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Site-directed mutagenesis of bacterial cellulose synthase highlights sulfur–arene interaction as key to catalysis

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ABSTRACT

Cellulose is one of the most abundant biological polymers on Earth, and is synthesized by the cellulose synthase complex in cell membranes. Although many cellulose synthase genes have been identified over the past 25 years, functional studies of cellulose synthase using recombinant proteins have rarely been conducted. In this study, we conducted a functional analysis of cellulose synthase with site-directed mutagenesis, by using recombinant cellulose synthase reconstituted in living *Escherichia coli* cells that we recently constructed (cellulose-synthesizing *E. coli*, CESEC). We demonstrated that inactivating mutations at an important amino acid residue reduced cellulose production. In this study, an interesting loss-of-function mutation occurred on Cys308, whose main chain carbonyl plays an important role for locating the cellulose terminus. Mutating this cysteine to serine, thus changing sulfur to oxygen in the side chain, abolished cellulose production in addition to other apparent detrimental mutations. This unexpected result highlights that the thiol side-chain of this cysteine plays an active role in catalysis, and additional mutation experiments indicated that the sulfur–arene interaction around Cys308 is a key in cellulose-synthesizing activity. Data obtained by CESEC shed light on the function of cellulose synthase in living cells, and will deepen our understanding of the mechanism of cellulose synthase.

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1. Introduction

Cellulose is a structural polysaccharide produced by plants, algae, protists, bacteria, and animal tunicates, and one of the most abundant biological polymers on Earth. Cellulose biosynthesis is actually a two-step reaction of (i) polymerization of β1→4-glucan and (ii) crystallization of the resulting polymers (microfibril formation). The enzyme responsible for cellulose biosynthesis is cellulose synthase, which is embedded in the lipid bilayer of cell membranes and is believed to be a heterosubunit complex. Therefore, it has been a difficult protein to analyze, and our biochemical understanding of cellulose biosynthesis remains limited.

The gene encoding the catalytic subunit of cellulose synthase is *cesA* [1,2]. The CesA protein is a glycosyltransferase of the GT-2 family according to the CAZy database. Other polysaccharide synthases are present in the GT-2 family including chitin synthase, β1→3-D-glucan synthase, mannan synthase, and hyaluronan synthase. Hydrophobic cluster analysis of amino acid sequences in an earlier study clarified that these β-glycosyltransferases have two domains (domains A and B), which contain D,D and D,QxxRW sequences, respectively [3]. The function of these highly conserved motif sequences was discussed in that study, for example, domain B was proposed to be important for chain elongation since it was found only in polysaccharide synthases. However, only a few studies [4–6] directly showed that these amino acid residues are involved in polysaccharide synthesis.

Recently, the X-ray crystallographic structure of cellulose synthase was reported from the minimally required subunits of bacterial cellulose synthase, CesA and CesB, of the purple bacterium *Rhodobacter sphaeroides* [7–9]; hereafter, we use the terminology proposed by Delmer (CesA) [1], instead of the original name (BcsA),

Abbreviations: DGC, diguanylate cyclase; c-di-GMP, cyclic-di-guanosine monophosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; FTIR, Fourier-transformed infrared spectroscopy; ATR, attenuated total reflection; PCR, polymerase chain reaction; DP, degree of polymerization.

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which was also used other recent studies [7–9]. This is the first three-dimensional structure determination, not only of cellulose synthase, but also for a GT-2 enzyme carrying domain B. This structural model clearly indicates that the two aforementioned aspartic acid residues in domain A are involved in the interaction with the UDP (uridine diphosphate) moiety of the substrate UDP-glucose, and the aspartic acid in domain B functions as the catalytic base in the glucosyltransfer reaction. The famous QxxRW motif in domain B, located in an interfacial α-helix (IF2) running on the inner surface of the lipid bilayer, plays an important role for keeping acceptor cellulose molecules in the correct position for the glucosyltransfer. In addition to these well-known motifs, the structural model of cellulose synthase indicated other important motifs in CesA, such as QTPH and FFCGS. Both of these are close to the acceptor cellulose chains and are proposed to play an important role in glucosyltransfer or chain translocation.

Despite such outstanding structural models that tell us new information about the mechanism of cellulose synthase, it is still important to test the molecular mechanism of cellulose synthase deduced from the X-ray crystallographic structure by observing its enzymatic activity. Site-directed mutagenesis is a clear way to evaluate the role of each amino acid residue in cellulose synthase. Although several previous studies have surveyed the effect of mutating the D,D,D,QxxRW motif in cellulose synthase [5] and chitin synthase [4,6], the mutations in these studies were actually designed based on amino acid sequence alignment [3].

In this study, we designed site-directed mutagenesis of the bacterial CesA protein with the aid of the recently published structural model of the RsCesAB complex [7–9]. Then, we determined the cellulose-synthesizing activity for each of these mutants by expressing bacterial cellulose synthase in recombinant *Escherichia coli*, which is designated as "CESEC" (cellulose-synthesizing *E. coli*) [10]. Our results were consistent with these structural studies, and further suggest that the sulfur–arene interaction around the cysteine residue in the FFCGS motif is key for CesA protein function.

