

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

Soo Youn Lee^{a,b,*}, Ji-Young Ahn^{b,*}, Mihye Kim^{b,c,*}, Simranjeet Singh Sekhon^b, Sung-Jin Cho^b, Young-Chang Kim^b and Yang-Hoon Kim^b

^aClimate Change Research Division, Korea Institute of Energy Research, Daejeon, South Korea; ^bSchool of Biological Sciences, Chungbuk National University, Cheongju, South Korea; ^cQuality Control Department, Medytox Inc., Cheongwon gu, Cheongju-si, South Korea

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 transportation 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.

ARTICLE HISTORY

Received 1 November 2016
Revised 27 December 2016
Accepted 29 December 2016

KEYWORDS

Gellan biosynthesis;
Sphingomonas elodea;
phenotypic analysis;
proteome analysis; *gel* cluster

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; Osmatek 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 metabolic intersections by UDP-D-glucose pyrophosphorylase

(UgpG) and dTDP-D-glucose 4,6-pyrophosphorylase (RhsA) (Figure 1(B)) (Sá-Correia et al. 2002). Consequentially, 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

CONTACT Yang-Hoon Kim  kyh@chungbuk.ac.kr; Young-Chang Kim  youngkim@chungbuk.ac.kr

*These authors contributed equally to this work.

© 2017 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

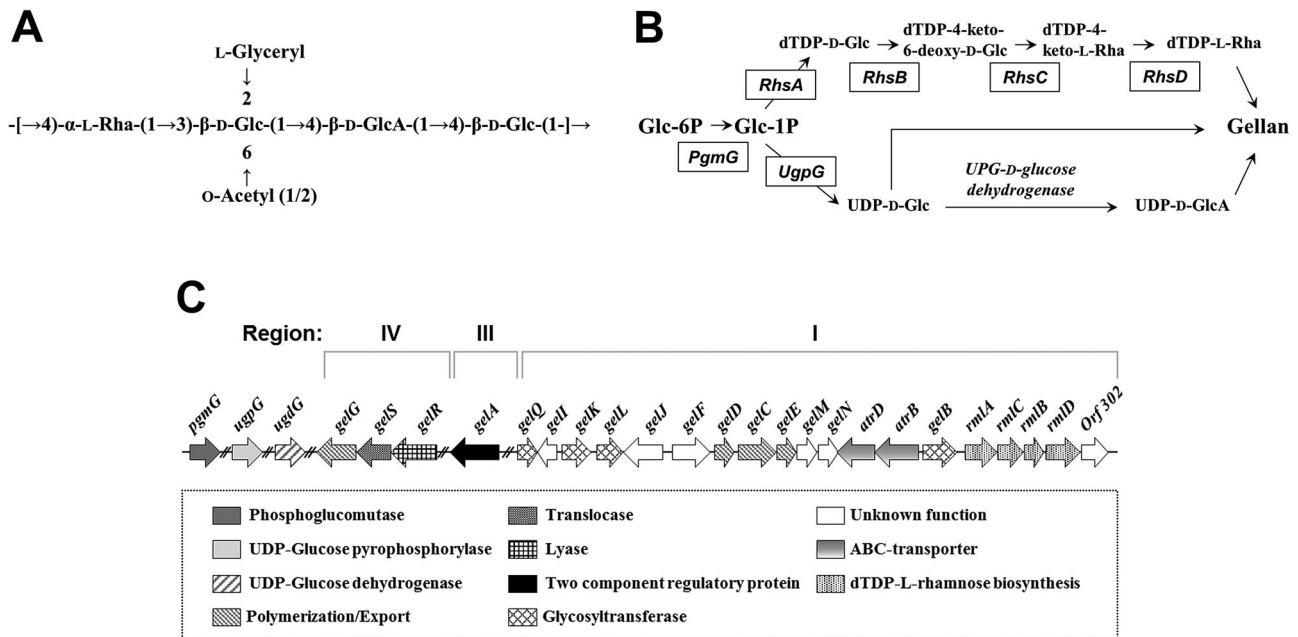


Figure 1. (A) Structure of the repeat unit of gellan. Glc: glucose; GlcA: glucuronic acid; Rha: L-rhamnose. (B) Gellan biosynthesis pathway in *S. elodea*. PgmG: phosphoglucomutase; UgpG: UDP-D-glucose pyrophosphorylase; RhsA: dTDP-D-glucose pyrophosphorylase; RhsB: dTDP-D-glucose 4,6-dehydratase; RhsC: dTDP-4-dehydrorhamnose 3,5 epimerase; RhsD: dTDP-4-dehydrorhamnose reductase; UDP-: uridinediphospho-; TDP-: thymidine diphospho- (Sá-Correia et al. 2002). (C) Organization of the gellan biosynthetic *gel* clusters (region I and IV) and gellan region III.

