Cellulose Accumulation and a Cellulose Synthase Gene are Responsible for Cell Aggregation in the Cyanobacterium Thermosynechococcus Vulcanus RKN

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A thermophilic cyanobacterium, Thermosynechococcus vulcanus RKN, exhibits cell aggregation under low temperature illuminated conditions as a means of physiological acclimation to avoid excess light stress. The cell aggregation was dispersed with cellulase treatment. We developed a method to quantify small amounts of cellulose by partial cellulose purification followed by quantitation of liberated glucose by cellulase. Under low temperature illuminated light conditions, cellulose accumulation was induced approximately 2-fold, to 10 μg (4 × 10^9 cells)^{-1}, and slightly preceded aggregation. Based on sequence similarity, three candidate genes for cellulose synthase (Tvtll0007, Tvtlr1795 and Tvtlr1930-33) were cloned from T. vulcanus. Genes disruption analysis showed that only Tvtll0007 was responsible for both the light- and low temperature-induced cell aggregation and the induction of cellulose accumulation. Gene expression analysis suggested that the low temperature illuminated conditions quickly induced expression of Tvtlr1795 and Tvtlr1930-33, while the induction of Tvtll0007 was slow. These results suggest that Tvtll0007 encodes a functional cellulose synthase whose activity may not be regulated at the transcriptional level.

**Keywords:** Cell aggregation ● Cellulose ● Cellulose synthase ● Low temperature acclimation ● Thermosynechococcus elongatus BP-1 ● Thermosynechococcus vulcanus RKN.

**Abbreviations:** c-di-GMP, 3’,5’-cyclic diguanylic acid; OD, optical density; RT–PCR, reverse transcription–PCR.

The nucleotide sequences reported in this paper have been submitted to the DNA Data Bank of Japan under the following accession numbers: Tvtll0007, AB571479; Tvtlr1795, AB571480; and Tvtlr1930-33, AB571481.

**Introduction**

Diverse families of bacteria can form multicellular aggregates known as biofilm, which is ubiquitous in natural environments. In biofilm formation, cells produce extracellular matrix to mediate cell–cell association. This morphological behavior has physiological significance such as colonization at liquid–solid and liquid–air interfaces, adherence to host cells and protection from physical, chemical and biological stresses (Branda et al. 2005). To date, various components in the extracellular matrix have been identified, e.g. proteins such as pili, curli and biofilm-related protein, and polysaccharides such as cellulose, poly-β-1,6-N-acetylglucosamine and alginate (Römling 2005, Las 2006).

Cyanobacterial biofilm formation often occurs in natural environments (e.g. cyanobacterial bloom in lakes and sea). Some free-living cyanobacteria also exhibit cell aggregation in response to various stresses (Price et al. 2002, Zilliges et al. 2008, Li et al. 2009). Under high light conditions, a rapid or slow aggregation process has been observed in several thermophilic cyanobacteria: the rapid process, completed in minutes, probably involves redox regulation (Koblìžek et al. 2000), and the slow process, completed in days, generally requires protein synthesis (Hirano et al. 1997).

This slow process was observed in light- and low temperature-induced cell aggregation of the thermophilic cyanobacterium, Thermosynechococcus vulcanus strain RKN, formerly known as Synechococcus vulcanus. It took approximately 24–48 h for full aggregation. The aggregation was accelerated under higher light irradiation and abolished in the dark. Low but physiological temperature (31–35°C) was effective for aggregation compared with the optimal growth temperature (57°C). The aggregated cells were more tolerant to...
photoinhibition than non-aggregated cells (Hirano et al. 1997). It was assumed that cell aggregation serves as a self-shading behavior to avoid excess light at low temperature. We determined the complete genome sequence of the closely related thermophilic cyanobacterium Thermosynechococcus elongatus BP-1 (Nakamura et al. 2002) and found three putative cellulose synthase genes. Some bacteria accumulate cellulose as an extracellular matrix used to construct an intercellular network during biofilm formation (Branda et al. 2005, Römling 2005, Lasa 2006). We hypothesized an analogous role for cellulose and cellulose synthase in the induction of cyanobacterial cell aggregation.

