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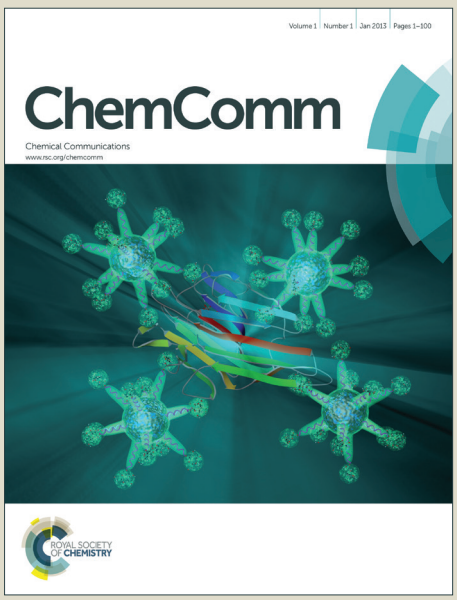

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Efficient Biological Conversion of Carbon Monoxide (CO) to Carbon Dioxide (CO_2) and for Utilization in Bioplastic Production by *Ralstonia eutropha* through the Display of an Enzyme Complex on the Cell Surface

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An enzyme complex for biological conversion of CO to CO_2 was anchored on the cell surface of the CO_2-utilizing *Ralstonia eutropha* and successfully resulted in a 3.3-fold increase in conversion efficiency. These results suggest that this complexed system may be a promising strategy for CO_2 utilization as biological tools for the production of bioplastics.

Although carbon monoxide (CO) is toxic to respiring organisms and is one of the pollutants in the atmosphere, it is also an energy and carbon source for microbes living in extreme environments.[1] The majority of CO is emitted by natural processes, such as atmospheric methane oxidation, natural hydrocarbon oxidation, volcanic activity, production by plants and photochemical degradation of organic matters in water, soil, and marine sediments.[2] Additionally, notable amounts of CO originate from anthropogenic processes, such as the incomplete combustion of fossil fuels and various industrial processes, and are emitted to the atmosphere.[3] Thus, CO can be utilized as a renewable resource with the assistance of CO-conversion enzymes, such as carbon monoxide dehydrogenase (CODH), which catalyzes the oxidation of CO with water to carbon dioxide (CO_2).[4] However, the application of this enzyme as a CO-conversion system remains economically unfeasible, and the development of a more effective and stable enzyme-based CO-conversion system is thus required to achieve the necessary marked reduction in the process costs. We developed a CO-conversion complexed system and immobilized it on the cell surfaces of CO_2-utilizing microorganisms for the consumption of CO as the sole carbon source. Nanoscale materials, such as nanoparticles, nanofibers, mesoporous materials, sol-gel silica and alginate-based microspheres or enzyme crosslinked aggregates (CLEAs) / crystals (CLECs), are conventionally used for immobilization tools.[5-9] In this study, rather than these conventional nanoscale materials, a microbial strain was selected as the support for enzyme immobilization because this approach utilizes the cell surface display technique and has certain advantages, such as its application as a bioactive reaction tool and its relation with the metabolic engineering and synthetic biology research fields.[10] Our methods should provide an effective whole-cell biocatalyst with enhanced CO-conversion efficiency.

The principle of enzyme complexes construction for CO conversion is illustrated in Fig. 1. The dockerin-cohesin interaction of the cellulosome was used to construct the designed system, which consisted of an enzyme complexed with a substrate-binding protein. Anaerobic bacteria, such as *Clostridium cellulovorans*, produce cellulosomes as complexed enzyme systems that involve the binding of the nonenzymatic scaffolding protein CbpA to a variety of cellulolytic subunits via dockerin-cohesin interactions.[11]

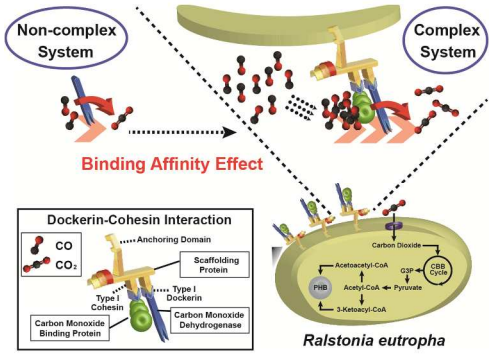

Fig. 1 Illustration of the enzyme complexes for CO conversion based on the dockerin-cohesin interaction of the cellulosome. The CO-conversion unit was based on carbon monoxide dehydrogenase (CooS) with a type I dockerin module. The CO-binding unit containing a carbon monoxide-binding protein (CooA) was fused to the type I dockerin module at the C terminus. The binding and conversion units were designed to connect the scaffolding protein with a cell surface-anchoring domain via the type I dockerin-cohesin interaction derived from the *C. cellulovorans* cellulosome system. The developed enzyme complexes exhibited enhanced CO-conversion efficiency due to a binding-affinity effect.

