Phenotypic and proteomic analysis of positively regulated gellan biosynthesis pathway in *Sphingomonas elodea*

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**ABSTRACT**

*Sphingomonas elodea* is a Gram-negative bacterium capable of producing ‘gellan gum’ exopolysaccharide that is the most extensively studied exopolysaccharides of microbial origin. In this study, we investigated the phenotypic and proteomic alterations in *S. elodea* by homogeneously expressing both *gelA* and *gelN* involved in positive regulation and extracellular secretion of metabolites in gellan biosynthesis, respectively. Expression of six histidine-tagged GelA and GelN was determined by Western blot analysis. Successful expression of GelA and GelN resulted in both morphological changes of colonies and enhanced secretion of gellan into the growth medium (GelA, 21.2% more and GelN, 48.3% more) overexpressed compared to the wild-type. Comparative two-dimensional gel electrophoresis analysis revealed a differential proteome expression in *S. elodea* overexpressing GelA and GelN. Proteins up- or down-regulated by GelA and GelN overexpression were found to be mainly sugar transport proteins, two-component regulatory proteins, and proteins involved in secretion pathways. The results suggest that the effect of GelA and GelN overexpression on gellan biosynthesis might be mainly caused by increased transportation of sugar units or enhanced exportation of gellan.

**INTRODUCTION**

*Sphingomonas elodea* is a Gram-negative bacterium capable of producing water-soluble and gelling agent called ‘gellan gum’ exopolysaccharide (EPS) (Kang et al. 1982). Because of its unique structure with repeating unit of heteropolysaccharides and excellent rheological characteristics, gellan gum has been used in diverse industries including the food and pharmaceutical industries (Fialho et al. 1999). The draft genome sequence of *S. elodea* ATCC 31461 strain has been reported (Gai et al. 2011). Since then, much efforts have been focused on the mechanical details of gellan biosynthesis in order to improve the productivity of bacterial gellan (Zhu et al. 2011; Li et al. 2014; Osmalek et al. 2014).

The repeating unit of gellan is composed of D-glucose (D-Glc), L-rhamnose (L-Rha), and D-glucuronic acid (D-GlcA). It forms the linear heteropolysaccharide structure as tetrasaccharide (Figure 1(A)) (Jansson et al. 1983; Sá-Correia et al. 2002). Enzymes involved or predicted to be involved in gellan biosynthesis pathway are two metabolite intersections by UDP-D-glucose pyrophosphorylase (UgpG) and dTDP-D-glucose 4,6-pyrophosphorylase (RhsA) (Figure 1(B)) (Sá-Correia et al. 2002). Consequently, gellan precursors UDP-D-glucose, UDP-D-glucuronic acid and dTDP-L-rhamnose are colocalized and assembled by genes involved in the *gel* cluster (Schmid et al. 2014).

As shown in Figure 1(C), gel clusters comprise at least 20 genes coding for enzymes involved in the synthesis of dTDP-L-rhamnose (*rmlABCD*), specific glycosyltransferases, and protein required for gellan polymerization and export (Harding et al. 2004). The *gel* cluster is categorized into three regions (regions I, III, and IV). Region I is involved in the assembly of tetrasaccharide repeat-units by *gelQ, gelK, gelL* and *gelB*. Region I and IV are involved in gel polymerization and export by *gelS, gelG, gelC, gelE*, and *geld*. Region III includes two component regulatory protein *gela* (Fialho et al. 2008). The *gelA*, encoding a two-component regulatory protein, is presumed to turn on gellan biosynthesis depending on environmental conditions that bacteria are exposed due to its regulatory cascade (Harding et al. 2004). Downstream components in gellan biosynthesis pathway...
might be directly affected by the expression level of gelA (Garg et al. 2000). In addition, gelN coding enzyme has been predicted to be involved in protein export (namely proteins targeted to the periplasm or outer membrane) by comparative sequence analysis between S. elodea ATCC 31641 and Sphingomonas sp. ATCC 31554 (S-88 strain, sps cluster) (Yamazaki et al. 1996; Haft et al. 2006; Fialho et al. 2008). Therefore, changes in intracellular expression of gelA and gelN gene might trigger the regulation of gellan biosynthesis, thus altering global responses in targeted metabolic pathway.