We hypothesized an analogous role for cellulose and cellulose synthase in the induction of cyanobacterial cell aggregation. Cellulose is an insoluble polysaccharide of linear β-1,4-glucan, which exhibits varying crystallinity according to the degree of inter- and intramolecular hydrogen bonding (O’ Sullivan 1997). Cellulose biosynthesis has been reported in plants, algae, metazoans (tunicates), fungi, cellular slime molds and bacteria (Ross et al. 1991, Römling 2002, Matthysse et al. 2004, Okuda et al. 2004, Grenville-Briggs et al. 2008, Mutwil et al. 2008, Taylor 2008). The first bacterial cellulose synthase gene was cloned and characterized from Gluconacetobacter xylinus (Saxena et al. 1990, Wong et al. 1990). Since then, several related cellulose synthase genes have been identified—mainly in proteobacteria (Römling 2002)—and homologous genes have been recognized in many bacterial genomes, including cyanobacteria (Nakamura et al. 2002, Nobles and Brown 2004, Nobles and Brown 2007). Cellulose synthase is usually located in the cytoplasmic membrane, and transfers glucose moieties from UDP-glucose to cellulose in the extracellular space.

Cellulose in cyanobacteria has been detected using X-ray diffraction and specific labeling (Nobles et al. 2001), although the amount and type of the cellulose is not known—presumably due to low abundance. It is not clear whether the putative cellulose synthase genes found in the cyanobacterial genome are indeed functioning in cellulose biosynthesis. To understand the relationship between cellulose and cell aggregation, we investigated cellulose accumulation in T. vulcanus under inducible conditions of low temperature in light. We also studied gene expression, and the disruption of the putative cellulose synthase genes.

**Results**

**Cell aggregation at low temperature**

* Thermosynechococcus vulcanus shows optimal growth at approximately 57°C. Cells were cultured at 45°C (normal temperature) or 31°C (low temperature) under light illumination. Cells grown at 31°C showed extensive cell aggregation, but not when grown at 45°C (Fig. 1A, B; Hirano et al. 1997). Small aggregates with several tens of cells were initially formed, some of which further aggregated together to form large clumps (Fig. 1B). These aggregates were mostly precipitated after settling for 30 min, but the non-aggregated cells grown at 45°C remained dispersed (Fig. 1A).

**Enzyme treatments to disperse cell aggregates**

Cells that aggregate at 31°C may have extracellular substances that mediate cell–cell attachment. Under microscopic observation, negative staining using India ink did not show a visible layer around the cell aggregates (data not shown). We tested various enzymatic treatments to disperse the aggregated cells (Fig. 1C). Cell aggregates were completely dispersed only with treatment with cellulase, and not at all with cellobiase, β-1,3-glucanase, α-amylase plus amyloglucosidase, or proteinase K. Cellobiase is known to hydrolyze β-1,4-glucosidic bonds, like cellulase, but the substrate only has short chains. These results suggest that long-chain β-1,4-glucan was secreted outside of the cells at 31°C, causing the cell aggregation.

**Aggregation and growth at normal and low temperature**

To quantify the aggregation, we defined the Aggregation Index as the fraction of total cells that were aggregated (see the Materials and Methods). At 31°C, cell aggregation began after 8 h and was almost completed, with a few free cells remaining, at 48 h (Fig. 2A). At 45°C, no clear aggregation was detected throughout growth. We also examined cell growth as the optical density of the total cells (ODtotal) (OD730 after cellulase treatment). At 31°C, T. vulcanus grew very slowly until 72 h (Fig. 2B). The growth at 31°C may be arithmetic due to nutrient limitation, consistent with a previous report (Sakamoto and Bryant 1998), while the growth at 45°C was exponential. We also investigated cell aggregation in *T. elongatus*, a closely related strain of *T. vulcanus*. Notably, cell aggregation at 31°C was not observed in *T. elongatus* (Fig. 2C), although the growth rate was comparable with that of *T. vulcanus* (Fig. 2D).

**Cellulose quantitation**

We developed a cellulose quantitation method that consists of its partial purification, followed by its detection. The partial purification method is similar to that used for other cyanobacteria (Nobles et al. 2001). Our technique includes cell solubilization using SDS, and digestion of protein and glycogen, using proteinase K and glucoamylase treatments, respectively, to recover insoluble cellulose. For cellulose detection, this insoluble material was treated with cellulase to liberate glucose, which was quantitated using a glucose oxidase kit. In the course of the cellulase treatment, glucose was mostly released within 24 h and its liberation was almost completed by 96 h (Supplementary Fig. S1); 96 h was therefore chosen as the duration of the cellulase treatment, and all liberated glucose was deemed to have originated from cellulose.