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 *Sphingomonad* 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
<i>Bacterial strains</i>		
<i>S. elodea</i>	Str ^r	ATCC 31461 ^a
<i>E. coli</i> BL21 (DE3)	<i>F_{ompThsdS_B(r_Bm_B) galdcm}</i> (DE3)	Novagen, Germany
<i>Plasmids</i>		
pET-21a	Ap ^r , T7 <i>lac</i> promoter, f1 origin; c-terminal six histidyl fusion vector	Novagen, Germany
pEGA	pET-21a carrying the <i>gelA</i> structural gene	This work
pEGN	pET-21a carrying the <i>gelN</i> structural gene	This work
pBBR122	Cm ^r , Km ^r , linearized at the unique <i>Eco</i> 72 I site and dephosphorylated	MoBiTec, Germany
pBGA	pBBR122 carrying the <i>gelA-His₆</i>	This work
pBGN	pBBR122 carrying the <i>gelN-His₆</i>	This work

^aAmerican 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⁻¹ 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

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* strains transformed with pBBR122. Culture growth in flasks was monitored by measuring the optical density at 640 nm (OD₆₀₀) using a UV/Vis spectrophotometer (Mecasys Co., Ltd., Korea).

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 *AcclII*/*SauI* for *gelA*-His₆ and *AgeI*/*SauI* for *gelN*-His₆ and ligated with plasmid pBBR122 digested with restriction enzymes of *AcclII*/*Bau36I* and *AgeI*/*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 FlukaAlcian Blue 8GS with 100 mL of 18 mM H₂SO₄) and incubated at 37°C for 30 min. At the end of incubation, the optical density of the sample was measured wavelength at 677 nm. The concentration of gellan was assayed using Alcian Blue dye stock solutions consisted 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

Table 2. Primers used in this study.

Primers	Sequences (5'–3') ^a	Restriction enzyme sites
<i>gelA</i> -F	TAGG/AATTCAGGCAGCATTGGCGTA	<i>EcoRI</i>
<i>gelA</i> -R	TAGA/AGCTTTTTGGCCACCAGGATC	<i>HindIII</i>
<i>gelN</i> -F	GCGG/AATTCGACTATCCGATCCTG	<i>EcoRI</i>
<i>gelN</i> -R	TAGA/AGCTTGGCGCGCTCGCGCGC	<i>HindIII</i>
<i>gelA</i> -His-F	T/CCGGGAAGATCCCGATCCCGGAAA	<i>AcclII</i>
<i>gelN</i> -His-F	TATA/ CCGGTAGATCTCGATCCCGGAAA	<i>AgeI</i>
<i>gelAN</i> -His-R	TAGCC/ TCAGGCAAAAACCCCTCAAGAC	<i>SauI</i> ^b

^aUnderline indicates restriction enzyme site for cloning with pET-21c or pBBR122.

^b*SauI* restriction enzyme site, CC/TNAGG (isoschizomer enzyme with *Bau36I*).

washed three times in an equal volume of ice-cold 0.2 M sucrose followed by washing with 5 mL ice-cold methanol. Cell pellets were resuspended in 10 mM Tris-EDTA buffer (pH 8.0) and disrupted by sonication. Cell debris in samples was moved by centrifugation at 13,000 rpm for 20 min at 4°C. To remove nucleic acids, 0.2% (v/v) DNase I (5 U μL^{-1}) and 0.2% (v/v) RNase (10 mg mL^{-1}) were added to each sample and incubated on ice for 30 min. After adding 100% ice-cold acetone and 100% trichloroacetic acid (1:8:1 ratio) followed by incubation at -20°C for 20 min, cell pellets were precipitated by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was decanted after the centrifugation. Pellets were washed with 1 mL ice-cold acetone and centrifuged again at the same condition. The supernatant was decanted and protein pellets were resuspended in 1 mL of lysis buffer [1 M Urea, 2 M Thiourea, 4% CHAPS, 40 mM Tris, Protease inhibitor cocktail] and mixed vigorously. Protein concentration was determined using 2-D Quant Kit (Amersham Biosciences, Sweden). Protein samples were then stored at -70°C .

