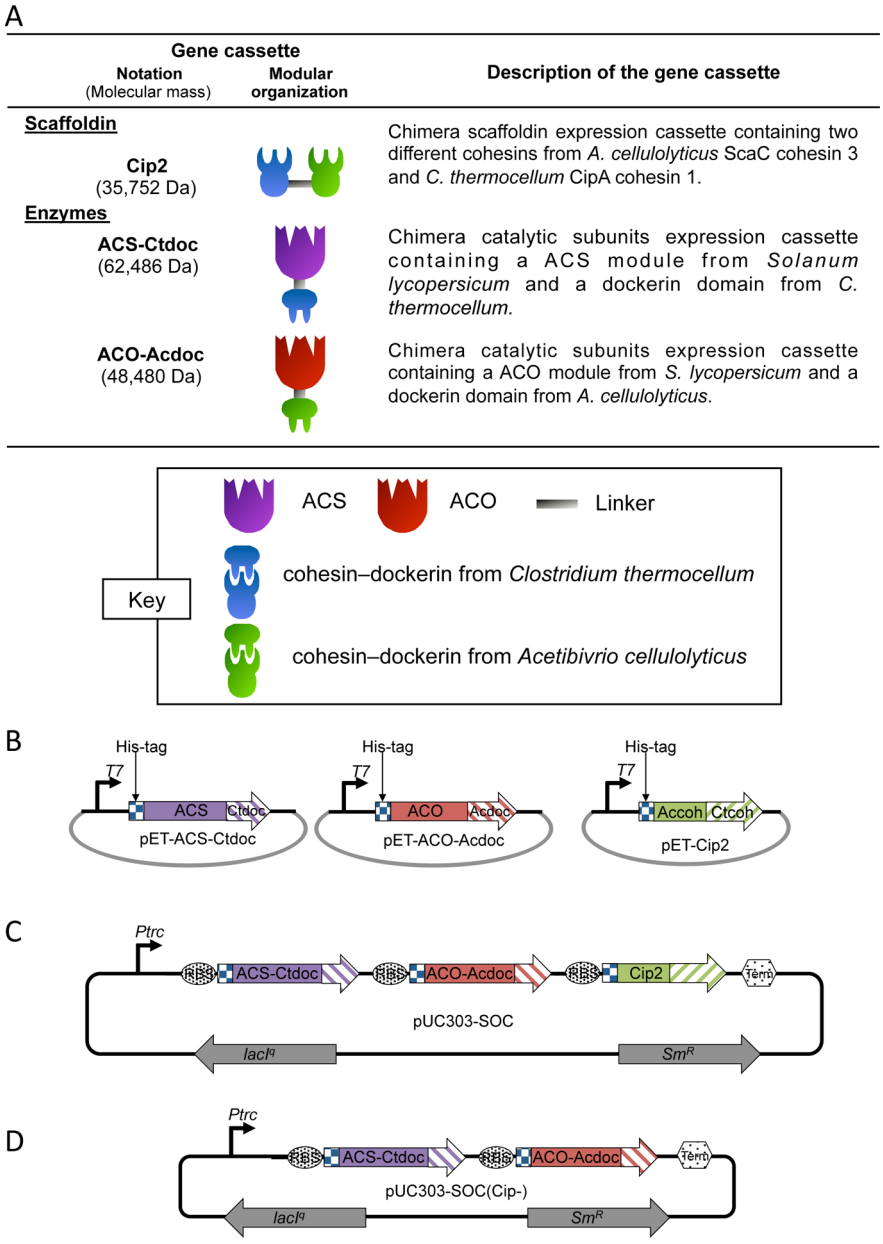

Figure 2. Schematic representation of the chimeric proteins and their expression vectors used in this study. (A) A cellulosome-based chimeric enzyme complex comprising two chimeric enzymes (ACS-Ctdoc and ACO-Acdoc) and one chimeric scaffoldin (Cip2) was designed. The two chimeric enzymes bind specifically to the chimeric scaffoldin via cohesion−dockerin interactions. ACC, 1-aminocyclopropane-1-carboxylate; Acdoc, dockerin domain of *Acetivibrio cellulolyticus*; ACO, aminocyclopropane carboxylate oxidase; ACS, ACC synthase; Cip2, chimeric scaffoldin constructed in this study; Ctdoc, dockerin domain of *Clostridium thermocellum*; SAM, *S*-adenosylmethionine. (B) A schematic of the three expression vectors for *Escherichia coli*. Three expression vectors, pET-ACS-Ctdoc, pET-ACO-Acdoc and pET-Cip2, were constructed as described in the Methods. (C) A schematic of the pUC303-SOC shuttle vector for *Synechococcus elongatus* PCC 7942. Three gene cassettes were introduced into pUC303, yielding plasmid pUC303-SOC, which encoded the three components of the enzyme complex, ACS-Ctdoc, ACO-Acdoc, and Cip2, and was regulated by the *trc* promoter. RBS, ribosomal binding site; Ptrc, *trc* promoter obtained from pTrc99A; Term, transcriptional terminator obtained from pTrc99A. (D) A schematic of the pUC303-SOC(Cip-) shuttle vector for *S. elongatus* PCC 7942. Two gene cassettes were introduced into pUC303, yielding plasmid pUC303-SOC(Cip-), which produced an enzyme complex without the scaffoldin protein, Cip2.

were lower than those of the previously reported mutant of ACS (K_m 280 μM, V_{max} 420 μmol/h·mg).[30]

ACO-Acdoc had a K_m value of 125 μM for ACC and a V_{max} of 80 nmol/h·mg at 30 °C and pH 7.5. These values were both higher than those of wild-type ACO (K_m 30 μM, V_{max} 25 nmol/h·mg).[31]

The activity of ACO-Acdoc was stable during storage for months at −80 °C. Conversely, ACS-Ctdoc was unstable and did not maintain activity through a freeze−thaw cycle.