2. Experimental procedures

2.1. Bacterial strains and plasmids

CESEC [10] was used for assaying recombinant cellulose synthase enzymatic activity. Briefly, the *E. coli* strain XL1-Blue was used for expressing cellulose synthase derived from *Gluconacetobacter xylinus* JCM9730 (GxCesA and GxCesB, the minimal requirement for cellulose-synthesizing activity in bacteria). The *gxcesA* and *gxcesB* genes, which are in two sequential open reading frames in the same operon, were inserted into a pQE-80L vector (Qiagen Inc.) for their expression. In addition, DGC (diguanylate cyclase) was expressed together with cellulose synthase. Diguanylate cyclase is an enzyme that synthesizes c-di-GMP (cyclic-di-guanosine monophosphate), an activator of bacterial cellulose synthase [11,12]. The *dgc* gene of *Thermotoga maritima* MSB8, TM1788 [13], was inserted into a pBAD33 vector for expression as a thioredoxin-fused protein. These two vectors were introduced into *E. coli* by chemical transformation, and the transformed *E. coli* were selected using the antibiotics ampicillin and chloramphenicol, resistance to which was induced by the pQE-80L and pBAD33 vectors, respectively.

2.2. Site-directed mutagenesis

A series of site-directed mutagenesis experiments were performed on the *gxcesA* gene in a sub-cloning vector pGEM-T easy (Promega Inc.) using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies Inc.), according to the manufacturer's protocol. DNA sequences were verified for the desired mutagenesis, and then the mutated sequence was cut out at the endogenous restriction sites (*Eco*RI sites for D188H, D188N, D189N, and D189Y, and *Bsr*GI and *Sac*II sites for the others). The expression vector containing cellulose synthase (GxCesA and GxCesB) was digested at the same restriction sites for each mutant. Mutated *cesA* DNA fragments were then ligated to the expression vector by using a DNA Ligation kit LONG (Takara Bio Inc., Japan). The ligated plasmid DNA was amplified in *E. coli* HST08 (Takara Bio Inc.) to prepare the expression vector of cellulose synthase containing a particular point mutation. The orientation of the ligated DNA fragment was verified by colony-direct PCR for D188H, D188N, D189N, and D189Y.

2.3. Expression of recombinant proteins

CESEC cells were cultured in 2 × YT medium supplemented with 12.5 μg/mL tetracycline, 100 μg/mL ampicillin, and 50 μg/mL chloramphenicol at 37 °C with orbital shaking at 190 rpm. Protein expression of cellulose synthase and DGC was induced with 0.4 mM IPTG and 0.2% L-arabinose, respectively, when the OD_{600} reached 0.5–0.7. The culture was then maintained with orbital shaking at 28 °C prior to harvesting by centrifugation. The wild type CesA was expressed alongside mutant proteins to compare protein expression and cellulose production for each mutant protein.

2.4. Western blot analysis

Expression of GxCesA and GxCesB was analyzed by SDS-PAGE and western blotting. Cultured cells were collected by centrifugation at 4 °C, and then incubated in SDS-PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 0.5% SDS (sodium dodecyl sulfate), 2% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) at 4 °C for at least 3 h. Proteins included in the cells in 24 μL culture medium were analyzed with a precast 10–20% polyacrylamide gel (SuperSep Ace, Wako Pure Chemicals Industries Ltd., Japan) alongside a molecular weight marker (Precision Plus Protein All Blue Standards, Bio-Rad Inc.), and transferred onto PVDF membranes (Immobilon-P, Millipore Inc.). The membrane was then incubated with a primary antibody against either GxCesA or GxCesB. The antibodies used were described in our previous studies [10,14] (a polyclonal antibody against a synthetic peptide corresponding to the carboxyl terminus of GxCesA and a loop in the CBD2 domain of GxCesB protein [7], respectively). Membranes were then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (Promega Inc.). Bands of CesA and CesB protein were visualized by a chemiluminescence method to quantitatively evaluate the expression of CesA and CesB proteins. ECL Select (GE Healthcare Inc.) was used for the luminescence reaction and digital images were taken by AE-9300H EZ-Capture MG (ATTO Inc., Japan). The intensity of CesA and CesB bands, which represents the amount of protein in the cells included in 24 μL of culture medium, was determined by ImageJ. Expression of GxCesA and GxCesB protein in each mutant cellulose synthase was evaluated as a percentage relative to wild type, hereafter referred to as the relative expression level.

To verify the correct expression of GxCesA and GxCesB protein, we examined samples prepared by alkaline fractionation [15], which separates the membrane-spanning proteins from other proteins. Briefly, cells treated by lysozyme were solubilized in 0.1 N NaOH solution on ice for 30 min and ultracentrifuged at 100,000 × g for 30 min. The pellet, containing membrane proteins, was washed with 5% trichloro acetic acid (TCA), whereas the proteins in the supernatant, including unfolded proteins and soluble proteins, are precipitated by adding TCA in a final concentration of 10%. These precipitated protein samples were air-dried after washing with cold acetone, and then were dissolved in 1% SDS,

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