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Efficient surface-display of autotransporter proteins in cyanobacteria

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ABSTRACT

The autotransporter protein antigen 43 from *Escherichia coli* was recombinantly expressed in the cyanobacterium *Synechocystis* sp. PCC6803, under the regulation of the green light-inducible promoter P_{cpcG2}. Antigen 43 expression was induced in response to green light, resulting in the constant accumulation of the protein for at least four days. The complete post-translational processing of the antigen 43 protein was confirmed by Western blot analysis. The resulting antigen 43 α chain was essentially all displayed on the cell surface, as confirmed by proteinase K treatment. Although microbial cell-surface-display technology has a wide range of biotechnological applications, this is the first report of an efficient method for the display of recombinant proteins in cyanobacteria.

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1. Introduction

Cyanobacteria are photosynthetic Gram-negative bacteria that play an important role in renewing the atmospheric oxygen and supporting the food web. The ability of cyanobacteria to grow in simple media and use sunlight to capture carbon dioxide has also made them an increasingly attractive target for industrial bioprocess applications. These applications include for the production of biofuels, such as alkane [1,2], butanol [3,4], isobutyraldehyde [5], and fatty acids [6], or for the production of other chemicals, such as polyhydroxybutyrate [7] and isoprene [8]. Although a number of researchers have successfully engineered cyanobacteria for various applications, there remains a need for tools to effectively display recombinant proteins on the surface of cyanobacterial cells. This can have a number of potential applications, such as for mobilizing cells on specific surfaces, inducing self-aggregation [9], displaying specific enzymes for extracellular bioconversions [10], or displaying specific ligands to capture valuable or toxic compounds from the environment [11].

Displaying recombinant proteins to the outer surface of cyanobacterial cells can be particularly challenging, considering the cells' complex internal thylakoid membrane system and their thick peptidoglycan layer. Attempts to use the truncated (containing only the amino and carboxyl transmembrane domains) ice nucleation protein INPNC from *Pseudomonas syringae* as an anchoring factor resulted in only a small fraction of the expressed protein being displayed on the surface of the cyanobacterium *Synechococcus* PCC 7942 [12,13]. Fusion with the *Synechococcus* outer membrane protein A (SomA), a transmembrane porin, resulted in only 25% of the expressed recombinant protein being digested when whole cells were treated with proteinase K, indicating that most of the expressed target proteins were not displayed on the cyanobacterial cell surface [14].

As an alternative approach to display proteins on the cell surface, we considered autotransporter proteins from Gram-negative bacteria. After crossing the inner cytoplasmic membrane via the Sec machinery, the transporter domain of a typical autotransporter forms a β-barrel structure in the outer membrane, through which the passenger domain is secreted to the outer surface [15]. One relatively well characterized autotransporter protein is the *Escherichia coli* antigen 43 (Ag43), which is involved in biofilm formation and cell–cell interaction [16,17]. After display on the outer surface, the passenger domain of Ag43 (α chain) is autocatalytically cleaved, but remains noncovalently attached to the integral membrane transporter β chain [18,19]. Ag43 has been used for the display of short peptides and an entire protein domain on the surface of *Salmonella* cells [20] and for the display of a functional enzyme on the surface of *E. coli* [21,22]. Because of the potential usefulness of Ag43, we set out to determine whether Ag43 can navigate through the unique cyanobacterial cell wall and be effectively displayed on the cell surface.

Abbreviations: Ag43, antigen 43; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline with Tween.

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2. Materials and methods

2.1. Plasmids and strains

All gene constructs were created by standard DNA manipulations using *E. coli* DH5α as the host. The *flu* gene, encoding antigen 43, was amplified by PCR from *E. coli* K12 and its nucleotide sequence confirmed. Six internal *Pst*I restriction endonuclease sites were disrupted by site-directed mutagenesis without changing the encoded amino acid sequence. The modified *flu* gene was then inserted into the *Pst*I sites of the RSF1010-derived broad-host-range vector pKT230 [23], removing the kanamycin resistance gene and keeping the streptomycin resistance gene. The green-light inducible promoter P_{cpcG2} from *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was inserted upstream of the *flu* gene to regulate its expression. Because the addition of the Shine Dalgarno-like sequence from the *Synechococcus* sp. PCC 7002 *cpcB* gene (5′-TATAAGTAGGAGATAAAAAT-3′) was found to increase the expression of genes regulated by P_{cpcG2} [24], this sequence was inserted upstream of the *flu* gene start codon to increase the expression level of antigen 43. The resulting plasmid was named pKT230-$P_{cpcG2\text{-}SD}$-*ag43*. The N-terminal signal peptide sequence of Ag43 (amino acids 1–52) was then exchanged with that of the *Synechococcus* sp. PCC 7942 somA (amino acids 1–27) to produce the plasmid pKT230-$P_{cpcG2\text{-}SD}$-$somA^{sig}$-$ag43^{\alpha\beta}$.

2.2. Transformation of Synechocystis

Transformation of *Synechocystis* was carried out as previously described [25]. Electrocompetent *Synechocystis* cells were prepared by washing a 40 mL culture (OD_{730} = 0.4) sequentially with 30 mL, 20 mL, and 15 mL of 1 mM HEPES buffer, and finally resuspending in approximately 200 μL of the remaining liquid. Inside a 2 mm cuvette, 40 μL of the suspended cells was mixed with 500 ng plasmid DNA and pulsed with a Bio-Rad Gene Pulser at 12 kV/cm with a time constant of 5 ms. Following the pulse, 1 mL of BG11 was immediately added and the suspended cells were transferred to 5 mL BG11 to undergo a one-day recovery at 30 °C under red-light illumination (20 μmol m^{-2} s^{-1}). The cells were then concentrated by centrifugation and spread onto a BG11-agar plate containing 50 μg/mL streptomycin. The plate was incubated at 30 °C under red light and transformed colonies were confirmed by PCR.