When an acetic/nitric acid treatment step was incorporated into the purification, the recovery of cellulose was reduced >70% (data not shown). This treatment has been widely used to remove most polysaccharides other than crystalline cellulose (Updegraff 1969), and can also extract non-crystalline cellulose (Lai-Kee-Him et al. 2002). This result suggests that the cellulose produced by *T. vulcanus* is largely non-crystalline,
Fig. 1 Cell aggregation of wild-type *T. vulcanus*. (A) Cell suspensions, grown at 31 and 45°C for 72 h, before (left) and after (right) settling for 30 min. Note that most of the aggregated cells grown at 31°C had precipitated at the bottom of the cuvette after settling. (B) Microscopic images of cells grown at 31 or 45°C for 72 h. Insets show higher magnifications. (C) Enzyme treatments (for 4 h) of cell aggregates grown at 31°C. Photographs were taken after leaving to stand for 10 min.

Fig. 2 Aggregation (A, C) and growth (B, D) curves of wild-type *T. vulcanus* (A, B) and *T. elongatus* (C, D) grown at 45 and 31°C. Aggregation Index indicates the percentage of aggregated cells. OD_total reflects the optical density of the total cells.
or of the low crystalline form, consistent with previous observations in other cyanobacteria (Nobles et al. 2001). We therefore omitted the acetic/nitric acid treatment in order to quantify all forms of cellulose.

**Cellulose accumulation during cell aggregation**

We investigated the time course of cellulose accumulation in *T. vulcanus* grown at 31°C in the light (Fig. 3A). The cellulose content before culture at 31°C (0 h) was approximately 5 μg (4 × 10^9 cells)^{-1}, accumulating to 10 μg (4 × 10^9 cells)^{-1} within 24 h, and remaining mostly unchanged with further cultivation at 31°C. Notably, the cellulose accumulation seemed to slightly precede cell aggregation, as reflected by the Aggregation Index (see ‘8 h’ data point in Figs. 2A and 3A). In *T. elongatus*, the induction of cellulose accumulation at 31°C did not occur (Fig. 3B), which is consistent with its having a non-aggregating phenotype (Fig. 2C). Cellulose accumulation induced at 31°C is therefore likely to be responsible for cell aggregation in *T. vulcanus*.

**Putative cellulose synthase genes in *T. vulcanus***

To investigate cell aggregation and cellulose biosynthesis at a genetic level, we searched for cellulose synthases in the protein database derived from the complete genome of the closely related *T. elongatus*. The cellulose synthase (BcsA) of *G. xylinus* was used as the query. The two genes till0007 and tlr1795 were detected with significant similarities, and homologous genes from the closely related *T. vulcanus* were cloned (designated Tvtll0007 and Tvtlr1795, respectively). Partial fragments of a putative cellulose synthase gene (tlr1930 and tlr1933) in *T. elongatus*, which was split by insertion of transposase genes (tlr1931 and tlr1932), were also identified. The homologous gene from *T. vulcanus* (designated Tvtlr1930-33), which was free from the transposase genes, was also cloned. Notably, another transposase gene (till1929), located in the promoter region of tlr1930 in *T. elongatus*, was also absent in *T. vulcanus*, suggesting that Tvtlr1930-33 is functional as the third putative cellulose synthase gene. The DNA sequences of the three genes were determined and deposited in the DNA Data Bank of Japan (see footnote).

The predicted domain architecture of the three putative cellulose synthases is almost identical to that of the known cellulose synthase BcsA of *G. xylinus* (Fig. 4A). It consists of a glycosyltransferase 2 (GT2) domain, several transmembrane helices, and a PilZ domain, which is not clearly detected in Tvtlr1930-33. They all harbor a highly conserved D, D, D, QXXRW motif, essential for catalytic activity in cellulose synthase (Saxena and Brown 1997, Saxena et al. 2001). The PilZ domain is known to bind to 3',5'-cyclic diguanylic acid (c-di-GMP), an intracellular second messenger molecule (Hengge 2009, Schirmer and Jenal 2009), and to activate cellulose synthase activity (Weinhouse et al. 1997, Amikam and Galperin 2006).