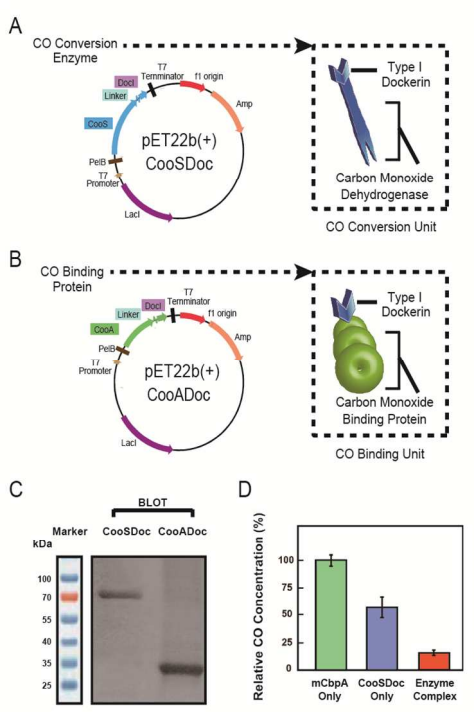

Fig. 2 Construction of plasmids for the CO-conversion complexes and the expression of each protein unit to analyze the conversion efficiency via measurements of the relative CO concentration after the enzyme reactions. The indicated plasmids were used to construct the following strains: (A) *E. coli* (pET22b (+) CooSDoc), and (B) *E. coli* (pET22b (+) CooADoc). PelB, signal peptide; CohI, type I cohesin module; DocI, type I dockerin module. (C) Electrophoretic analysis of each unit by SDS-PAGE and western blot analysis. The protein bands were visualized by western blotting with luminal reagents using a rabbit anti-6x-His antibody and a goat anti-rabbit IgG-HRP antibody as the primary and secondary antibodies, respectively. (D) The CO-conversion efficiencies of the non-complexed (dockerin-fused CooSDoc) and complexed (CO-conversion complexes) systems were compared using same amounts of enzyme. The differences between the two systems reflect the binding-affinity effect through the carbon monoxide-binding protein.

The dockerin-cohesin interaction between enzymes with duplicated sequences (dockerin modules) and a non-catalytic scaffolding protein with repeated sequences (cohesin modules) is a high-affinity protein-protein interaction.[12] Additionally, in an enzyme complex system, a substrate-binding module, such as the carbohydrate-binding module (CBM), of a scaffolding protein plays a crucial role in the effective reaction by facilitating the strong binding of the scaffolding protein to the substrate.[13] Thus, a carbon monoxide dehydrogenase and a carbon monoxide-binding protein were applied as a catalytic enzyme and enhanced substrate for binding, respectively, to construct a CO-conversion complexed system with enhanced efficiency. To assemble the complexed system, both CO-related enzymes were fused with the type I dockerin module and bound to the scaffolding protein mCbpA containing two type I cohesin modules. For whole-cell biocatalyst application, a scaffolding protein fused with a cell-surface-anchoring domain was also developed. To design the CO conversion complexes via the dockerin-cohesin interaction of the cellulosomes, we constructed a chimeric carbon monoxide dehydrogenase from *Acetobacterium woodii* that contained a type I dockerin module from *C. cellulovorans* at the C terminus (CooSDoc unit) as the catalytic enzyme subunit for CO conversion (Fig. 2A). Additionally, to enhance the conversion efficiency by inducing a binding-affinity effect, the carbon monoxide-binding protein from *Rhodospirillum rubrum* was used as the substrate-binding module, similar to the CBM in the cellulosome system. To construct a carbon monoxide-conversion complex with enhanced enzyme efficiency, a chimeric carbon monoxide-binding unit (CooADoc unit) was generated based on the type I dockerin module from *C. cellulovorans* (Fig. 2B). Dockerin-fused protein units were designed to connect the scaffolding protein mCbpA that contained two type I cohesin modules from the *C. cellulovorans* cellulosome system via a type I cohesin-dockerin interaction. The genetic fusion of the type I dockerin module in each protein unit was performed using a multistep PCR strategy with overlapping primers.[14] To fuse the different fragments, each of the overlapping primers possessed a 10-nucleotide-long 5' extension that was complementary to the end of the adjacent fragment. The final PCR products of the CooSDoc and CooADoc units were separated and ligated into the pET22b (+) *Escherichia coli* vector (Novagen, San Diego, CA, USA), resulting in the pET22b (+) CooSDoc and pET22b (+) CooADoc plasmids, respectively. After construction of the plasmids, DH5α and BL21 (DE3) cells were used as hosts for recombinant DNA manipulation and gene expression, respectively.