The objective of this study was to investigate the responses of S. elodea strain overexpressed with two pivotal genes (gelA and gelN) involved in gellan biosynthesis and secretion. First, gelA and gelN genes were cloned into broad-host-range vector pBBR122 and expressed homogeneously in S. elodea. Phenotypic changes of this bacterium were compared by quantifying the amount of gellan produced with morphological observations. Moreover, comparative proteomic analysis was performed between wild-type S. elodea and S. elodea everexpressing gelA and gelN to determine the effect of everexpression of gelA and gelN on gellan biosynthesis. This might shed light on the molecular mechanisms underlying the regulation of gellan biosynthesis and the target metabolic.

### Table 1. Bacterial strains and plasmids used in this study.

| Bacterial strains and plasmids | Relevant characteristics | Source or reference |
|-------------------------------|-------------------------|--------------------|
| S. elodea                     | Str<sup>a</sup>          | ATCC 31461*       |
| E. coli BL21 (DE3)            | F ompThsdSa<sub>(rpsL)</sub> galdcm(DE3) | Novagen, Germany |
| Plasmids                      |                         |                   |
| pET-21a                       | Ap<sup>+</sup>, T7 lac promoter, f1 origin; c-terminal six histidyl fusion vector | Novagen, Germany |
| pEGA                          | pET-21a carrying the gelA structural gene | This work |
| pEGN                          | pET-21a carrying the gelN structural gene | This work |
| pBRR122                       | Cm<sup>+</sup>, Km<sup>+</sup>, linearized at the unique Eco72 I site and dephosphorylated | MoBiTec, Germany |
| pBGA                          | pBRR122 carrying the gelA-His<sub>6</sub> | This work |
| pBGN                          | pBRR122 carrying the gelN-His<sub>6</sub> | This work |

<sup>a</sup>American Type Culture Collection

### Materials and methods

#### Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are summarized in Table 1. Wild-type S. elodea ATCC 31461 was cultivated in Luria-Bertani (LB) medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/w) NaCl] at 30°C for 72 h. Escherichia coli BL21 (DE3) strain was used as host for genetic manipulation and cultured in LB medium at 37°C. LB solid medium contained 15 g L<sup>-1</sup> agar. For gellan production, one loop full of S. elodea slant culture was transferred to 50 mL of LB medium in Erlenmeyer flasks. Flasks were incubated at 30°C in a
Genetic manipulations

Genomic DNA extractions, plasmid preparations, PCR reactions, ligations, transformations, and other standard molecular biology techniques were carried out as described elsewhere (Sambrook & Russell 2012) or following the instructions of the supplier. PCR experiments were carried out by using T Gradient thermocycler (Biometra, Germany) and Ex Tag DNA polymerase (Takara Bio, Inc., Japan). Genomic DNA extracted from S. elodea was used as the template. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany). E. coli and S. elodea cells were transformed by electroporation using Electro Cell Manipulator (BTX Technologies Inc., USA).

Construction of recombinant plasmids

All oligonucleotide sequences used in this study are listed in Table 2. Coding regions of gelA and gelN were amplified from the genomic DNA of S. elodea by PCR. Primer pairs gelA-F/gelA-R were used for the amplification of gelA gene and gelN-F/gelN-R primers were used for the amplification of gelN gene. PCR products of gelA and gelN genes were digested with EcoRI/HindIII and ligated with plasmid pET-21a digested with the same restriction enzymes. The resulting plasmids named ‘pEGA’ and ‘pEGN’, respectively, were transformed into E. coli BL21 (DE3). Subsequently, gelA and gelN coding regions with N-terminal six histidine (His6) tag sequence were amplified from pEGA and pEGN plasmids using primer pairs gelA-His-F/gelAN-His-R and gelN-His-F/gelAN-His-R, respectively. These secondary PCR products were digested with AcclI/Saul for gelA-His6 and Agel/Saul for gelN-His6 and ligated with plasmid pBBR122 digested with restriction enzymes of AcclI/Bau36I and Agel/Bau36I, respectively. The resulting plasmids named ‘pBGA’ and ‘pBGN’, respectively, were transformed into S. elodea.