Binding Analysis within the Chimeric Enzyme Complex. To observe the cohesin−dockerin binding activity between the two chimeric enzymes and one scaffoldin protein, complex formation in the presence of calcium was verified using two different techniques: gel filtration and nondenaturing PAGE. First, the recombinant proteins were mixed and subjected to gel filtration analysis (Figure 4). Each of the proteins eluted as a single peak in the chromatogram. In Figure 4A, the eluted Cip2 was approximately 35 kDa, matching the

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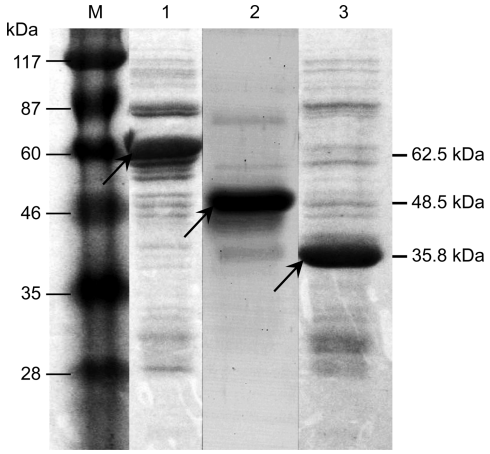

Figure 3. SDS-PAGE analysis of the purified recombinant proteins. Purified recombinant proteins expressed in *Escherichia coli* BL21-(DE3)RIL were resolved in a 10% SDS polyacrylamide gel followed by CBB-R250 staining. Lane M, protein markers; lane 1, ACS-Ctdoc; lane 2, ACO-Acdoc; lane 3, Cip2.

predicted molecular size of the Cip2 monomer (35.7 kDa). The size of the eluted ACO-Acdoc was approximately 50 kDa, matching the size of the ACO-Acdoc monomer (48.5 kDa). A mixture of the free proteins ACO-Acdoc and Cip2 resulted in a single band with a higher molecular weight of approximately 90 kDa that corresponded to the expected sizes of the desired chimera protein complex of ACO-Acdoc and Cip2. In Figure 4B, the eluted Cip2 was approximately 70 kDa, matching the size of a dimer of Cip2 (35.7 kDa). The eluted ACS-Ctdoc was approximately 130 kDa, corresponding to a dimer of ACS-Ctdoc (62.5 kDa). When a mixture of ACS-Ctdoc and Cip2 was analyzed, the peaks corresponding to the free components were much smaller and were replaced by a major peak of higher molecular mass (approximately 190 kDa). A minor peak was also observed, suggesting that a low level of oligomerization may have also occurred. Notably, Cip2, ACS-Ctdoc, and the chimeric complex eluted at higher positions than expected based on their molecular masses because of the known tendency of both cohesins in Cip2 and ACS catalytic domain in ACS-Ctdoc to dimerize.[30,32]

Binding experiments were done with nondenaturing PAGE for the same enzyme combinations as analyzed by gel filtration (Supporting Information Figure S1). Band shifts relative to the individual enzymes were observed in both binary mixtures, indicating that both ACO-Acdoc plus Cip2 and ACS-Ctdoc plus Cip2 could bind and form chimeric enzyme complexes (Figure S1, lanes 5 and 6). Finally, purified ACS-Ctdoc, ACO-Acdoc, and Cip2 were mixed together and subjected to nondenaturing PAGE. The cohesins and dockerins allowed ACS-Ctdoc and ACO-Acdoc to bind to Cip2 together, forming a synthetic two-enzyme chimeric complex (Figure S1, lane 7).

Coexpression of the Three Component Proteins of the Chimeric Enzyme Complex in Cyanobacteria. We reconstructed the plant pathway for ethylene synthesis in *S. elongatus* PCC 7942 strain R2-SPc (hereafter, referred to as *S. elongatus* PCC 7942) by expressing a chimeric enzyme complex comprising two chimeric enzymes from *S. lycopersicum* (ACS-Ctdoc and ACO-Acdoc) and a scaffoldin protein from bacterial cellulosomes (Cip2). Plasmid pUC303, which can replicate in *S. elongatus* PCC 7942, was selected as a shuttle vector.[33] A gene cluster encoding two gene cassettes of chimeric enzyme

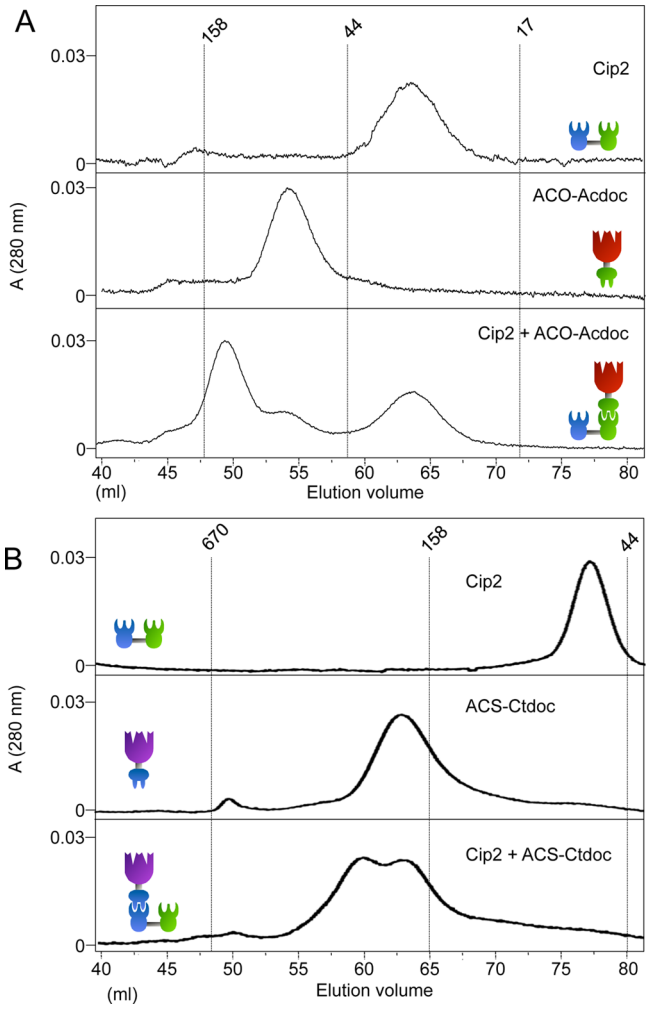

Figure 4. Gel filtration analysis of the recombinant proteins expressed in *Escherichia coli* and their assembled chimeric complexes. Cip2-based chimeric enzyme complex formation with ACO-Acdoc (A) or with ACS-Ctdoc (B), and their components, were analyzed by either Superdex 75pg or Superdex 200pg gel filtration chromatography. Injected proteins are indicated on each chromatogram. Vertical lines denote the positions of molecular mass markers: thyroglobulin (bovine), 670 kDa; γ-globulin (bovine), 158 kDa; ovalbumin (chicken) 44 kDa, and myoglobin (horse), 17 kDa. In part B, note that Cip2, ACS-Ctdoc, and the chimeric complex eluted at higher-than-expected molecular masses because both cohesin in Cip2 and ACS catalytic domain in ACS-Ctdoc tended to dimerize in 1 week after purification of proteins.

and one gene cassette of scaffoldin protein were introduced into pUC303 as presented in Figure 2C; pUC303-SOC was then transformed into *S. elongatus* PCC 7942. The transformed strain was named strain SOC.