The expression of the induced subunit proteins in the recombinant *E. coli* strains harboring the pET22b (+) CooSDoc and pET22b (+) CooADoc plasmids was confirmed by SDS-PAGE and western blotting (Fig. 2C). The calculated molecular masses of the CooSDoc and CooADoc subunits were 74.4 kDa and 32.1 kDa, respectively (66.9-kDa CooS and 24.6-kDa CooA plus 7.5-kDa residues for the linker peptide, type I dockerin and His-tag, respectively). A single 32-kDa band and a single 74-kDa band were observed in each lane on the SDS-PAGE gels and corresponded to the molecular sizes predicted by the nucleotide sequences. After the confirmation of the expression of proteins with correct sizes, the enzyme activity was measured to ensure that there was no effect on protein folding due to the dockerin fusion. According to previous research, carbon monoxide dehydrogenase from *A. woodii* requires electron-transferring cofactors for CO conversion reactions.[15] Among electron-transferring molecules, artificial electron acceptors, including methyl viologen, provide a strategy for solving the problem of cofactor regeneration.[16] Moreover, methyl viologen is the preferred artificial electron acceptor for metabolic studies because it has no effect on crucial metabolic pathways.[17] In this study, 20 mM methyl viologen was used as the electron acceptor to substitute for natural electron acceptors, such as rubredoxin and ferredoxin, in all experiments related to the carbon monoxide dehydrogenase reaction. Thus, the utilization of methyl viologen as the electron acceptor do not require an additional supply of any cofactors at any time throughout the reaction of carbon monoxide dehydrogenase-mediated conversion of CO to CO_2. The analysis of enzyme activity revealed that the specific activity of the recombinant dockerin-fused CooSDoc was approximately 480 μmol of CO oxidized min^{-1} mg^{-1}, which is in good agreement with the measured value for the specific activity of same enzyme isolated from *A. woodii*.[15] These results indicated that the dockerin-fused CooSDoc was expressed in an active form and that the dockerin domain at the C-terminus of the enzyme did not interfere with the carbon monoxide dehydrogenase activity.

After the expression of the individual subunits, the formation of the CO-conversion complexes was confirmed through an analysis of the CO-conversion efficiency via measurements of the relative CO concentrations after the enzyme reactions. To demonstrate the effect of complexed system on CO transfer from the binding protein to the catalytic site, CO-occupied CooADoc was directly assembled with one cohesin site of mCbpA, and the conversion of the CO occupying the CooADoc was then analyzed after the additional assembly of CooSDoc. Half-assembled complexes containing only CO-occupied CooADoc remained in the CO-occupied state for long periods. However, as soon as the CooSDoc was assembled with another cohesin site of mCbpA, the CO-occupied state of CooADoc was changed to a CO-emptied state for notably short time. Moreover, CO_2 was detected in the reaction bottle after the addition of CooSDoc. These results demonstrated that CO bound with CooADoc was successfully transferred to the catalytic site of CooSDoc and converted to CO_2 via the carbon monoxide dehydrogenation enzymatic reaction. Compared with the dockerin-fused CooSDoc protein as the non-complexed system, the complexed conversion system exhibited a 3.1-fold increase in CO conversion efficiency (Fig. 2D). These results indicated that the CooSDoc and CooADoc units were assembled into a complex and that the dockerin-fused proteins in the designed CO-conversion complex exhibited enhanced CO-conversion efficiency due to the binding-affinity effect. The enhanced efficiency clearly suggested that the CooSDoc and CooADoc units were all correctly folded and that their high-affinity interactions were sufficient to direct the assembly of the complexes.