Homogeneous expression of GelA and GelN proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses were used to analyze proteins of E. coli and S. elodea strains using standard protocols (Sambrook & Russell 2012). Western blot analyses of GelA and GelN protein were performed using His-Tag monoclonal antibody (Novagen, Madison, WI, USA).

Quantification of extracellular polysaccharides

Cultures were centrifuged at 13,000 rpm for 30 min at 25°C. One volume of cell-free supernatant was added to three volumes of 95% ethanol (v/v) to precipitate gellan gum. The precipitate was recovered by centrifugation at 13,000 rpm for 10 min at 25°C and dried in a hot-air oven (60°C) for 24 h. The precipitate was stained with Alcian blue (1 g Fluka Alcian Blue 8GS with 100 mL of 85% ethanol (v/v) and incubated for 30 min. At the end of incubation, the optical density of the sample was measured at 677 nm. The concentration of gellan was assayed using Alcian Blue dye stock solutions of 1, 1/10, 1/100, and 1/1000 dilutions of the original dye solution. The concentration of glucose in the broth medium was assayed using a Glucose Assay Kit (Sigma-Aldrich) according to the manufacturer’s protocol. All experimental data given below are mean values obtained from three independent determinations.

Sample preparation for proteome analysis

S. elodea cells were grown in 100 mL of S-media until late stationary-phase (72 h). They were harvested by centrifugation at 6000 rpm for 20 min at 4°C. Cell pellets were

rotary shaker (150 rpm) for 24 h for inoculum development. Then 1 mL of the broth was used as inoculum for 100 mL of S-medium [10 g Na2HPO4, 3 g KH2PO4, 1 g K2SO4, 0.2 g MgSO4·7H2O, 10 mg CaCl2, 1 mg FeSO4·7H2O, 1 g casamino acid, 1 g yeast extract, and 20 g glucose per 1 L of distilled water] in 500 mL of Erlenmeyer flasks followed by incubation at 30°C in a rotary shaker (150 rpm) for 24–72 h. When necessary, antibiotics were used at the following concentrations: 25 mg L−1 streptomycin for S. elodea, 25 mg L−1 ampicillin for E. coli strains transformed with pET-21a and 50 mg L−1 kanamycin for S. elodea. GelA strains transformed with pBGA and pBGN were grown in 100 mL of S-medium [10 g Na2HPO4, 3 g K2HPO4, 1 g K2SO4, 0.2 g MgSO4·7H2O, 10 mg CaCl2, 1 mg FeSO4·7H2O, 1 g casamino acid, 1 g yeast extract, and 20 g glucose per 1 L of distilled water] in 500 mL of Erlenmeyer flasks followed by incubation at 30°C in a rotary shaker (150 rpm) for 72 h. When necessary, anti-shaker (150 rpm) for 24 h for inoculum development. Then 1 mL of the broth was used as inoculum for 100 mL of S-medium [10 g Na2HPO4, 3 g KH2PO4, 1 g K2SO4, 0.2 g MgSO4·7H2O, 10 mg CaCl2, 1 mg FeSO4·7H2O, 1 g casamino acid, 1 g yeast extract, and 20 g glucose per 1 L of distilled water] in 500 mL of Erlenmeyer flasks followed by incubation at 30°C in a rotary shaker (150 rpm) for 24–72 h. When necessary, antibiotics were used at the following concentrations: 25 mg L−1 streptomycin for S. elodea, 25 mg L−1 ampicillin for E. coli strains transformed with pET-21a and 50 mg L−1 kanamycin for S. elodea.
Protein samples were then stored at 20°C under 200 V, 200 mA for 6 h. SDS-PAGE was performed in PROTEAN ii xi Cell (Bio-Rad, Hercules, CA, USA) using 12% SDS-polyacrylamide gels (20 × 20 cm) at a constant temperature of 20°C with a total of 35,500 Vh.

