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Engineering of *Clostridium thermocellum* for Heterologous Gene Expression and Secretion

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Abstract

Recent studies have shown that a cellulolytic bacterium, *Clostridium thermocellum*, can be genetically modified. However, strong expression of heterologous genes and secretion of their proteins in this organism are difficult. In the current study, we report that a gene expression cassette including the cellobiose phosphorylase gene promoter, the hypoxanthine phosphoribosyl transferase gene terminator from *C. thermocellum*, and the ribosome-binding site and histidine-tag sequence from pQE30, led to the expression of a heterologous gene: aldehyde deformylating oxygenase. Additionally, fusion of the signal peptide and cohesin domain of a cellulosome-scaffolding protein was sufficient to cause extracellular secretion of aldehyde deformylating oxygenase.

Key Words: Biorefinery, *Clostrdium thermocellum*, heterologous gene expression, protein secretion

Introduction

Biorefinery, the production of fuels, high value chemicals, and materials from cellulosic biomass, is hoped to relieve the problems caused by the use of petroleum (i.e., global warming and the depletion of fossil resources)[1)]. Recent synthetic biology approaches have developed microorganisms that produce various fuels, high value chemicals, and materials from glucose[2)]. Economical glucose production from cellulosic biomass is therefore essential for successful biorefinery. Lignocellulose-degrading enzymes are used to convert cellulosic biomass into glucose; however, the efficiency of the enzymatic degradation process is low[3)].

The *Clostridia* are anaerobic soil bacteria, and some species can degrade cellulosic biomass without supplementation of lignocellulose-degrading enzymes[4, 5)]. Heterologous genes have been expressed in the *Clostridia* to enhance their capacity to degrade lignocellulose. For example, *Clostridium cellulolyticum* engineered with the Cel9A gene from *Clostridium phytofermentans* degraded pure cellulose 1.5 times faster than the wild type strain[6)]. Prawitwong *et al.* reported that *Clostridium thermocellum* could be used to produce glucose from cellulosic biomass[7)]. In this study, to enhance the lignocellulose degradation activity of *C. thermocellum*, we sought to construct gene expression cassettes for heterologous gene expression and secretion in *C. thermocellum*. To date, there have been no reports on the secretion of heterologous lignocellulose degrading enzymes from *C. thermocellum*. Ichikawa and Karita reported that the expression level of the heterologous gene aldehyde-deformylating oxygenase (ADO) from *Synechococcus elongatus* was

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higher than that of the other heterologous genes, the fatty acyl acyl carrier protein reductases[8]. The amount of the ADO protein expression was sufficient to detect via western blot analysis. Therefore, we chose the ADO gene as a model gene for the construction of gene expression cassettes for heterologous gene expression and secretion in *C. thermocellum*.

Materials and Methods

Strains and growth conditions

C. thermocellum DSM 1313 was cultured anaerobically in CTFUD medium with 0.5% cellobiose at 55℃ [9]. Transformed *C. thermocellum* cells were cultured in CTFUD medium supplemented with 6 μg mL^{-1} of thiamphenicol. *Escherichia coli* BL21(DE3) (TOYOBO) was cultured in Luria broth supplemented with 20 μg mL^{-1} of chloramphenicol at 37℃.

Plasmid construction

The ADO gene from *S. elongatus* PCC 7492 (WP 011378104.1) was isolated by polymerase chain reaction (PCR), and ligated into pMU102, containing an origin of replication that functions in *C. thermocellum*[9, 10]. Then, the terminator region of the hypoxanthine phosphoribosyl transferase gene from *C. thermocellum* was inserted downstream of the ADO gene[11]. The promoter region of the cellobiose phosphorylase gene from *C. thermocellum* and the signal peptide sequence of the CipA gene from *C. thermocellum* were inserted into the upstream region of the ADO gene to construct "Sig-ADO-His" plasmid DNA (Fig. 1).

The CipA signal peptide sequence was replaced with the ribosome-binding site and the histidine-tag sequence from pQE30 (QIAGEN) to construct "His-ADO" plasmid DNA (Fig. 1). The DNA fragment,