After the CO-conversion complexed system was developed, the system was immobilized on the cell surface of CO_2-utilizing microorganisms, such as *Ralstonia eutropha*, to enable the consumption of CO as the sole carbon source. *R. eutropha* is an industrially relevant, facultative chemolithoautotrophic bacterium that is able to grow with organic substrates or CO_2 under aerobic conditions and synthesize poly [(R)-3-hydroxybutyrate] (PHB), a biopolymer that is stored in intracellular granules.[18] *R. eutropha* has type IV pili (T4P), which are polymers of the major pilin protein that are displayed on the surfaces of many Gram-negative bacteria.[19] The type IV pilus biogenesis-stability protein PilF from *R. eutropha* and a green fluorescence protein (GFP) from the jellyfish *Aequorea victoria* were used to attach the protein to the cell surface and to confirm its cell surface display through optical analysis, respectively.

The pET22b (+) PilF GFP plasmid containing the anchoring module of PilF fused with the GFP at the C terminus. The nucleotide sequence of the PilF GFP-coding region is 1545 bp and encodes a polypeptide of 515 amino acids (Fig. 3A). The pET22b (+) PilF GFP plasmid was transformed into *E. coli* BL21 (DE3) cells, and the resulting recombinant strain was named *E. coli* (pET22b (+) PilF GFP). After the expression and purification of the PilF GFP protein from *E. coli* (pET22b (+) PilF GFP) as mentioned above, the purified PilF GFP protein exhibited fluorescence at 488 nm. Additionally, PilF was used as the anchoring protein for the attachment of the scaffolding protein mCbpA to the cell surface of *R. eutropha* to immobilize the CO-conversion complexes. PilF was fused at the N terminus of mCbpA, and this chimeric gene (PilF mCbpA) of 2472-bp-nucleotide was inserted into the pET22b (+) vector, resulting in the pET22b (+) PilF mCbpA vector (Fig. 3B).