Two-dimensional gel electrophoresis and image analysis

A total of 60 μg protein was resuspended in 350 μL rehydration solution [7 M Urea, 2 M Thiourea, 4% CHAPS, 20 mM Tris, 0.5% IPG buffer (Amersham Bioscience)], 200 mM TBP and 0.02% bromophenol blue. Protein samples were applied to 18 cm-length (pH 4-7) IPG Dry Strips (Amersham Biosciences) followed by rehydration at 20°C for 12 h. Isoelectric focusing was conducted using an EttanIPGphor II (Amersham Biosciences) at a constant temperature of 20°C with a total of 35,500 Vh as follows: 500 V for 1 h; 1000 V for 1 h; 8000 V until 32,000 Vh was reached. IPG strips were incubated with equilibration solution [6 M urea, 50 mM Tris-Cl (pH 8.8), 30% (v/v) glycerol, 2% SDS, 5 mM TBP, 0.02% bromophenol blue] for 15 min at room temperature with gentle shaking. SDS PAGE was performed in PROTEAN II xi Cell (Bio-Rad, Hercules, CA, USA) using 12% SDS-polyacrylamide gels (20 \times 20 cm) at a constant temperature of 20°C under 200 V, 200 mA for 6 h.

Image analysis

To visualize protein electrophoresis, silver staining was carried out using PlusOne Silver Staining Kit (Amersham biosciences). Stained-2DE gels were scanned using PowerLook 1100 (UMAX, Korea). Comparative image analysis was carried out using Progenesis software (Non-linear Dynamics, Newcastle, UK). Gels were stained with silver to select spots of interest. Meanwhile, Coomassie

brilliant blue staining was performed for spot identification by mass spectrometry. For protein identification, proteins in gel were fixed by soaking in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for 1 h with gentle agitation. Fixed gels were stained by colloidal Coomassie staining solution (ProteomeTechInc, Korea).

MALDI-TOF mass spectrometric analysis and protein identification

Enzymatic digestions were performed overnight at 37°C in a stationary incubator using 10–15 $\mu\text{g mL}^{-1}$ of sequencing-grade modified trypsin (Proma, Wisconsin, USA) in 25 mM ammonium bicarbonate (pH 8.0). In-gel-digested peptide fragments were extracted from gel pieces using a solution containing 5% (v/v) trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile (ACN) by vortexing for 1 h. After repeating this extraction procedure three times, solute materials including peptide fragments were dried using vacuum evaporator. The peptide solution was prepared with an equal volume of saturated α -cyano-4-hydroxy-cinnamic acid solution in 50% ACN/0.1% TFA on a sample plate of MALDI-TOF mass spectrometer. Protein analyses were performed using MALDI-TOF mass spectrometry system (Voyager DE-STR, Applied Biosystems). Spectra were calibrated using matrix and Tryptic autodigestion ion peaks were used as internal standards. Peptide mass fingerprints were analyzed using MASCOT peptide fingerprint supported by Matrix Science (<http://www.matrixscience.com/>). The identification of protein with respective theoretical parameters (pI, molecular mass) was accepted if the peptide mass matched with a mass tolerance within 10 ppm.

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 (pBBR122 carrying each *gelA* and *gelN* structural gene with His₆-tag) were transformed and expressed in wild-type *S. elodea* strain as described earlier. SDS-PAGE and Western blotting results of homogeneous expression of His₆-tagged GelA and GelN in *S. elodea* are shown in Figure 2. Since both proteins belong to a family of integral membrane proteins, excessive expression might be harmful to the cells (Shin et al. 2010; Haft et al. 2012). Therefore, pBBR122-based low-copy-number 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)).