Results

Homogeneous expression of GelA and GelN in S. elodea

The lengths of gelA and gelN structural gene of S. elodea are 2391 bp and 699 bp, respectively. Recombinant plasmids pBGA and pBGN were more appropriate for their expression. Successful expression of GelA-His6 and GelN-His6 were clearly observed based on western blot (Figure 2(B) and 2(D)).
Protein bands corresponding to their expected sizes (88.9 kDa for GelA-His$_6$ and 26.8 kDa for GelN-His$_6$) were observed from cell lysates carrying the two recombinant plasmids (lanes 3 and 4). Such bands were absent from cell lysate without the vector (lane 1) or carrying an empty vector (lane 2). The amounts of expression products of GelA-His$_6$ and GelN-His$_6$ after 72 h cultivation (lane 4, Figure 2(B) and 2(D)) were increased significantly (up to 1.5 folds and 4.8 folds, respectively) compared to products after 24 h cultivation (lane 3).

Phenotypic changes and gellan biosynthesis in S. elodea

Homogenous and overexpression of GelA and GelN affected the phenotype of S. elodea. As shown in Figure 3(A), colony morphologies of the two S. elodea strains transformed with pBGA and pBGN were more viscous than the wild-type strain. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses revealed that saccharide-like lumps or precipitates were released around the extracellular surfaces of these bacteria (Figure 3(B) and 3(C)). The morphologic changes of S. elodea strains resulted in enhanced gellan biosynthesis as expected (Table 3). Cell growth, glucose consumption, and gellan production were observed for different S. elodea strains grown in S-media containing 20 mg mL$^{-1}$ glucose as carbon source. There was no significant difference in bacterial growth. However, the amount of extracellular gellan was enhanced by S. elodea with homologous expression of GelA (21.2%) and GelN (48.3%) compared to S. elodea strain carrying empty pBBR122.

Proteomic responses in S. elodea to expression of GelA and GelN

To determine the influence of overexpression of GelA and GelN proteins on proteomic profiled of S. elodea, two-dimensional gel electrophoresis (2-DE) experiments of S. elodea strains transformed with pBGA and pBGN were performed. Results are shown in Figure 4. After 72 h cultivation, total intracellular proteins were prepared from cultured after disrupting cell pellets. For each protein sample, 2-DE was repeated at least five times. After silver staining, average 2-DE image was used for comparative image analysis. Figure 4(A) shows protein expression profile of S. elodea transformed with empty pBBR122 vector as a control. Through comparative image analysis, overexpression of GelA resulted in four differentially expressed protein spots (spot A1 to A4, Figure 4(B)) compared to control. Similarly, overexpression of GelN resulted in four differentially expressed protein spots (spot N1 to N4, Figure 4(C)). These eight protein spots were subjected to peptide fragmentation. Results of MALDI-TOF mass spectrometric analysis and protein identification are shown in Table 4.
As shown in Figure 4(B), overexpression of GelA resulted in up-regulation of EPS transport protein (spot A1), sugar-binding exported protein (spot A2), and two-component system regulatory protein (spot A3). On the other hand, sugar epimerase-related protein (spot A4) was down-regulated as a result of GelA overexpression. Epimerization of sugar unit might have altered the physicochemical properties of the polymer, thus affecting bacterial pathogenicity, virulence, and environmental adaptability (Whitfield et al. 2015). 2-DE results of S. elodea (pBGN) (Figure 4(C)) showed that two protein spots, mannose-1-phosphate guanylyltransferase/ mannose-6-phosphate isomerase (spot N2) and general secretion pathway protein L (spot N3), were up-regulated as a result of overexpression of GelN. The identified proteins are related to the transfer of sugar monomer (i.e. mannose) to guanosine triphosphate (GTP) and extracellular secretion (Sandkvist et al. 1999; Zakrzewska et al. 2003). Intracellular glycosylation might have been activated by GelN overexpression, resulting in enhanced secretion in S. elodea. Meanwhile, UDP-a-acetylglucosamine 1-carboxyvinttransferase (N1) and glycosyltransferase group family