**Cell aggregation of the putative cellulose synthase disruptants**

To examine whether the putative cellulose synthase genes were really involved in the light- and low temperature-induced cell aggregation, we disrupted each cellulose synthase gene separately, and also constructed a triple disruption mutant (Fig. 4B). These mutants completely segregated, which was confirmed by PCR (Fig. 4C). Cell aggregation at 31°C in light was abolished in ΔTvtll0007 and the triple disruptant, while aggregation was not affected in ΔTvtlr1930-33 (Fig. 5B). ΔTvtlr1795 showed a slightly quicker aggregation phenotype than the wild type. At 45°C, no aggregation was observed in any disruptants or the wild type (Fig. 5A). Growth of these disruptants was similar to that of the wild type at 31°C (Fig. 5D) and 45°C (Fig. 5C). These results show that only Tvtll0007 is essential for the light- and low temperature-induced cell aggregation.
Cellulose accumulation in the putative cellulose synthase disruptants

The cellulose content of the disruptants, which were grown at 31°C in light for 72 h, was measured (Fig. 6). Cellulose accumulation in ΔTvTll0007 was clearly lower than that in the wild type, in agreement with the non-aggregating phenotype (Fig. 5B). Cellulose accumulation in ΔTvTlr1795 and ΔTvTlr1930-33 was comparable with that in the wild type, while cellulose accumulation in the triple disruptant was comparable with that in ΔTvTll0007. When grown at 45°C, cellulose accumulation in all the disruptants was comparable with that in the wild type. These results strongly suggest that TvTll0007 contributes to the light- and low temperature-induced cellulose accumulation, corresponding to approximately 50% of the total cellulose content. In contrast, the other two enzymes appear not to contribute to cellulose accumulation under these experimental conditions—thus the remaining 50% of the total cellulose content was not derived from these three enzymes.

Transcriptional regulation of the putative cellulose synthase genes at low temperature

We measured transcript levels of the putative cellulose synthase genes by quantitative real-time reverse transcription–PCR (RT–PCR) (Fig. 7). The levels of TvTlr1795 and TvTlr1930-33 were quickly up-regulated (within 2 h) after shifting to 31°C, and remained high after 24 h. The level of TvTll0007 was essentially unchanged at 2 h but was up-regulated 2-fold at 24 h. For all genes, at 45°C the transcript levels barely changed. These results suggest that slow transcriptional induction of TvTll0007 may contribute to maintenance of the high level of cellulose synthase during long-term acclimation.

Discussion

To our knowledge, this is the first report to describe that the light- and low temperature-induced cyanobacterial cell aggregation is due to accumulation of cellulose, which was estimated by our newly developed method. We also identified the cellulose synthase experimentally for the first time in cyanobacteria: the gene TvTll0007 is responsible for cellulose accumulation and cell aggregation.

Cluster analysis of putative bacterial cellulose synthases revealed that they are roughly divided into seven clades, four of which contain the PilZ domain, while the other three do not (Supplementary Fig. S2). Cellulose synthase and cellulose synthase-like proteins from plants and other eukaryotes are not included because they are distantly related to the bacterial group. While the known cellulose synthases from G. xylinus, Escherichia coli and Agrobacterium tumefaciens (Matthysse et al. 1995, Zogaj et al. 2001) are clustered in one clade, the cyanobacterial proteins including TvTll0007, TvTlr1795 and TvTlr1930-33 are clustered in another previously unknown clade. There are other putative cyanobacterial cellulose
synthases which do not have the PilZ domain (e.g. ALR3757 of Anabaena sp. PCC 7120). Nobles et al. (2001) mentioned that ALR3757 is similar to the plant cellulose synthase. They also detected cellulose in Anabaena cells after extraction with acetic/nitric acid. Using our method, we measured a small but significant amount of cellulose in Anabaena cells [0.24 mg (mg dry cell weight)^{-1}]. This could be further examined by gene disruption analysis.