To assess the expression levels of three recombinant proteins in strain SOC exposed to different concentrations of IPTG, Western blot analysis of His-tagged proteins using anti His-tag antibodies was performed. The results indicated that strain SOC successfully produced ACS-Ctdoc, ACO-Acdoc, and Cip2. The mobilities of the proteins were consistent with the calculated molecular weights of the recombinant proteins. The proteins were also expressed in the absence of IPTG. Most of the three chimeric proteins were recovered in the soluble fraction of *S. elongatus* PCC 7942 cell extract; however, small amounts occurred in the insoluble fraction. In the wild-type

dx.doi.org/10.1021/sb400197f | *ACS Synth. Biol.* 2014, 3, 487−496

cells, no cross-reaction occurred between anti His-tag antibodies and any proteins of the *S. elongatus* PCC 7942 soluble and insoluble extracts (Figure 5A).

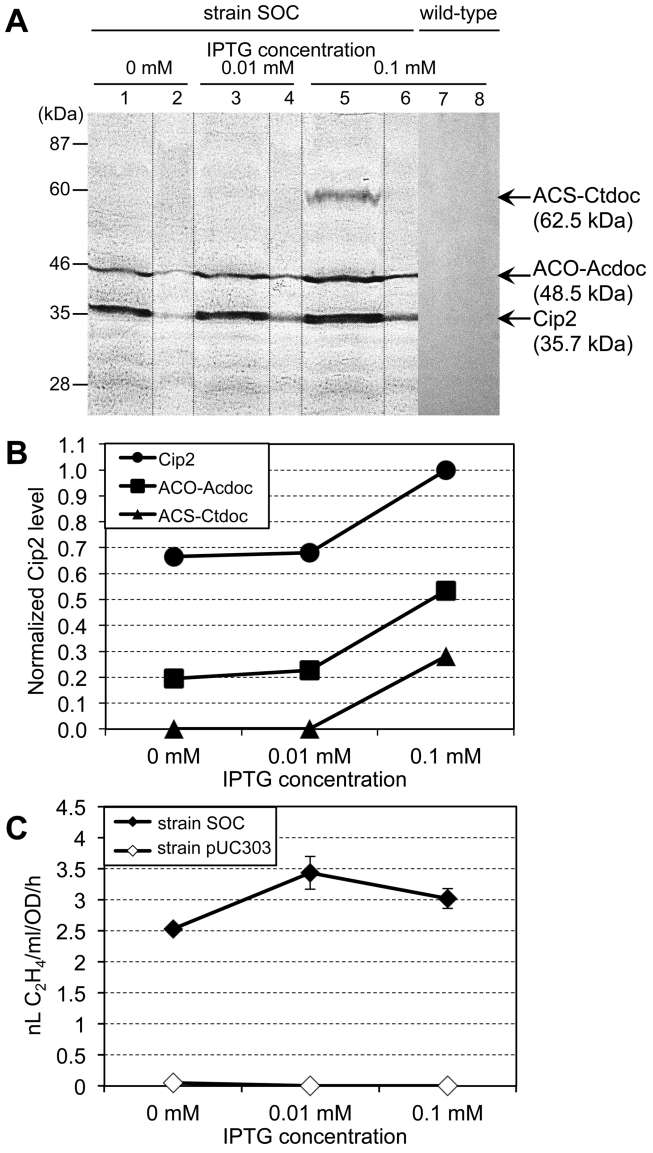

Figure 5. Effects of IPTG concentrations on the enzyme complex expression and on ethylene production in *Synechococcus elongatus* PCC 7942 strain SOC. Strain SOC and wild-type of *S. elongatus* PCC 7942 were grown in BG-11 medium until the optical density (at 730 nm) was 0.6. To induce protein expression, IPTG (0.01 or 0.1 mM) was added. (A) Western blotting analysis of the proteins in soluble (lanes 1, 3, 5, and 7) and insoluble (lanes 2, 4, 6, and 8) fractions of *S. elongatus* PCC 7942 cells and identification of His-tagged chimeric proteins. Lanes 1 and 2, without IPTG; lanes 3 and 4, 0.01 mM IPTG; lanes 5, 6, 7, and 8, 0.1 mM IPTG. (B) Semiquantitative analysis of the protein bands detected in Western blotting. The optical density of the bands were determined using National Institute of Health ImageJ software (Bethesda, MD, U.S.A.). The amount of the protein is plotted relative to the amount of Cip2 induced with 0.1 mM IPTG. (C) The rate of *in vivo* ethylene synthesis in recombinant *S. elongatus* PCC 7942 cells. The *in vivo* ethylene accumulation by *S. elongatus* PCC 7942 strain SOC and strain pUC303 (transformed with the empty vector pUC303) was analyzed by gas chromatography. Each reaction was carried out in triplicate; the averages and standard deviations are plotted as a function of IPTG concentration.

We semiquantitatively calculated the band intensities of the soluble fraction in Western blots and found that the expression levels were 1.5−2.4 times higher at 0.1 mM IPTG than at 0.01 mM (Figure 5B).

Ethylene Production in Cyanobacteria Expressing a Chimeric Enzyme Complex. To determine whether the expressed chimeric enzymes were metabolically competent, the *in vivo* ethylene accumulations of strain SOC and *S. elongatus* PCC 7942 strain pUC303 (transformed with empty pUC303 vector) was assessed.

In strain SOC, but not in strain pUC303, an ethylene-specific peak was clearly evident, showing that the expressed chimeric enzymes were indeed active in strain SOC. In strain SOC, ethylene production reached 3.4 nL ml^{-1} OD^{-1} h^{-1} at 0.01 mM IPTG induction and occurred both with and without IPTG induction (Figure 5C). Strain SOC continued to produce ethylene at a similar rate for more than 6 months and after at least 12 successive generations of batch culture transfer.

Enhanced Ethylene Production With or Without Forming a Chimeric Enzyme Complex in Cyanobacteria. To observe the synergistic effects of a chimeric enzyme complex on ethylene production, we prepared plasmid pUC303-SOC (Cip-), in which the *cip2* gene was removed from the gene cluster (Figure 2D). *S. elongatus* PCC 7942 transformed with pUC303-SOC(Cip-) was named as strain SOC(Cip-). This strain expressed ACS-Ctdoc and ACO-Acdoc as free enzymes, without forming an enzyme complex.

In both strains SOC and SOC(Cip-), but not in strain pUC303, ethylene production was observed both with and without IPTG induction (0.1 mM). Without IPTG, strain SOC produced 3.7 times more ethylene than strain SOC(Cip-), while no significant difference ($p > 0.5$) was observed in the assay with 0.1 mM IPTG (Figure 6).