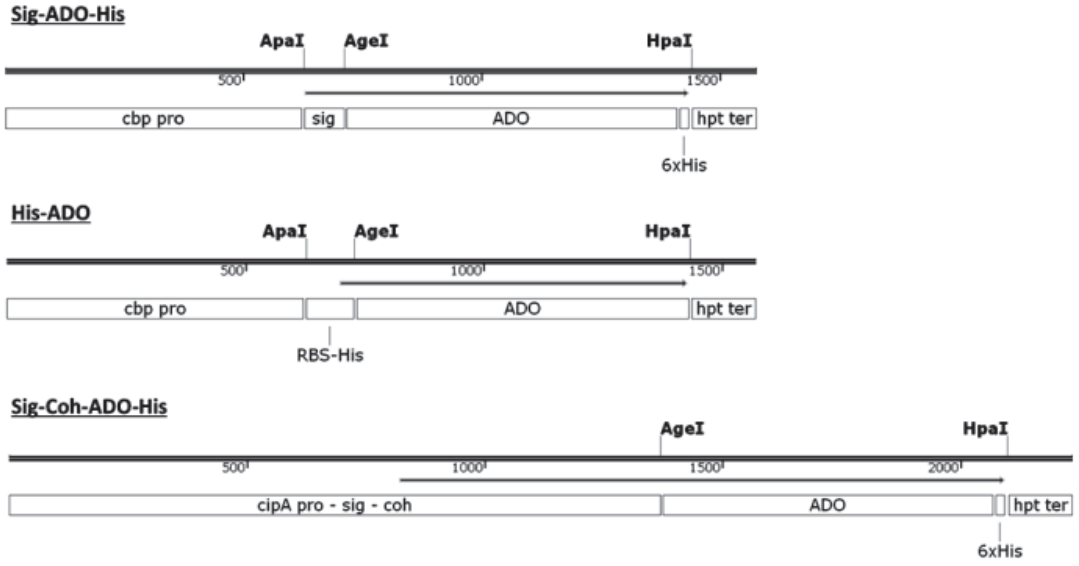

Fig. 1 Gene expression cassettes for ADO gene expression in *C. thermocellum*

ADO: aldehyde deformylating oxygenase gene from *S. elongatus*. cbp pro: promoter of the cellobiose phosphorylase gene from *C. thermocellum*. sig: signal peptide sequence of the CipA gene from *C. thermocellum*. 6xHis: histidine tag sequence. hpt ter: terminator of the hypoxanthine phosphoribosyl transferase gene from *C. thermocellum*. RBS-His: ribosome-binding sites and histidine tag sequence from pQE30. cipA pro - sig - coh: CipA gene promoter and a part of the CipA gene from the signal peptide sequence to the cohesin domain. These DNA fragments were inserted into the vector pMU102 at the restriction site of SphI and XhoI. Bold characters show the restriction sites used to construct these plasmids. Arrows indicate the regions of coding sequences in these plasmids. Numerals show the length of the DNA fragments.

including the promoter, signal peptide sequence, and cohesin domain of the CipA gene, was inserted instead of the promoter region and the signal peptide sequence of the Sig-ADO-His plasmid DNA to construct "Sig-Coh-ADO-His" plasmid DNA (Fig. 1). The primers used in this study are listed in Table 1.

Transformation of *C. thermocellum*

The plasmids were constructed and purified from *E. coli* BL21 (DE3)[12]. *C. thermocellum* DSM 1313 was cultivated, and the culture broth was centrifuged at 6,500 × g for 12 min at 4℃. The supernatant was discarded, and the pellet was washed three times with autoclaved pure water. The washed cells were suspended in 100 μL of autoclaved pure water. A total of 20 μL of cell suspension and 1 μg of plasmid DNA were mixed in a 1 mm electroporation cuvette. A square electrical pulse was applied using a Gene Pulser Xcell Electroporation System (Bio-Rad). The voltage and pulse length were 1500 V and 1.5 ms, respectively. The pulsed cells were suspended in 5 mL of CTFUD medium with 0.5% cellobiose. The cells were incubated at 51℃ in a dry bath incubator for 18 h. A total of 100 μL of the recovered cell culture was plated onto CTFUD solid medium containing 1% agarose and 6 μg mL^{-1} of thiamphenicol. The cells were incubated at 55℃ for 3–5 days[9]. The transformed cells were isolated and cultured in CTUFD medium with 0.5% cellobiose and 6 μg mL^{-1} of thiamphenicol.

Protein expression analysis

Expression of the heterologous proteins in transformed *C. thermocellum* strains were analyzed via western blot. The transformed *C. thermocellum* strains were cultured in CTFUD with 6 μg mL^{-1} of thiamphenicol at 55℃ to an OD_{600} = 0.8. Equal volumes of the culture broths including cells and the sample preparation solution (1% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol

Table 1 Primers used in this study

Isolated region	Sequence (5'->3')	Template
cbp pro	TAA**GCATGC**GAGTCGTGACTAAGAACGTCAAAGTAA	Genomic DNA of *C. thermocellum*
	TAA**GGGCCC**CTTTCGTCCTCCTTAAAATTTTCG	
sig	ATT**GGGCCC**ATGAGAAAAGTCATCAGTATGCTCTTAGT	
	AGT**ACCGGT**CGCCGATACTGTCTGCG	
hpt ter	TAA**GTTAAC**AAAATATACAAAGGTTTCTTGTGTTTTTAATAC	
	TAA**GTCGAC**TTCAACTAGTTCCCTCCTCATTCTT	
cipA pro - sig - coh	TAA**GCATGC**TAATAATTTCATTGCAAATAGTAATTTTGTTAGTA	
	TAA**ACCGGT**ACCGTCTGACGGAACATTTG	
ADO	ATT**ACCGGT**ATGCCGCAGCTTGAAGC	Genomic DNA of *S. elongatus*
	ATT**AGATCT**AACGGCCGCAAGG	
	ATT**GTTAAC**TCAAACGGCCGCAAGG	
RBS-His	ATTCCCGGGATT**GGGCCC**AATTATAATAGATTCAATTGTGAGCGGA	pQE30
	ATT**ACCGGT**GTGATGGTGATGGTGATGCG	

The names of the isolated regions are linked to those of the DNA fragments in Fig. 1. The restriction sites are indicated by bold characters.

blue, and 62.5 mM Tris-HCl pH 6.8) were mixed and boiled for 5 min. Eight microliters of the solutions were applied to SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes using a CompactBLOT apparatus (ATTO). Protein expression was detected using an anti-His-tag antibody conjugated with horseradish peroxidase (HRP) (QIAGEN) and chemiluminescence regents, e.g., Chemi-Lumi One L (Nacalai Tesque) and Luminata Forte Western HRP substrate (Merck Millipore).

Results and Discussion

In this study, we sought to construct gene expression cassettes for heterologous gene expression and extracellular protein secretion from *C. thermocellum*. Sig-ADO-His, the ADO gene fused with the signal peptide sequence of the CipA gene, was expressed under the cellobiose phosphorylase gene promoter (Fig. 1). Olson *et al*. reported that the expression levels of the heterologous genes LacZ and AdhB, under the cellobiose phosphorylase gene promoter, were the highest compared with the other promoters they tested. However, in the present study, it was difficult to detect the Sig-ADO-His protein in the engineered *C. thermocellum* strain [13] (Fig. 2).

To increase the expression level of the ADO gene, the signal peptide sequence was replaced with the ribosome-binding site and the histidine-tag sequence from pQE30 [8] (Fig. 1). The expression of the His-ADO protein was detected more obviously via western blot analysis than Sig-ADO-His (Fig. 2). This indicated that the cellobiose phosphorylase gene promoter fused with the ribosome-binding site could lead to the expression of a heterologous gene, but the presence of the ribosome-binding site was expected in the cellobiose phosphorylase gene promoter [13].

Transcriptomic analyses showed that the expression level of the CipA gene was higher in *C. thermocellum* [14, 15].

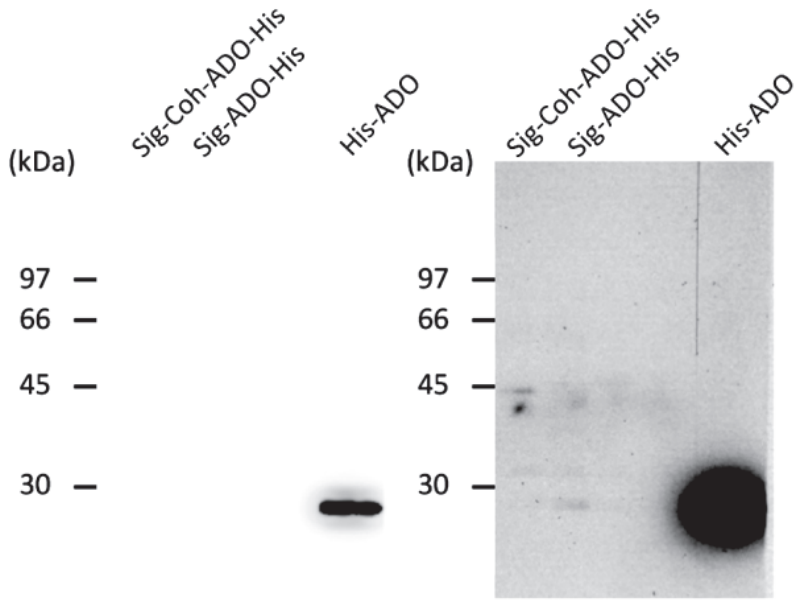

Fig. 2 ADO protein expression in the engineered *C. thermocellum* strains

Eight microliters of the sample solutions prepared from the culture broths including the engineered cells were analyzed by western blot to evaluate the expression level of the ADO protein (see Materials and Methods). The ADO protein was detected with Chemi-Lumi One L (Nacalai Tesque). The exposure times were 5 and 60 min in the left and right panels, respectively. The molecular weights of Sig-Coh-ADO-His, Sig-ADO-His, and His-ADO were 46.6, 30.6, and 27.8 kDa, respectively.