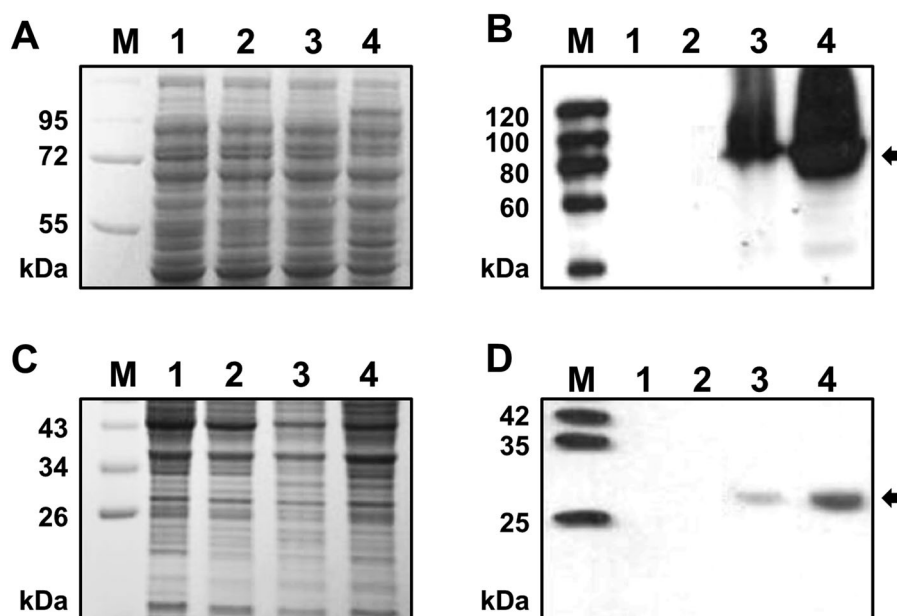


Figure 2. SDS-PAGE (A, C) and western blot analysis (B, D) results of homogenous expression of *gelA* (A, B) and *gelN* (C, D) gene in *S. elodea*. Lane M: protein size marker; lane 1: whole proteins of wild-type *S. elodea* (72 h); lane 2: whole proteins of *S. elodea* transformed with pBBR122 (72 h); lane 3: whole proteins of *S. elodea* transformed with pBBR122 carrying *gelA* (pBGA); lane 4: whole proteins of *S. elodea* transformed with pBBR122 carrying *gelN* (pBGB); lane 3: protein samples were prepared from mid-log phase cultures of each cell (24 h); lane 4: protein samples were prepared from stationary phase cultures of each cell (72 h).

Protein bands corresponding to their expected sizes (88.9 kDa for GelA-His₆ and 26.8 kDa for GelN-His₆) 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-His₆ and GelN-His₆ 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 profile 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.