![Figure 3. Phenotype comparison of empty pBBR122 (control) and pBGA (gelA) of pBGN (gelN) transformed S. elodea. (A) Colony morphologies (72 h of cultivation on S-media plate at 30°C) of three-types of S. elodea strains. (B) SEM and (C) TEM images of cultured cells in liquid S-media for 72 h at 30 °C, 150 rpm. Arrows indicate extracellular gellan lumps.](image)

|                | Cell growth (OD<sub>600</sub>) | Residual glucose (mg mL<sup>−1</sup>) | Gellan (mg mL<sup>−1</sup>) |
|----------------|-------------------------------|----------------------------------|-----------------------------|
|                | 24 h  | 48 h  | 72 h  | 24 h  | 48 h  | 72 h  | 24 h  | 48 h  | 72 h  |
| S. elodea      | 1.8 ± 0.1 | 2.3 ± 0.1 | 2.4 ± 0.2 | 16.2 ± 0.2 | 13.2 ± 0.5 | 10.5 ± 0.9 | 4.8 ± 1.2 | 7.3 ± 1.7 | 12.3 ± 0.8 |
| S. elodea (pBBR122) | 1.6 ± 0.2 | 2.1 ± 0.2 | 2.3 ± 0.1 | 16.8 ± 0.3 | 13.3 ± 0.8 | 10.9 ± 0.0 | 3.2 ± 1.0 | 6.8 ± 0.5 | 11.8 ± 1.1 |
| S. elodea (pBGA) | 1.3 ± 0.5 | 1.6 ± 0.7 | 1.9 ± 0.6 | 18.2 ± 0.8 | 13.0 ± 0.5 | 8.4 ± 0.2 | 2.5 ± 0.7 | 6.6 ± 1.1 | 14.3 ± 1.0 |
| S. elodea (pBGN) | 1.9 ± 0.3 | 2.4 ± 0.4 | 2.5 ± 0.4 | 16.5 ± 0.7 | 12.9 ± 1.0 | 7.9 ± 0.3 | 2.4 ± 0.9 | 6.7 ± 0.8 | 17.5 ± 1.2 |

<sup>a</sup>Initial glucose concentration was 20 mg mL<sup>−1</sup>.
2 protein (N4), two proteins related to cell wall biosynthesis, were down-regulated by GelN overexpression (Wanke et al. 1992; Goldman et al. 2006).

**Discussion**

Over the last decade, EPS synthesized by microbes have received great interest with possibility to replace fossil based polymers (Rehm 2010). Gellan is one of the most extensively studied EPS of microbial origin due to its advantageous properties such as biodegradability, non-toxicity, rapid gelation in the presence of cations, high water holding capacity, and mucoadhesive potential (Osmalek et al. 2014).

Recently, the draft genome sequence of *S. elodea* ATCC 31461 has been revealed using Illumina paired-end technology and Velvet software (Gai et al. 2011). At least 42 enzymes related to metabolism of monosaccharides such as mannose, D-galactonate, D-gluconate, ketogluconates, D-galactonate and D-glucuronate have been predicted (Gai et al. 2011). However, genes involved in the biosynthesis, regulation, and modification of gellan have not been fully characterized. The process of gellan biosynthesis follows several steps: (i)

![Figure 4. Protein expression profile of (A) empty pBBR122 transformed *S. elodea*. Circles (spot A1 to A4) indicate significantly changed spot intensities comparing to *gelA*-carrying pBBR122 (pBGA) transformed *S. elodea* strain. Squares (spot N1 to N4) indicate significantly changed spot intensities comparing to *gelN*-carrying pBBR122 (pBGN) transformed *S. elodea* strain. The spot numbers in (B) and (C) correspond to those in (A). Black color indicates up-regulated protein spots. Red color indicates down-regulated protein spots by homogenous expression of *gelA* or *gelN* gene in *S. elodea*.