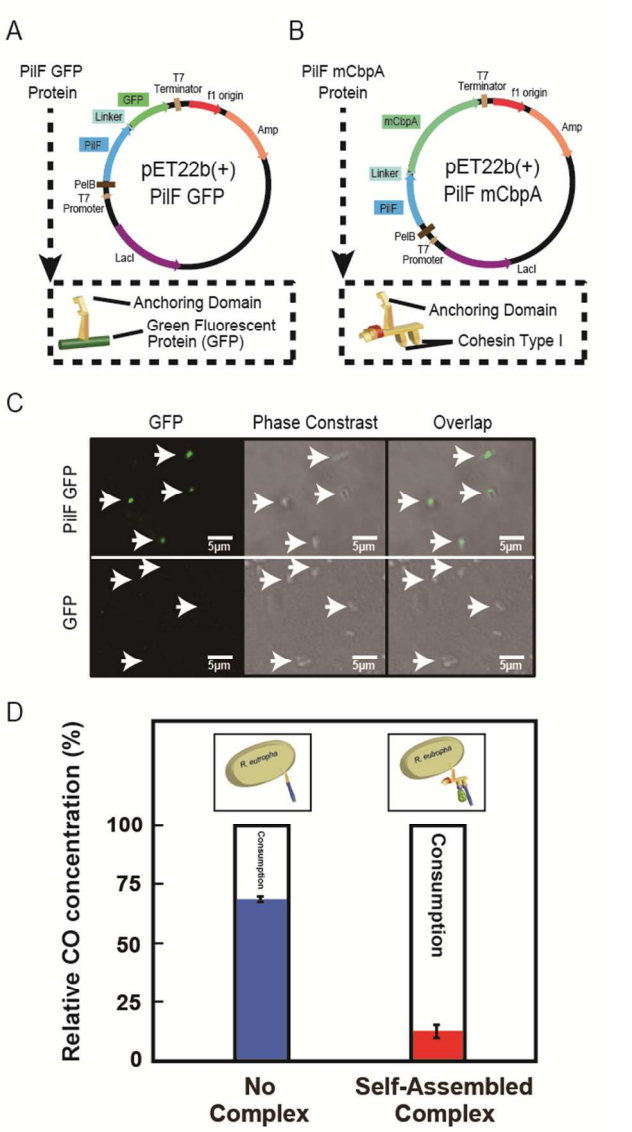

Fig. 3 Construction of plasmids for the expression of cell surface-anchoring proteins and the display of the proteins on the cell surfaces of *R. eutropha*. The indicated plasmids were used to construct the strains as follows: (A) *E. coli* (pET22b (+) PilF GFP), and (B) *E. coli* (pET22b (+) PilF mCbpA). PelB, signal peptide; GFP, green fluorescent protein. (C) Fluorescence micrographs of the surface-displayed anchoring protein. Green fluorescence protein (GFP) was used as a fluorescence tag. The displayed anchoring protein was fused with GFP at the C terminus. The images of the green fluorescent probes on the cell surface of *R. eutropha* strains were obtained with the GFP-fused anchoring protein. The images of *R. eutropha* cells were obtained under transmitted light (phase contrast). (D) The CO conversion efficiencies of the non-complexed anchoring system and the cell-surface-displayed complexed system were compared (the systems were analyzed at the same cell density). The differences between the non-complexed and complexed results reflect the binding-affinity effect through the pull down of the substrates. The analysis was recorded by comparing the results obtained with PilF CooS anchored in *R. eutropha* (blue) and the CO-conversion system displayed on *R. eutropha* (red).

The attachment of the anchoring protein on the cell surface of *R. eutropha* was observed by fluorescence microscopy (Fig. 3C). Only the green fluorescence-labeled anchoring protein PilF GFP appeared on the cell surface on phase-contrast images. These results indicate that the anchoring module PilF was displayed on the same cell surface, that the fused protein was expressed in a non-degraded, active form and that the fusion of the different domains at the C or N terminus did not interfere with the correct folding of the proteins. Because the chimeric PilF GFP-anchoring protein was displayed on the cell surface, the PilF-fused scaffolding protein (PilF mCbpA) was also successfully attached to the cell surface of *R. eutropha*. Thus, the CO-conversion complexes based on PilF mCbpA on the cell surface of *R. eutropha* play a potential role as a whole-cell biocatalyst for the consumption of CO as the sole carbon source. The display of enzyme complexes on industrial strains was studied to develop a whole-cell biocatalyst that exhibits enhanced enzyme efficiency based on the effects of the proximity of the enzyme to the cell surface.[20] In this study, the displayed CO-conversion complexes exhibited greater conversion efficiency with CO as the sole carbon source than the non-complexed anchoring enzyme (PilF CooS). The enhanced conversion efficiency values of the displayed CO-conversion complexes were 3.3-fold higher compared with the CO consumption level of the non-complexed system (Fig. 3D). The introduction of the CO-conversion complexes to the cell surface resulted in enhanced CO-conversion efficiency. These data indicate that the enzyme complex-displaying technology significantly increased the CO-conversion efficiency of *R. eutropha*.

Conclusions

We constructed a CO-conversion system consisting of a complex between one mCbpA scaffolding protein and two carbon monoxide-related proteins, such as CooSDoc and CooADoc. Additionally, this complex was attached to the cell surface of *R. eutropha* to develop a whole-cell biocatalyst. The designed cell-surface-displayed CO-conversion system successfully increased the conversion efficiency of CO through the substrate-binding-affinity effect. The carbon monoxide-binding protein in the CO-conversion complex brought CO, which acted as the substrate of the carbon monoxide dehydrogenase, near the cell surface, and the CO_2 converted from CO was then consumed by the CO_2-utilizing strains, such as *R. eutropha*, as a sole carbon source through facultative chemolithoautotrophic metabolism. *R. eutropha* served as a useful living support for enzyme immobilization because this strain already has the sophisticated metabolism to synthesize PHB. This thermoplastic is biodegradable and biocompatible thermoplastic and can be made into films, fibers, and sheets and can even be molded into the shapes of bags and bottles. This successfully developed microbial cell display technique could easily be linked with metabolic engineering or synthetic biology research to produce more valuable products. These results suggest that the developed whole-cell biocatalyst for CO conversion may be a promising strategy for utilizing CO as a renewable resource.

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Notes and references

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