Conclusion. The C terminus of ACS was shown to be dispensable for maintaining activity.[30] Addition of a dockerin domain to the C terminus of both ACS and ACO catalytic domains did not abolish the catalytic functionality of the two chimeric enzymes, ACS-Ctdoc and ACO-Acdoc. The kinetic analysis showed that the K_m values of both ACS-Ctdoc and

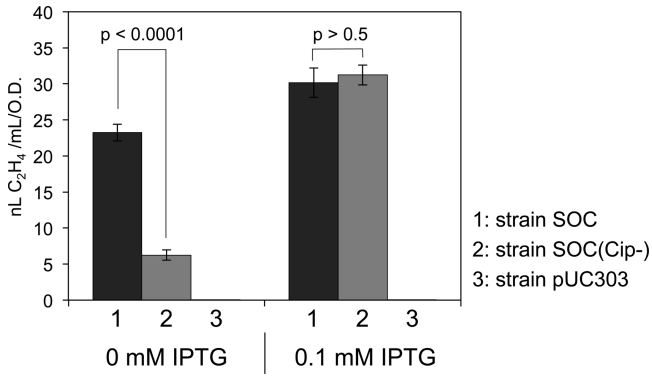

Figure 6. Comparative analysis of the *in vivo* ethylene formation of *Synechococcus elongatus* PCC 7942 strains SOC and SOC(Cip-). *S. elongatus* PCC 7942 strains SOC, SOC(Cip-), and pUC303 were cultured in the presence of 0 or 0.1 mM IPTG to induce low or high protein levels, respectively. The *in vivo* ethylene accumulation of the cells was analyzed by gas chromatography. Presented data were averages of triplicates and standard errors were drawn on the plot. Student's *t*-test was used to calculate the *p* values in the statistical analysis. Statistical significance was declared when the *p* value was less than 0.0001.

dx.doi.org/10.1021/sb400197f | *ACS Synth. Biol.* 2014, 3, 487−496

ACO-Acdoc were slightly lower than those of their wild-type homologues. Notably, the V_{max} value of ACO-Acdoc was much lower than that of ACS-Ctdoc in the chimeric enzyme. This disparity was consistent with previous reports that the wild-type ACO had much lower V_{max} value than the wild-type ACS.[30,31]

Analysis of gel filtration and nondenaturing PAGE demonstrated that complex formation could be achieved by mixing the desired components *in vitro*. However, the interaction was not complete in the gel filtration analysis (Figure 4). This may be because the incubation time of 30 min between Cip2 and the chimeric enzyme was not sufficient. The results of the present work also provide experimental verification that a chimeric enzyme complex can be constructed by combining appropriate dockerin-containing enzymes and recombinant cohesin-containing scaffoldins as described before.[34]

In this study, protein expression in *S. elongatus* PCC 7942 was controlled by the *trc* promoter, a well-established tunable promoter.[35] Because the complex proteins was observed even without IPTG, the *trc* promoter had poor repression ability, as has been previously reported.[36] On the other hand, McEwen et al. showed that the ability of the *trc* promoter to induce protein expression was similar at both 0.1 mM and 1 mM IPTG.[37] Consistent with this observation, our experiment also showed similar levels of protein expression in *S. elongatus* PCC 7942 at 0.1 mM and 1 mM IPTG concentrations. Thus, we assumed that protein induction was saturated at 0.1 mM IPTG for this construct.

The cellulosomal enzymatic subunit constructed in this study had a 6 × His tag at the N-terminus, followed by a catalytic domain. The catalytic domain was separated from a dockerin domain by a 5−7 amino-acids linker (TYKVP for Ctdoc, LITPPGT for Acdoc). When a cellulosomal enzymatic subunit is expressed in *E. coli*, proteolytic cleavage of the cellulosomal enzymatic subunit between the catalytic and dockerin domains is frequently observed.[38,39] In the expressed protein derived from *S. elongatus* PCC 7942, we were concerned about such cleavage between the catalytic and dockerin domains in both ACS-Ctdoc and ACO-Acdoc but did not observe cleavage in the Western blots (Figure 5A). In this case, the enzymes with a fused dockerin domain were more stable when expressed in *S. elongatus* PCC 7942 than in *E. coli*. Therefore, we concluded that cyanobacteria appear to be a better host for the exogenous expression of dockerin-containing proteins than *E. coli*.

The level of the ethylene production did not increase significantly as IPTG concentration rose from 0.01 mM to 0.1 mM (Figure 5C). However, our data indicated that protein induction increased significantly as IPTG concentration rose (Figure 5A). The higher protein concentrations at 0.1 mM IPTG did not affect ethylene production, suggesting that total chimeric protein level in the cytosol was saturated at 0.1 mM IPTG and was not the main rate-limiting factor for ethylene synthesis. We suspect that the levels of endogenous SAM might be limited in the *S. elongatus* PCC 7942. The actual rate-limiting factor for ethylene synthesis in this engineered *S. elongatus* PCC 7942 awaits further investigation.

The rate of ethylene synthesis by strain SOC observed in this study was 3.4 nL C_2H_4 ml^{-1} OD^{-1} h^{-1}, much less than in previous studies in which other bacterial *EFE* genes were expressed in *S. elongatus* PCC 7942 (451 nL C_2H_4 mL^{-1} OD^{-1} h^{-1},[16] 323 nL C_2H_4 mL^{-1} OD^{-1} h^{-1}[15]) and in *Synechocystis* (approximately 170 nL C_2H_4 mL^{-1} OD^{-1} h^{-1}[14]). A possible reason could be limitation of the intracellular substrate, SAM. To overcome this issue, another enzyme, such as SAM synthetase, could be introduced into the pathway to enhance substrate supply. Another possible explanation could be the low V_{max} of ACO, which could be circumvented by constructing a scaffoldin that could accommodate multiple ACOs in a single enzyme complex.

This work presents a synthetic bioethylene production system constructed by introducing the plant EFEs ACS and ACO into a cyanobacterium (Figure 5C). A recent study attempted to produce ethylene by coexpressing ACS and ACO from *Arabidopsis thaliana* in *Synechocystis*, but no ethylene accumulated.[14] Li and co-workers[40] attempted to synthesize ethylene by introducing ACS from *S. lycopersicum* and ACO from soybean into *E. coli* as a fusion enzyme. Ethylene was produced *in vitro* with purified enzymes, but no information regarding *in vivo* synthesis in *E. coli* cultures was provided. Although these two studies explored ethylene productions using plant enzymes *in vitro*, no reports using cyanobacteria have been published.

In this study, the synergistic effects of chimeric enzyme complexes on enzyme activity were observed at low enzyme concentrations, probably because the clustering of enzymes in the complex increases their local concentrations where the reactions occur.[28] However, at high levels of induction, the overall enzyme concentration equals or exceeds the local enhancement of the enzyme complexes, erasing the synergistic effect. The enhanced *in vivo* ethylene production demonstrated in this study at low enzyme concentrations clearly demonstrated the synergistic effects of the chimeric enzyme complex (Figure 6). The reaction rate enhancements observed in this study indicated strong substrate channeling within the chimeric enzyme complex due to the close proximity of the chimeric enzymes in the cascade.[41] This work presents a synthetic enzyme complex that mimics a cellulosome system to increase the catalytic efficiency of a reaction cascade.