2.3. Expression of Ag43 and proteinase K treatment

Synechocystis transformed with pKT230-$P_{cpcG2\text{-}SD}$-*ag43* or pKT230-$P_{cpcG2\text{-}SD}$-$somA^{sig}$-$ag43^{\alpha\beta}$ were grown in BG11 liquid medium at 30 °C with gentle shaking (100 rpm) and overhead illumination by red-light (620 nm, 20 μmol m^{-2} s^{-1}) LED (Panasonic, Osaka, Japan). When the cultures reached OD_{730} = 0.5 additional illumination was provided by green-light (520 nm, 15 μmol m^{-2} s^{-1}) LED (NK System, Osaka, Japan) from the bottom while maintaining the overhead red-light illumination. As a negative control, cells were illuminated only with red light throughout. After four days, the culture was centrifuged (5000 *g*, 5 min) and resuspended in 1/5 volume of phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). The resuspended cells were divided into 200 μL aliquots and incubated for 30 min at 37 °C with 0, 0.1, 0.2, 0.5, 1, or 1.5 U of proteinase K (Roche Diagnostics, Switzerland). The proteinase K inhibitor Pefabloc SC (Roche Diagnostics, Switzerland) was then added to a final concentration of 4 mM to stop further proteolysis.

Cells were centrifuged at 10,000 *g* for 3 min and the pellet was washed twice with 1 mL PBS. The washed cell pellet was finally resuspended in 20 μL PBS containing 4 mM Pefabloc SC and an equal volume of SDS sample buffer and heated at 95 °C for 10 min. Each sample underwent SDS-PAGE on two separate gels, one visualized by Coomassie Brilliant Blue staining and the other was subjected to Western blot analysis.

2.4. Western blotting

The oligopeptide GLEYGPDNEANTGG, corresponding to amino acid residues 88–101 of Ag43 within the α chain, was used as antigenic epitope. Peptide synthesis and rabbit immunization were performed by Eurofins Genomics (Tokyo, Japan). The resulting polyclonal antibody was used for Western blot analysis of *Synechocystis* expressing Ag43. Whole-cell extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked by incubation with 10% skim milk in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.5% Tween 20) and then incubated with the above polyclonal antibody to the synthetic peptide of the Ag43 α chain with shaking for 1 h. The membrane was washed three times for 5 min and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Promega, USA) for 1 h. Blots were washed with TBS-T three times and visualized with the Immobilon Western chemiluminescent HRP substrate (Merck Millipore, USA), according to the manufacturer's instructions, on an ImageQuant LAS 4000 (GE Healthcare Life Sciences, USA).

3. Results and discussion

The *E. coli*-derived *flu* gene, encoding the autotransporter Ag43, was expressed in *Synechocystis* by inserting it into the broad-host-range vector pKT230. To regulate Ag43 expression, we chose the endogenous green-light regulation system of *Synechocystis*, which controls the expression of cpcG2, and is expected to be repressed under red light and induced under green light illumination. The promoter P_{cpcG2} was introduced upstream of the *flu* gene in an attempt to regulate the expression of Ag43 with green-light illumination. *Synechocystis* transformed with pKT230-$P_{cpcG2\text{-}SD}$-*ag43* were grown under red-light illumination until they reached an OD_{730} of 0.5, at which point growth was continued for 48 h under either red light alone or simultaneous red-light and green-light illumination. Because Ag43 is derived from *E. coli*, its N-terminal signal peptide might not be ideally suited to distinguish between the cytoplasmic and thylakoid membranes. We therefore also tested the expression of the flu gene in which the region encoding the signal peptide was substituted to that of the signal peptide of the *Synechococcus* sp. PCC 7942 SomA, a cyanobacterial outer membrane protein.

Western blot analysis using an antibody against a partial peptide of the Ag43 α chain shows a clear induction in response to green light, resulting in the appearance of a single intense band of a size consistent with that expected from the Ag43 α chain after cleavage from the β domain (Fig. 1). A faint band of the same size was barely visible in cells grown under red light alone. Although *Synechocystis* transformed with pKT230-$P_{cpcG2\text{-}SD}$-*ag43* or pKT230-$P_{cpcG2\text{-}SD}$-$somA^{sig}$-$ag43^{\alpha\beta}$ produced very similar Western blot results (Fig. 1), a longer exposure time was used for the latter, indicating that its expression was significantly lower than that of the wild-type Ag43. These results indicate that both gene constructs produce Ag43 α chains that appear to have undergone the correct post-translational processing.

Time dependence of the Ag43 induction was also investigated by taking samples immediately prior to green-light induction and at different times after starting induction for up to four days. Western blot analysis showed that the α chain production increased in a linear fashion during the four days of green-light induction for both constructs (Supplementary Fig. S1). The Ag43 expression level of cells harboring the pKT230-$P_{cpcG2\text{-}SD}$-*ag43* plasmid was approximately 6-fold greater than that of cells harboring pKT230-$P_{cpcG2\text{-}SD}$-$somA^{sig}$-$ag43^{\alpha\beta}$. No significant difference was observed in the growth rate of *Synechocystis* transformed with either of the two constructs or with the empty vector (pKT230) under red light as well as under simultaneous red and green light (data not shown).

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