The cellulose detection method was designed to quantify relatively small amounts of cellulose accumulated in the cyanobacterial cells. The detection limit of this method is approximately 1 mg (4 x 10^7 cells)^{-1}, which corresponds to 0.003% (w/w) of cell dry weight. The glycogen removal by repetitive glucoamylase treatments, the glucose release by cellulase treatment and the specific glucose assay are critical components of our detection method. Under low temperature in light, cells accumulated a large amount of glycogen (data not shown), which may interfere with subsequent glucose detection. Indeed, glucoamylase treatment of intact cells liberated some glucose, probably due to partial cell breakage. We used a chromatographically purified grade of fungal cellulase to avoid non-specific digestion of other polysaccharides. The glucose oxidase assay was also highly specific to glucose and essentially insensitive to galactose, maltose, cellobiose, etc. (data not shown). In summary, we were able to detect small amounts of cellulose in T. vulcanus cells. The insoluble material that was subjected to cellulase treatment clearly contained other polysaccharides as well—its total polysaccharide content, determined by the phenol–sulfuric acid method, was approximately 40 times greater than could be accounted for from cellulose alone. Further purification is needed, however, for a more detailed analysis, for example to confirm the glucose linkage type by methylation analysis or to estimate cellulose crystallinity by spectroscopic analysis.

The triple disruptant contained a significant amount of cellulose before and after induction (Fig. 6). There may therefore be additional active cellulose synthase gene(s) which are not homologous to known genes. Alternatively, glucose may be liberated from cellular components such as other polysaccharides, glycoproteins or glycolipids by the cellulase treatment. Although the cellulase used in this study was a chromatographically purified grade, it may have other glucosidase activity.

Bacterial cell aggregation is generally related to biofilm formation, in which a large amount of extracellular polysaccharides, such as cellulose, accumulates and protects the cells embedded in the biofilm from various environmental stresses. Biofilm formation in bacteria is generally regulated by the
c-di-GMP signaling system (Hengge 2009). We previously reported that the light- and low temperature-induced cell aggregation of the thermophilic cyanobacterium *T. vulcanus* is indeed an acclimation response to avoid excess light stress at suboptimal temperatures (Hirano et al. 1997). Here, we showed that the induced aggregation of *T. vulcanus* cells is due to cellulose accumulation, and further showed that the cellulose synthase gene is responsible for this accumulation. The slow transcriptional activation of *Tvtll0007* (Fig. 7) may contribute to maintenance of the high level of cellulose synthase during long-term acclimation. Alternatively, c-di-GMP-mediated activation of *Tvtll0007* via its PilZ domain would be more plausible because the genome of the closely related *T. elongatus* BP-1 contains many signaling proteins for c-di-GMP metabolism, such as diguanylate cyclase and phosphodiesterase. Of these, there are three photoreceptor-type genes that may be involved in the light regulation of cellulose biosynthesis. Molecular characterization of these genes is now in progress.

The light- and low temperature-induced cellulose accumulation and cell aggregation were defective in the closely related strain *T. elongatus* BP-1 (Figs. 2C, 3B). Comparison of *T. vulcanus* and *T. elongatus* cellulose synthase genes showed substitutions at three nucleotide positions in the *tll0007* coding region, resulting in a single substitution of Ala654 with threonine in the PilZ domain, while the other two were synonymous. We also sequenced the promoter region in *T. vulcanus* but did not find any substitution. At present, it is not clear whether the defect in *T. elongatus* was due to this point mutation in the PilZ domain or an as yet unidentified mutation in c-di-GMP signaling or other regulatory system(s). In contrast, there are many more substitutions in *tll1795* (184 nucleotides and 54 amino acid residues). The gene *tll1930-33* was inactivated by two independent insertion events of ISEL4p and ISEL2h, as shown in the genome analysis (Nakamura et al. 2002), in addition to substitutions (eight nucleotides and one amino acid residue). We recently found a new variant of *T. elongatus* BP-1 that shows cell aggregation similar to the light- and low temperature-induced cell aggregation of *T. vulcanus* described here. This finding suggests that cell aggregation is a common environmental response in thermophilic cyanobacteria to avoid photoinhibition by self-shading effects. Similar cell aggregation of some cyanobacteria under suboptimal conditions (e.g. Sakamoto and Bryant 1998) may be found to be due to accumulation of cellulose or other extracellular substances. Further studies on photosynthesis-driven cellulose accumulation can be applied to biomass production through genetic engineering, because thermophilic cyanobacteria are suitable for large-scale open culture.