Overall, this work demonstrated that cyanobacteria can be engineered to photosynthetically convert CO_2 into ethylene. The transformed cyanobacteria produced sufficient amounts of ethylene under photosynthetic conditions without any constraints on growth. The two cascade enzymes were successfully expressed in a cyanobacterium. At low enzyme concentrations, chimeric enzyme complexes were more active at producing ethylene than free enzymes. Thus, these engineered complexes may be useful tools for the renewable production of valuable chemicals by photosynthetic organisms.

The enzyme system described here can be refined by optimizing the cyanobacterial growth conditions, such as the temperature, medium, and/or light parameters, and elaborated in several ways. In a broader context, the capacity to incorporate specific enzymatic components into a defined chimeric enzyme complex that can be transformed into cyanobacteria will have considerable biotechnological value for producing valuable chemicals, including ethylene.

METHODS

Chemicals and Reagents. All chemicals were purchased from Wako Chemicals (Osaka, Japan). Restriction enzymes were purchased from New England BioLabs (Ipswich, MA, U.S.A.). The PrimeSTAR MAX DNA polymerase, In-fusion HD cloning kit, and oligonucleotides were purchased from Takara (Tokyo, Japan).

Bacterial Strains and Culture Conditions. The *S. elongatus* PCC 7942 strain was grown on BG-11 medium[42]

dx.doi.org/10.1021/sb400197f | ACS Synth. Biol. 2014, 3, 487−496

Table 1. Primers Used in This Study

name	nucleotide sequence	comments
ACS-Ctdoc-Fwd1	AATGGGTCGCGGATCCGGATTTGAGATTGCAAAGAC	pET-ACS-Ctdoc, ACS
ACSdelC2-Ctdoc-R1	ATAAATCGAGTTCGATGGAAAAGAAGACATATAAAGTACCTGGTACTCCTT	pET-ACS-Ctdoc, ACS
ACSdelC2-Ctdoc-F2	ATAAATCGAGTTCGATGGAAAAGAAGACATATAAAGTACCTGGTACTCCTT	pET-ACS-Ctdoc, Ctdoc
ACSdelC23-Ctdoc-R2	TAGATACATTGCCGTACAAGAACTAAGGATCCGAATTCGAGCTCCG	pET-ACS-Ctdoc, Ctdoc
ACO-AcBdoc-Fwd1	AATGGGTCGCGGATCCGAGAACTTCCCAATTATCAAC	pET-ACO-Acdoc, ACO
ACO-AcBdoc-Rev1	GCTCGAATTCGGATCCTTATTCTTCTTTCTCTTCAACAGG	pET-ACO-Acdoc, ACO
ACO-AcBdoc-Fwd2	CAAATTGCAAGTGCTTTAATAACACCGCCAGGTACC	pET-ACO-Acdoc, Acdoc
ACO-AcBdoc-Rev2	TGGCGGTGTTATTAAAGCACTTGCAATTTGATCAAC	pET-ACO-Acdoc, Acdoc
Cip2Ac-Fwd-InF	AATGGGTCGCGGATCCGATTTACAGGTTGACATTGGAAG	pET-Cip2, AcCoh
Cip2Ac-Rev-InF	GCTCGAATTCGGATCCTTAGATAGCGCCATCAGTAAGAG	pET-Cip2, AcCoh
pETCip2B-Fwd1	AACACACCGAATGCAATAAAGATTAAGGTGG	pET-Cip2, CtCoh
pETCip2B-Rev1	TATTGCATTCGGTGTGTTTGTCGGTGTGTTTG	pET-Cip2, CtCoh
303ACS-F	CACACAGGAAACAGATCATGAGCAGCAGCCATC	ACS-Ctdoc ORF derived from pET-ACS-Ctdoc
303ACS-R	GGCTGCTGCCCATGATCTGTTTCCTGTGGTCGACTTACGCTTGAGGAAGTGTGATGTTAGTT CTTGTACGGCAATG	
303ACO-F	CATTGCCGTACAAGAACTAACATCACACTTCCTCAAGCGTAAGTCGACCACAGGAAACAGATC ATGGGCAGCAGCC	ACO-Acdoc ORF derived from pET-ACO-Acdoc
303ACO-R	TGATGATGATGGCTGCTGCCCATGGTCTGTTTCCTGTGAAGCTTTTATTCTTCTTTCTCTTC	
303 Cip2-F	GAAGAGAAAGAAGAATAAAAGCTTCACAGGAAACAGACCATGGGCAGCAGCCATCATCATCA	Cip2 ORF derived from pET-Cip2
303 Cip2-R	GCATGCCTGCAGGTCGACTCTAGATTATGTTGTATCTCCAACATTTAC	
303Trc99lactrc-F	GAATGCTCATCCGGAATTCATTCGCGCGCGAAGGCGAAGCGGC	fragment containing a *lacI* gene and *trc* promoter sequence derived from pTrc99A
303Trc99lactrc-R	GTGATGATGGCTGCTGCTCATGATCTGTTTCCTGTGTG	
303Trc99term-F	GTTGGAGATACAACATAATCTAGAGTCGACCTGCAGGCATGC	fragment containing a transcriptional terminator sequence derived from pTrc99A
303Trc99term-F	CTTTCATTGCCATACGGAATTCGGATACATATTTGAATGTATTTAG	

with reciprocal shaking at 27 °C under light. Plates containing 1.5% (w/v) agar were prepared for solid culture. For ethylene production, 50 mL of culture was grown in a 250-mL Erlenmeyer flasks under 100 μE s^{-1} m^{-2} light at 27 °C. Cell growth was monitored by measuring OD_{730}. *E. coli* DH5α and BL21(DE3)RIL strains were grown in LB medium at 37 °C.

Construction of Plasmids. All cloning and plasmid preparation were done using *E. coli* DH5α cells (Toyobo, Osaka, Japan). Detailed information about plasmids and primers used in this study can be found in Figure 2 and Table 1. The cDNA clones which encoded the sequence of the wild-type enzymes of both the *ACS* and *ACO* genes from *S. lycopersicum* were obtained from Kazusa DNA Research Institute (accession nos. AK323740 and AK324411).