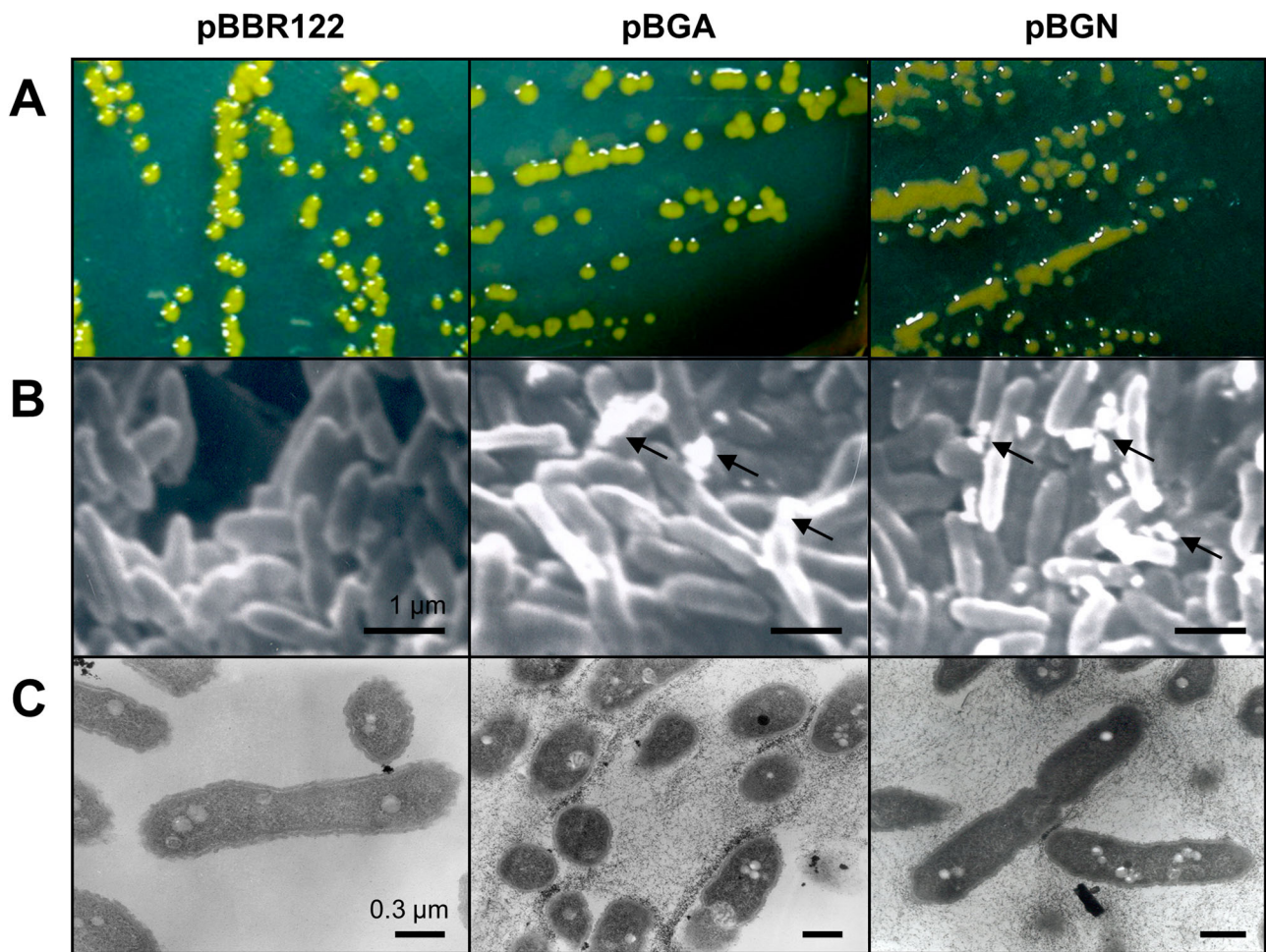


Figure 3. Phenotype comparison of empty pBBR122 (control) and, pBGA (*gelA*) of pBGN (*gelN*) transformed *S. elodea*. (A) Colony morphologies (72 h of cultivation on S-media plate at 30°C) of three-types of *S. elodea* strains. (B) SEM and (C) TEM images of cultured cells in liquid S-media for 72 h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

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-carboxyvinyltransferase (N1) and glycosyltransferase group family

Table 3. Quantification of residual glucose and extracellular gellan concentrations during cultivation of *S. elodea* strains.

	Cell growth (OD ₆₄₀)			Residual glucose (mg mL ⁻¹) ^a			Gellan (mg mL ⁻¹)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
<i>S. elodea</i>	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
<i>S. elodea</i> (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
<i>S. elodea</i> (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
<i>S. elodea</i> (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

^aInitial glucose concentration was 20 mg mL⁻¹.