Thus, it is speculated that the CipA gene promoter may have higher promoter activity, similar to the cellobiose phosphorylase gene promoter. In the case of *Clostridium acetobutylicum*, the Cel48F or Cel9G gene from *C. cellulolyticum* fused with the signal peptide, and the family 3a carbohydrate-binding module of the cellulosome scaffolding protein CipC, was successfully expressed and secreted extracellularly[16]. The CipA gene was successfully overexpressed under the CipA gene promoter and the secreted CipA protein exhibited a 2.5-fold increase in *C. thermocellum*[17]. Therefore, it is expected that a heterologous gene product, fused with a signal peptide and a carbohydrate-binding module or a cohesion module, may be secreted extracellularly. In this study, the ADO gene (fused with part of the CipA gene, the signal peptide and the cohesin module) was expressed under the CipA gene promoter (Fig. 1). Sig-Coh-ADO-His was only slightly detected in the engineered *C. thermocellum*, and the expression level was significantly lower than that of His-ADO (Fig. 2).

There have been no reports on extracellular secretion of heterologous gene products from *C. thermocellum*. Because the Sig-Coh-ADO-His protein contains a signal peptide, it was expected that it would be secreted into the culture broth from the engineered cells. The culture broths of the engineered *C. thermocellum* strains were centrifuged and the supernatants were analyzed by western blot. The His-ADO protein was not detected in the supernatant of the culture broth, while the Sig-Coh-ADO-His protein was detected (Fig. 3). This result indicates that the fusion of the CipA signal peptide and the cohesin domain was sufficient to cause secretion of the ADO protein.

In this study, we sought to construct gene expression cassettes for heterologous gene expression and protein secretion in *C. thermocellum*. We showed that the expression level of the heterologous gene, aldehyde deformylating oxygenase, was highest with the gene expression cassette including the cellobiose

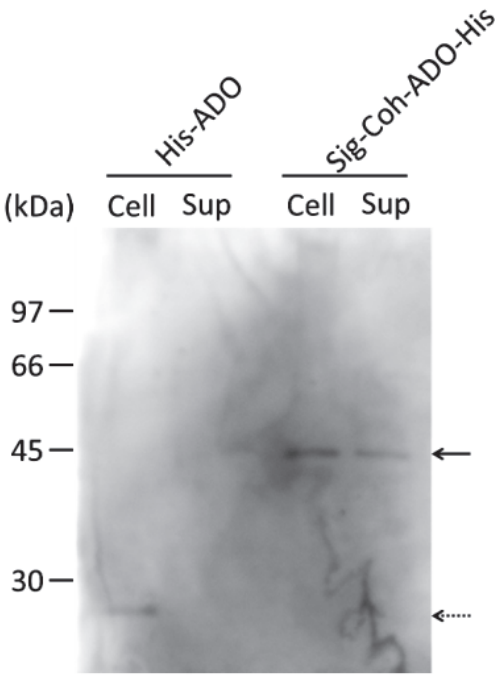

Fig. 3 Secretion of the recombinant proteins from the engineered *C. thermocellum*

Eight microliters of the sample solutions prepared from the culture broths, including the engineered cells (Cell) or the supernatants of the culture broths after centrifugation (Sup), were analyzed by western blot to evaluate the expression level of the *ADO* gene (see Materials and Methods). The objective proteins were detected with Luminata Forte Western HRP substrate (Merck Millipore). The exposure time was 4 minutes. Solid and dotted arrows indicate Sig-Coh-ADO-His (46.6 kDa) and His-ADO (27.8 kDa), respectively. Note that Sig-Coh-ADO-His was detected in the supernatant of the culture broth.

phosphorylase gene promoter and the ribosome-binding site from pQE30. We also demonstrated that ADO fused with the signal peptide and the cohesin domain of the cellulosome scaffolding protein and was successfully secreted extracellularly.

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Clostridium thermocellum による異種遺伝子発現と発現タンパク質の分泌

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要　旨

セルロース系バイオマス分解細菌 *Clostridium thermocellum* の異種遺伝子発現事例はいくつか報告されているが，発現タンパク質の分泌は達成できていない。私たちは，*C. thermocellum* ゲノム DNA 由来のセロビオースホスホリラーゼ遺伝子のプロモーター，ヒポキサンチンホスホリボシルトランスフェラーゼ遺伝子のターミネーター，プラスミド pQE30 由来のリボソーム結合部位，ヒスチジンタグからなる遺伝子発現カセットによって，アルデヒドデホルミレーティングオキシゲナーゼ遺伝子を *C. thermocellum* で発現させることができることを明らかにした。加えて，*C. thermocellum* のセルロソーム骨格タンパク質のシグナルペプチドとコヘシンを付与することによって，発現したアルデヒドデホルミレーティングオキシゲナーゼを細胞外へ分泌させることができることを示した。