The cDNA encoding the catalytic domain of ACS was amplified from a cDNA provided by Kazusa DNA Research Institute using the ACS-Ctdoc-Fwd1 and ACSdelC2-Ctdoc-R1 primers. The nucleotide sequence encoding the 36 unconserved amino acids at the COOH terminus of ACS were removed,[30] resulting in a cDNA sequence encoding an enzymatically functional protein. The ACS gene was fused to the dockerin-encoding region of *C. thermocellum* Cel48A, which was amplified from the genomic DNA of *C. thermocellum* using the primers ACSdelC2-Ctdoc-F2 and ACSdelC23-Ctdoc-R2. The two resultant overlapping fragments were mixed, and a combined fragment was synthesized using the external primers. The fragment was cloned into linearized pET28a, which has a 6 × His-tag epitope at the N-terminus, using the In-fusion HD cloning kit (Takara, Shiga, Japan), thereby generating pET-ACS-Ctdoc.

pET-ACO-Acdoc was constructed similarly. The cDNA encoding the full-length catalytic domain of ACO was amplified from the cDNA clone using the forward ACO-AcBdoc-Fwd1 and reverse ACO-AcBdoc-Rev1 primers. DNA encoding an *A. cellulolyticus* ScaB dockerin-domain was amplified from the genomic DNA of *A. cellulolyticus* using the primers ACO-AcBdoc-Fwd2 and ACO-AcBdoc-Rev2. The two resultant overlapping fragments were mixed, and a combined fragment was synthesized using the external primers. The fragment was cloned into linearized pET28a, thereby generating pET-ACO-Acdoc.

pET-Cip2 was constructed by amplification of two fragments. The ScaC cohesin 3 encoding DNA was amplified from the genomic DNA of *A. cellulolyticus* using the primers Cip2Ac-Fwd-InF and Cip2Ac-Rev-InF. The DNA encoding CipA cohesin1 was amplified from the genomic DNA of *C. thermocellum* using the primers pETCip2B-Fwd1 and pET-Cip2B-Rev1.

Three plasmids, pET-ACS-Ctdoc, pET-ACO-Acdoc and pET-Cip2, were independently transformed and expressed in *E. coli* BL21(DE3)RIL.

Production and Purification of Recombinant Proteins. The plasmids pET-ACS-Ctdoc, pET-ACO-Acdoc, and pET-Cip2 were transformed into *E. coli* BL21(RIL) cells. The transformants were cultured at 37 °C in 500 mL Terrific broth[43] supplemented with 50 mg/L kanamycin. When the absorbance at 600 nm of the cells reached 0.5, isopropyl ß-D-1-thiogalactopyranoside (IPTG, 0.5 mM) was added to induce gene expression, and cultivation was continued at 16 °C for an additional 16 h.

The cells were collected by centrifugation and subsequently resuspended in 50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl at a ratio of 1 g wet pellet to 4.5 mL buffer solution. The suspension was kept on ice during sonication, after which it was centrifuged and the supernatant collected. The expressed His-tagged protein was isolated by metal-chelate affinity chromatography using His-accept resin (Nacalai Tesque, Kyoto, Japan). Fast protein liquid chromatography (FPLC) was performed using a Hi-TrapQ column and AKTA Prime system (GE Healthcare, Little Chalfont, UK). The purified proteins

dx.doi.org/10.1021/sb400197f | *ACS Synth. Biol.* 2014, 3, 487−496

were subjected to SDS-PAGE according to the method of Laemmli.[44] Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA, U.S.A.) or by measuring the absorbance at 280 nm.[45]

Gel Filtration. Gel filtration was performed by FPLC using either HiLoad 16/60 Superdex 200 pg or 75 pg (GE Healthcare) columns equilibrated with 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 1 mM $CaCl_2$ at a flow rate of 2 mL/min. Samples were diluted in the same buffer (300 μM final concentration), allowed to stand at room temperature for 30 min, and 250 μL were loaded onto the column. Chromatographic data were recorded at 280 nm.

Nondenaturing PAGE. Nondenaturing PAGE was performed according to the method of Ornstein and Davis.[46,47] All the solutions and buffers for SDS-PAGE analysis was prepared without SDS or reducing agent. Protein samples (5 μM final concentration) were mixed in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 1 mM $CaCl_2$, allowed to stand at room temperature for 30 min, loaded onto the 15% gel at 4 °C, and visualized by CBB-R250.

Enzyme Activity Assays. For the ACS activity assay, the amount of ACC was measured by amino acid analysis. Purified ACS-Ctdoc was mixed with a standard reaction mixture containing 20 mM Tris-HCl (pH 7.5) and 20 mM pyridoxal phosphate. Different concentrations of SAM (0.01−1 mM) were added to the mixture (total volume 100 μL) and incubated at 30 °C for 10 min. The reaction was stopped by adding 400−800 μL of ethanol. The reaction products were analyzed using an HPLC LC-VP Amino Acid Analyzing System equipped with a Shim-pack Amino Li column (Shimadzu Science, Osaka, Japan). The eluted amino acids were converted to *o*-phthalaldehyde derivatives and detected with a fluorescence detector (RF-10AXL, Shimadzu Science).

To assess ACO activity, the amount of ethylene was measured by gas chromatography. Purified ACO-Acdoc was mixed with a standard reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 30 μM sodium ascorbate, 20 mM sodium bicarbonate, 20 mM ferrous sulfate, and 20% glycerol in a 2 mL syringe vial. Different concentrations of ACC (0.02−1 mM) were added to the mixture (total volume 1 mL) and incubated at 30 °C for 10 min. Five hundred microliters of the gas phase were subjected to gas chromatography.

The kinetic parameters, K_m and V_{max} were determined from the substrate saturation kinetic data using a linear least-squares fitting of a Lineweaver−Birk plot of the Michaelis−Menten equation.

Gas Chromatography. To assay ethylene production, gas from the headspace of sealed cultures was sampled and analyzed by gas chromatography using a Shimadzu 2012A gas chromatograph equipped with a flame ionization detector and a capillary column (Rt Q-Bond, Shimadzu Scientific) selected to detect short-chain hydrocarbons. The amounts of ethylene produced were estimated by comparison with a pure ethylene standard (GL Sciences Inc., Tokyo, Japan).

Cloning of *ACS-Ctdoc*, *ACO-Acdoc*, and *Cip2* genes into the pUC303 Plasmid. Plasmid pUC303-SOC was prepared using plasmid pUC303. A fragment containing the *trc* promoter fused to the ORFs of ACS-Ctdoc, ACO-Acdoc, and Cip2 derived from pET expression vectors, with 12 nucleotides containing a ribosomal binding site sequence (AGGAAA) between the each of three ORFs, was cloned into *Eco*RI digested plasmid pUC303 by directional cloning method using In-Fusion HD Cloning kits (Takara, Tokyo, Japan). A *trc* promoter, transcriptional terminator, *lac*I gene and a streptomycin-resistant gene obtained from pTrc99A were also inserted along with the operon to achieve inducible gene expression and facilitate antibiotic selection, respectively. Primers used for this construct was listed in Table 1. After transformation of *S. elongatus* PCC 7942 with pUC303-SOC, the transformants were named strain SOC.