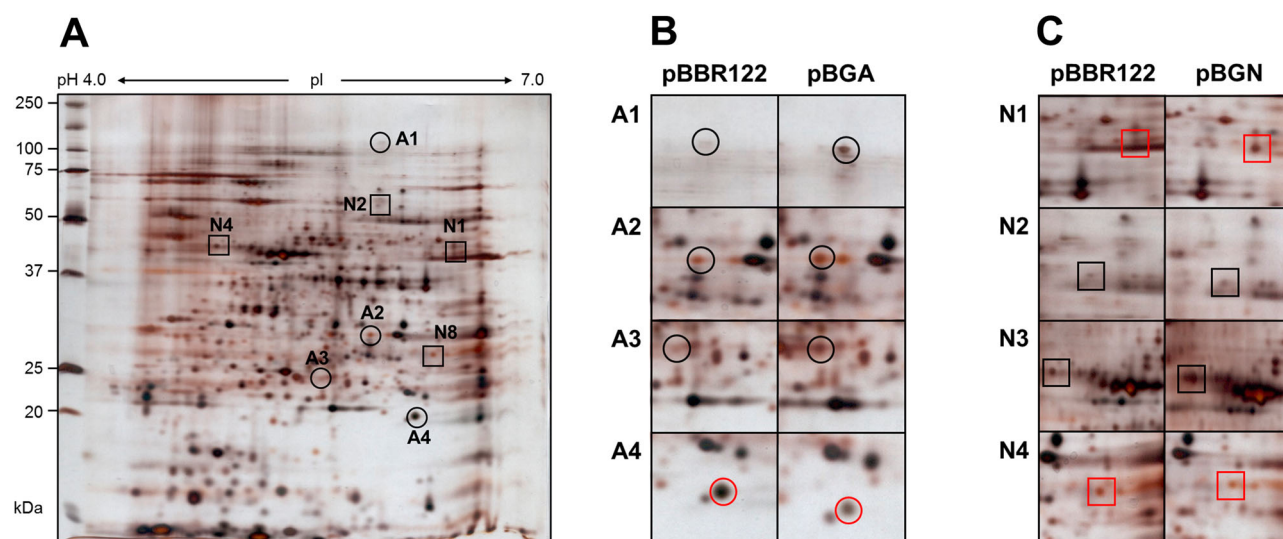


Figure 4. Protein expression profile of (A) empty pBBR122 transformed *S. elodea*. Circles (spot A1 to A4) indicate significantly changed spot intensities comparing to *gela*-carrying pBBR122 (pBGA) transformed *S. elodea* strain. Squares (spot N1 to N4) indicate significantly changed spot intensities 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*.

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 (Osmałek 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)

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 ^a	Protein name	pI	MW (kDa)	Accession number ^b	% Sequence coverage ^c	Changes in spot intensity ^d
A1	Becp1808	EPS transport protein in <i>Burkholderia vietnamiensis</i> G4	6.52	93.2	ABO59421	41	+3.61
A2	BPSL1793	Putative sugar-binding exported protein in <i>Burkholderia pseudomallei</i>	5.99	32.6	CAH35792	50	+2.57
A3	MED92_00345	Two-component system regulatory protein in <i>Neptuniibacter caesariensis</i>	5.32	26.03	EAR60333	64	+2.11
A4	BAV1250	Putative sugar epimerase/dehydratase in <i>Bordetella avium</i> 197N	6.54	38.27	CAJ48858	76	-2.10
N1	Xaut_3131	UDP-n-acetylglucosamine 1-carboxyvinyltransferase in <i>Xanthobacter</i> sp. Py2	6.12	45.41	ABS68361	51	-3.81
N2	BTH_I0522	Mannose-1-phosphate guanylyltransferase/ mannose-6-phosphate isomerase in <i>Burkholderia thailandensis</i>	5.50	57.38	ABC38076	48	+2.70
N3	Mlg_2385	General secretion pathway protein L in <i>Alkalilimicola ethrlichei</i> MLHE-1	5.05	46.06	ABI57725	58	+3.23
N4	MXAN_1944	Glycosyltransferase, group 2 family protein in <i>Mycococcus xanthus</i> DK1622	6.38	26.42	ABF91903	59	-3.53

^aGene name or locus_tag from NCBI reference sequences.

^bAccession code refers to the SWISS-2DPAGE database.

^cMass tolerance in protein identification through PMF experiments was 10 ppm.

^dProtein 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.

Funding

The present work was supported by 'Cooperative Research Program for Agriculture Science and Technology Development (Project title: Risk Assessment Research and Development of Rapid Diagnostic Method for Biological, Chemical and Environmental Animal Disease, Project No: PJ01052301)' funded by Rural Development Administration, Republic of Korea.