**Table 4.** Results of MALDI-TOF mass spectrometric analysis and protein identification for the eight protein spots of *S. elodea* transformed with pBGA or pBGN showing significant differences in intracellular protein level.

| No. | Gene ID  | Protein name                                                                 | pl | MW (kDa) | Accession number | % Sequence coverage | Changes in spot intensity |
|-----|----------|------------------------------------------------------------------------------|----|----------|------------------|---------------------|--------------------------|
| A1  | Becp1808 | EPS transport protein in *Burkholderia vietnamensis* G4                      | 6.52| 93.2     | ABOS9421         | 41                  | +3.61                    |
| A2  | BPSL1793 | Putative sugar-binding exported protein in *Burkholderia pseudomallei*        | 5.99| 32.6     | CAH35792         | 50                  | +2.57                    |
| A3  | MED92_00345 | Two-component system regulatory protein in *Neptunibacter caesaris*    | 5.32| 26.03    | EAP60333         | 64                  | -2.11                    |
| A4  | BAV1250  | Putative sugar epimerase/dehydratase in *Bordetella avium* 197N             | 6.54| 38.27    | CAJ48858         | 76                  | -3.81                    |
| N1  | Xaut_3131 | UDP-n-acetylglucosamine 1-carboxyvinyltransferase in *Xanthobacter sp.* Py2 | 6.12| 45.41    | ABSS8361         | 51                  | -3.81                    |
| N2  | BTH_I0522 | Mannose-1-phosphate guanylyltransferase/ mannose-6-phosphate isomerase in *Burkholderia thailandica* | 5.50| 57.38    | ABC38076         | 48                  | +2.70                    |
| N3  | Mlg_2385 | General secretion pathway protein L in *Alkalimicrobium* ML-1                 | 5.05| 46.06    | ABIS7725         | 58                  | +3.23                    |
| N4  | MXAN_1944 | Glycosyltransferase, group 2 family protein in *Mycococcus xanthus* DK1622 | 6.38| 26.42    | ABF91903         | 59                  | -3.53                    |

*Gene name or locus_tag from NCBI reference sequences.

Accession code refers to the SWISS-2DPAGE database.

Mass tolerance in protein identification through PMF experiments was 10 ppm.

Protein expression levels were compared with wild-type *S. elodea*. 
nucleotide precursors, (ii) formation of tetrasaccharide repeating unit, (iii) backbone modification, and (iv) polymerization and export (Harding et al. 2004). Deep understanding of gellan biosynthesis pathway and regulatory mechanism is crucial to enhance the productivity and create tailor-made polysaccharide variants by genetic engineering (Schmid et al. 2014).

In this study, we reported the cloning of GelA and gelN genes from gellan-producing bacterium S. elodea ATCC 31461 and homogeneously expressed them in order to investigate global cellular responses (Figure 2). GelA has been predicted to a two-component regulatory protein (positive regulator), not linked to the main gellan biosynthetic gene cluster (Harding et al. 2004). In addition, Harding et al. (2004) have reported that mutant S. elodea deleted with gelN gene has reduced gellan productivity compared to its wild-type strain. The effect of overexpression of GelA and GelN on gellan biosynthesis might be due to increased transportation of sugar units or enhanced exportation of gellan.

Phenotypic analysis of this study revealed that overexpression of both GelA and GelN changed colony morphologies due to enhanced gellan production and release compared to wild-type and control (empty-vector contained) strains (Figure 3 and Table 3). Proteomic analysis results suggest that GelA and GelN might have affected biosynthesis pathways related to EPS biosynthesis and exportation (Figure 4 and Table 4). However, these results showed little information about the functional characteristics of gellan biosynthetic pathways. Further studies are needed to determine the regulatory roles and mechanisms of gel cluster to provide additional useful information to help elucidate the mechanisms involved in gellan gum biosynthesis in S. elodea.

Disclosure statement
No potential conflict of interest was reported by the authors.

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Ethical approval
This article does not contain any studies involving human participants or animals performed by any of the authors.

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