Transformation and Selection of Cyanobacteria. *S. elongatus* PCC 7942 cells were transformed as previously described by Kuhlemeier et al.[48] The transformed cells were spread onto BG-11 plates (1.5% agar) containing 20 μg/mL streptomycin and incubated under constant illumination of 3000 lx (i.e., 38 μE m^{-2} s^{-1}) to select for recombinants. Colonies were verified by PCR and inoculated into liquid BG-11 medium containing 20 μg/mL streptomycin for further experiments.

Detection of Recombinant Proteins in Cyanobacteria (Western Blot Analysis). Crude extracts of *S. elongatus* PCC 7942 cells were prepared by sonication of cells in phosphate buffered saline (PBS) buffer. After sonication, the insoluble fraction was removed by centrifugation. The soluble protein fraction was separated on SDS-PAGE and blotted onto PVDF membranes (GE Healthcare) according to standard procedures.[49] The membranes were blocked with 0.4% bovine serum albumin in PBS prior to probing with a 1:500 dilution of the horseradish peroxidase conjugated anti His-tag monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, U.S.A.). Cross-reactions between protein bands and antibodies were visualized with 3,3′-diaminobenzidine.

***In Vivo* Assay of Ethylene-Forming Activity by Cyanobacteria.** A single colony of *S. elongatus* PCC 7942 cells transformed with pUC303-SOC was selected and resuspended in a Roux flask containing 100 mL BG-11 liquid medium containing 20 μg/mL streptomycin. The culture was incubated at 27 °C with aeration (0.5 L/min) using an air pump under continuous exposure to white fluorescent light until the optical density of the culture was approximately $OD_{730\ nm}$ = 1.0. Cells were cultured and the proteins were induced with different concentrations of IPTG. Ten milliliters of the culture were then transferred into a 20 mL syringe vial. Five millimoles of sodium ascorbate were added, the head space of the vial was replaced with CO_2 gas, and the vial was sealed with a gastight screw cap. The culture was then incubated at 27 °C with shaking (115 rpm) on a rotary shaker overnight. Five hundred microliters of gas were extracted from the headspace using a syringe and analyzed with a gas chromatography.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 for the binding experiments with nondenaturing PAGE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Masayoshi Matsuoka for their kind gift of *S. elongatus* PCC 7942 and pUC303 plasmid. We also thank Drs.

Edward A. Bayer and Raphael Lamed for their generous gift of the DNA resources from *C. thermocellum* and *A. cellulolyticus*. This work was supported by the fund for Ministry of Education, Culture, Sports, Science, and Technology (MEXT) supported program for strategic research foundation at private universities, 2008−2012 and The Biorematerial Center Project from the Research Institute of Meijo University, Japan. Additional support was obtained by a grant issued to SJ by Meijo University, Japan.

REFERENCES

(1) Ghanta, M., Darryl, F., and Subramaniam, Bala (2013) Environmental impacts of ethylene production from diverse feedstocks and energy sources. *Appl. Petrochem. Res.*, DOI: 10.1007/s13203-013-0029-7.

(2) Vasant, R., Choudhary, B. S. U., and Shafeek, A. R. Mulla. (1995) Coupling of endothermic thermal cracking with exothermic oxidative dehydrogenation of ethane to ethylene using a diluted SrO/La_2O_3 catalyst. *Angew. Chem., Int. Ed. 34*, 665−666.

(3) Ungerer, J., Tao, L., Davis, M., Ghirardi, M., Maness, P.-C., and Yu, J. (2012) Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium Synechocystis 6803. *Energy Environ. Sci. 5*, 8998−9006.

(4) Rottmann, W. H., Peter, G. F., Oeller, P. W., Keller, J. A., Shen, N. F., Nagy, B. P., Taylor, L. P., Campbell, A. D., and Theologis, A. (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J. Mol. Biol. 222*, 937−961.

(5) S F Yang, a. N. E. H. (1984) Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol. 35*, 155−189.

(6) Albers, E. (2009) Metabolic characteristics and importance of the universal methionine salvage pathway recycling methionine from 5′-methylthioadenosine. *IUBMB life 61*, 1132−1142.

(7) Backlund, P. S., Jr., Chang, C. P., and Smith, R. A. (1982) Identification of 2-keto-4-methylthiobutyrate as an intermediate compound in methionine synthesis from 5′-methylthioadenosine. *J. Biol. Chem. 257*, 4196−4202.

(8) Cone, M. C., Marchitto, K., Zehfus, B., and Ferro, A. J. (1982) Utilization by *Saccharomyces cerevisiae* of 5′-methylthioadenosine as a source of both purine and methionine. *J. Bacteriol. 151*, 510−515.

(9) Marchitto, K. S., and Ferro, A. J. (1985) The metabolism of 5′-methylthioadenosine and 5-methylthioribose 1-phosphate in *Saccharomyces cerevisiae*. *J. Gen. Microbiol. 131*, 2153−2164.

(10) Trackman, P. C., and Abeles, R. H. (1983) Methionine synthesis from 5′-S-Methylthioadenosine. Resolution of enzyme activities and identification of 1-phospho-5-S methylthioribulose. *J. Biol. Chem. 258*, 6717−6720.

(11) Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y., and Fukuda, H. (1991) Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. phaseolicola PK2. *J. Gen. Microbiol. 137*, 2281−2286.

(12) Ducat, D. C., Way, J. C., and Silver, P. A. (2011) Engineering cyanobacteria to generate high-value products. *Trends Biotechnol. 29*, 95−103.

(13) Atsumi, S., Higashide, W., and Liao, J. C. (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. biotechnol. 27*, 1177−1180.

(14) Guerrero, F., Carbonell, V., Cossu, M., Correddu, D., and Jones, P. R. (2012) Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp. PCC 6803. *PloS One 7*, e50470.

(15) Sakai, M., Ogawa, T., Matsuoka, M., and Fukuda, H. (1997) Photosynthetic conversion of carbon dioxide to ethylene by the recombinant cyanobacterium, *Synechococcus* sp. PCC 7942, which harbors a gene for the ethylene-forming enzyme of *Pseudomonas syringae*. *J. Ferment. Bioeng. 84*, 434−443.

(16) Takahama, K., Matsuoka, M., Nagahama, K., and Ogawa, T. (2003) Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbAI locus. *J. Biosci. Bioeng. 95*, 302−305.

(17) Lamed, R., Setter, E., and Kenig, R. (1983) Cellulosomes—A discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding, and various catalytic activities. *Biotechnol. Bioeng. Symp. 13*, 6838299.