Ethical approval

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

References

- Fialho AM, Martins LO, Donval ML, Leitao JH, Ridout MJ, Jay AJ, Morris VJ, Sa-Correia I. 1999. Structures and properties of gellan polymers produced by *Sphingomonas paucimobilis* ATCC 31461 from lactose compared with those produced from glucose and from cheese whey. *Appl Environ Microbiol.* 65:2485–2491.
- Fialho AM, Moreira LM, Granja AT, Popescu AO, Hoffmann K, Sá-Correia I. 2008. Occurrence, production, and applications of gellan: current state and perspectives. *Appl Microbiol Biotechnol.* 79:889–900.
- Gai Z, Wang X, Zhang X, Su F, Wang X, Tang H, Tai C, Tao F, Ma C, Xu P. 2011. Genome sequence of *Sphingomonas elodea* ATCC 31461, a highly productive industrial strain of gellan gum. *J Bacteriol.* 193:7015–7016.
- Garg RP, Huang J, Yindeeyoungyeon W, Denny TP, Schell MA. 2000. Multicomponent transcriptional regulation at the complex promoter of the exopolysaccharide i biosynthetic operon of *Ralstonia solanacearum*. *J Bacteriol.* 182:6659–6666.
- Goldman BS, Nierman WC, Kaiser D, Slater SC, Durkin AS, Eisen JA, Ronning CM, Barbazuk WB, Blanchard M, Field C, et al. 2006. Evolution of sensory complexity recorded in a myxobacterial genome. *Proc Natl Acad Sci U S A.* 103:15200–15205.
- Haft DH, Paulsen IT, Ward N, Selengut JD. 2006. Exopolysaccharide-associated protein sorting in environmental organisms: the PEPCTERM/EpsH system. Application of a novel phylogenetic profiling heuristic. *BMC Biol.* 4:1–16.
- Haft DH, Payne SH, Selengut JD. 2012. Archaeosortases and exosortases are widely distributed systems linking membrane transit with posttranslational modification. *J Bacteriol.* 194:36–48.
- Harding NE, Patel YN, Coleman RJ. 2004. Organization of genes required for gellan polysaccharide biosynthesis in *Sphingomonas elodea* ATCC 31461. *J Ind Microbiol Biotechnol.* 31:70–82.
- Jansson PE, Lindberg B, Sandford PA. 1983. Structural studies of gellan gum, an extracellular polysaccharide elaborated by *Pseudomonas elodea*. *Carbohydr Res.* 124:135–139.
- Kang K, Veeder G, Mirrasoul P, Kaneko T, Cottrell I. 1982. Agar-like polysaccharide produced by a *Pseudomonas* species: production and basic properties. *Appl Environ Microbiol.* 43:1086–1091.
- Li O, Liu A, Lu C, Zheng DQ, Qian CD, Wang PM, Jiang XH, Wu XC. 2014. Increasing viscosity and yields of bacterial exopolysaccharides by repeatedly exposing strains to ampicillin. *Carbohydr Pol.* 110:203–208.
- Osmatek T, Froelich A, Tasarek S. 2014. Application of gellan gum in pharmacy and medicine. *Int J Pharm.* 466:328–340.
- Rehm BHA. 2010. Bacterial polymers: biosynthesis, modifications and applications. *Nat Rev Microbiol.* 8:578–592.
- Sá-Correia I, Fialho AM, Videira P, Moreira LM, Marques AR, Albano H. 2002. Gellan gum biosynthesis in *Sphingomonas paucimobilis* ATCC 31461: genes, enzymes and exopolysaccharide production engineering. *J Ind Microbiol Biotechnol.* 29:170–176.
- Sambrook J, Russell DW. 2012. *Molecular cloning: a laboratory manual*. 4th ed. New York: Cold Spring Harbor Laboratory Press.
- Sandkvist M, Hough LP, Bagdasarian MM, Bagdasarian M. 1999. Direct interaction of the EpsL and EpsM proteins of the

- general secretion apparatus in vibrio cholera. *J Bacteriol.* 181:3129–3135.
- Schmid J, Sperl N, Sieber V. 2014. A comparison of genes involved in sphingan biosynthesis brought up to date. *Appl Microbiol Biotechnol.* 98:7719–7733.
- Shin HD, McClendon S, Vo T, Chen RR. 2010. *Escherichia coli* binary culture engineered for direct fermentation of hemi-cellulose to a biofuel. *Appl Environ Microbiol.* 76:8150–8159.
- Wanke C, Falchetto R, Amrhein N. 1992. The UDP-N-acetylglucosamine 1-carboxyvinyl-transferase of *Enterobacter cloacae*. *FEBS Lett.* 301:271–276.
- Whitfield GB, Marmont LS, Howell PL. 2015. Enzymatic modifications of exopolysaccharides enhance bacterial persistence. *Front Microbiol.* 6:1–21.
- Yamazaki M, Thorne L, Mikolajczak M, Armentrout RW, Pollock TJ. 1996. Linkage of genes essential for synthesis of a polysaccharide capsule in *Sphingomonas* strain S88. *J Bacteriol.* 178:2676–2687.
- Zakrzewska A, Palamarczyk G, Krotkiewski H, Zdebska E, Saloheimo M, Penttilä M, Kruszewska JS. 2003. Overexpression of the gene encoding GTP: mannose-1-phosphate guanyltransferase, mpg1, increases cellular GDP-mannose levels and protein mannosylation in *Trichoderma reesei*. *Appl Environ Microbiol.* 69:4383–4389.
- Zhu L, Wu X, Li O, Chen Y, Qian C, Teng Y, Tao X, Gao H. 2011. Cloning and knockout of phytoene desaturase gene in *Sphingomonas elodea* ATCC 31461 for economic recovery of gellan gum. *J Ind Microbiol Biotechnol.* 38:1507–1513.