(18) Pages, S., Belaich, A., Tardif, C., Reverbel-Leroy, C., Gaudin, C., and Belaich, J. P. (1996) Interaction between the endoglucanase CelA and the scaffolding protein CipC of the Clostridium cellulolyticum cellulosome. *J. Bacteriol. 178*, 2279−2286.

(19) Doi, R. H., Goldstein, M., Hashida, S., Park, J. S., and Takagi, M. (1994) The Clostridium cellulovorans cellulosome. *Crit. Rev. Microbiol. 20*, 87−93.

(20) Kakiuchi, M., Isui, A., Suzuki, K., Fujino, T., Fujino, E., Kimura, T., Karita, S., Sakka, K., and Ohmiya, K. (1998) Cloning and DNA sequencing of the genes encoding *Clostridium josui* scaffolding protein CipA and cellulase CelD and identification of their gene products as major components of the cellulosome. *J. Bacteriol. 180*, 4303−4308.

(21) Felix, C. R., and Ljungdahl, L. G. (1993) The cellulosome: The exocellular organelle of Clostridium. *Annu. Rev. Microbiol. 47*, 791−819.

(22) Jindou, S., Soda, A., Karita, S., Kajino, T., Beguin, P., Wu, J. H., Inagaki, M., Kimura, T., Sakka, K., and Ohmiya, K. (2004) Cohesin-dockerin interactions within and between *Clostridium josui* and *Clostridium thermocellum*: Binding selectivity between cognate dockerin and cohesin domains and species specificity. *J. Biol. Chem. 279*, 9867−9874.

(23) Bayer, E. A., Moraq, E., and Lamed, R. (1994) The cellulosome—A treasure-trove for biotechnology. *Trends Biotechnol. 12* (9), 379−386.

(24) Zhang, Y. H. (2011) Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnol. Adv. 29*, 715−725.

(25) Fierobe, H. P., Mechaly, A., Tardif, C., Belaich, A., Lamed, R., Shoham, Y., Belaich, J. P., and Bayer, E. A. (2001) Design and production of active cellulosome chimeras. Selective incorporation of dockerin-containing enzymes into defined functional complexes. *J. Biol. Chem. 276*, 21257−21261.

(26) Caspi, J., Irwin, D., Lamed, R., Li, Y., Fierobe, H. P., Wilson, D. B., and Bayer, E. A. (2008) Conversion of *Thermobifida fusca* free exoglucanases into cellulosomal components: Comparative impact on cellulose-degrading activity. *J. Biotechnol. 135*, 351−357.

(27) Tsai, S. L., DaSilva, N. A., and Chen, W. (2013) Functional display of complex cellulosomes on the yeast surface via adaptive assembly. *ACS Synth. Biol. 2*, 14−21.

(28) You, C., and Zhang, Y. H. (2013) Self-assembly of synthetic metabolons through synthetic protein scaffolds: One-step purification, co-immobilization, and substrate channeling. *ACS Synth. Biol. 2*, 102−110.

(29) Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene 77*, 51−59.

(30) Li, N., and Mattoo, A. K. (1994) Deletion of the carboxyl-terminal region of 1-aminocyclopropane-1-carboxylic acid synthase, a key protein in the biosynthesis of ethylene, results in catalytically hyperactive, monomeric enzyme. *J. Biol. Chem. 269*, 6908−6917.

(31) Bidonde, S., Ferrer, M. A., Zegzouti, H., Ramassamy, S., Latche, A., Pech, J. C., Hamilton, A. J., Grierson, D., and Bouzayen, M. (1998) Expression and characterization of three tomato 1-aminocyclopropane-1-carboxylate oxidase cDNAs in yeast. *Eur. J. Biochem. FEBS 253*, 20−26.

(32) Noach, I., Levy-Assaraf, M., Lamed, R., Shimon, L. J., Frolow, F., and Bayer, E. A. (2010) Modular arrangement of a cellulosomal scaffoldin subunit revealed from the crystal structure of a cohesin dyad. *J. Mol. Biol. 399*, 294−305.

(33) Kuhlemeier, C. J., Thomas, A. A., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A., and van Arkel, G. A. (1983) A host−vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. *Plasmid 10*, 156−163.

(34) Morais, S., Barak, Y., Caspi, J., Hadar, Y., Lamed, R., Shoham, Y., Wilson, D. B., and Bayer, E. A. (2010) Cellulase−xylanase synergy in designer cellulosomes for enhanced degradation of a complex cellulosic substrate. *mBio 1* (5), e00285-10 DOI: 10.1128/mBio.00285-10.
(35) Amann, E., Brosius, J., and Ptashne, M. (1983) Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene 25*, 167−178.
(36) Huang, H.-H., Casmund, D., Lindblad, P., and Heidorn, T. (2010) Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res. 38*, 2577−2593.
(37) McEwen, J. T., Machado, I. M., Connor, M. R., and Atsumi, S. (2013) Engineering *Synechococcus elongatus* PCC 7942 for continuous growth under diurnal conditions. *Appl. Environ. Microbiol. 79*, 1668−1675.
(38) Foong, F. C., and Doi, R. H. (1992) Characterization and comparison of *Clostridium cellulovorans* endoglucanases−xylanases EngB and EngD hyperexpressed in *Escherichia coli*. *J. Bacteriol. 174*, 1403−1409.
(39) Murashima, K., Chen, C. L., Kosugi, A., Tamaru, Y., Doi, R. H., and Wong, S. L. (2002) Heterologous production of *Clostridium cellulovorans* engB, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. *J. Bacteriol. 184*, 76−81.
(40) Li, N., Jiang, X. N., Cai, G. P., and Yang, S. F. (1996) A novel bifunctional fusion enzyme catalyzing ethylene synthesis via 1-aminocyclopropane1-carboxylic acid. *J. Biol. Chem. 271*, 25738−25741.
(41) You, C., and Zhang, Y. H. (2013) Annexation of a high-activity enzyme in a synthetic three-enzyme complex greatly decreases the degree of substrate channeling. *ACS Synth. Biol.*, DOI: 10.1021/sb4000993.
(42) Williams, J. G. (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in Synechocystis 6803. *Methods Enzymol. 167*, 766−778.
(43) Tartof, K., and Hobbs, C. (1987) Improved media for growing plasmid and cosmid clones. *Focus 9*, 12.
(44) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.
(45) Gill, S. C., and Von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319−326.
(46) Ornstein, L. (1964) Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci. 121*, 321−349.
(47) Davis, B. J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci. 121*, 404−427.
(48) Kuhlemeier, C., and Van Arkel, G. (1987) Host−vector systems for gene cloning in cyanobacteria. *Methods Enzymol. 153*, 199−215.
(49) Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. 76*, 4350−4354.