**Materials and Methods**

**Strains and cultures**

The thermophilic cyanobacteria *T. vulcanus* strain RKN (Koike and Inoue 1983) and the closely related *T. elongatus* strain BP-1 (Yamaoka et al. 1978, Nakamura et al. 2002) were cultured at 45°C (normal temperature) or 31°C (low temperature) in BG11 medium (Stanier et al. 1971), supplemented with 20 mM...
HEPES-KOH, pH 8.2, under continuous illumination with white fluorescent lamps (50 μmol photons m$^{-2}$ s$^{-1}$), and bubbled with air containing 1.0% (v/v) CO$_2$. Optical density was monitored at 730 nm (OD$_{730}$) using a UV-VIS spectrophotometer (model UV-2400, Shimadzu, Japan). We assumed that a reading for OD$_{730}$ of 1 corresponded to $4 \times 10^9$ cells ml$^{-1}$.

Measurements of cell aggregation and growth curves
During the exponential growth phase, cells grown at 45°C were diluted to give an OD$_{730}$ of approximately 0.25, and transferred to an incubation temperature of 31°C to initiate cell aggregation. In order to measure the Aggregation Index (defined below), cell suspensions were thoroughly mixed, and aliquots were transferred to cuvettes. Aggregated cells had mostly precipitated to the bottom of the cuvette after standing for 30 min at room temperature. The OD$_{730}$ of the remaining supernatant was measured as OD$_{NA}$ (i.e. OD$_{730}$ of non-aggregated cells). Cellulase (chromatographically purified cellulase complex, derived from Trichoderma reesei ATCC26921, Worthington Biochemical Corporation) was added to the suspension to give a final concentration of 12.5 U ml$^{-1}$, and the mixture was incubated for 20 min at 37°C to disperse the aggregated cells completely. The OD$_{730}$ of the resulting cell suspension without aggregation, OD$_{total}$, was measured. OD$_{total}$ was also used for analyses of cell growth. The Aggregation Index was defined as follows:

$$\text{Aggregation Index (\%) = \left( \frac{\text{OD}_{total} - \text{OD}_{NA}}{\text{OD}_{total}} \right) \times 100}$$

Testing enzyme treatments to disperse cell aggregations
The following enzymatic treatments were tested on the aggregated cells that had been grown at 31°C for 2 d: 0.60 U ml$^{-1}$ cellulase; 2.0 U ml$^{-1}$ β-1,3-glucanase (from Arthrobacter sp., Wako); 0.22 U ml$^{-1}$ α-amylase (from Bacillus sp., Sigma) plus 0.24 U ml$^{-1}$ amyloglucosidase (from Aspergillus niger, Sigma); 4.0 U ml$^{-1}$ protease K (Wako); and 1.57 $\times$ 10$^{-4}$ U ml$^{-1}$ cellobiase (from Aspergillus niger, Sigma). Enzymatic treatments were allowed to progress for 4 h under cell cultivation conditions, i.e. 31°C. Suspensions were photographed after allowing to stand for 10 min.

Cellulose quantitation
Cells were harvested by centrifugation at 8,270 $\times$ g for 10 min, resuspended in water and were immediately frozen at $-80°C$ until needed for analysis. After thawing, cells (approximately 4 $\times$ 10$^{10}$) were precipitated by centrifugation at 20,400 $\times$ g at 4°C for 10 min and treated with 1 ml of 5 mg ml$^{-1}$ lysozyme (Wako) in TE buffer (10 mM Tris–HCl at pH 8.2, 1 mM EDTA) at 37°C for 30 min with gentle shaking. After SDS was added to a final concentration of 1% (w/v), cells were disrupted by three cycles of sonication (10 W, 30 s), and the insoluble material, including cellulose, was collected by centrifugation at 20,400 $\times$ g for 10 min at room temperature. The precipitate was resuspended in 1 ml of 0.5% (w/v) SDS in TE buffer, sonicated for 30 s, then digested overnight at 55°C with gentle shaking with proteinase K, at a final concentration of 0.53 mg ml$^{-1}$. The insoluble materials were collected by centrifugation, washed in 1 ml of 50 mM sodium acetate buffer at pH 5.0 and resuspended in 1 ml of the same buffer by sonication for 10 s. Glucoamylase (from Rhizopus sp., Seikagaku Biobusines) was then added to a final concentration of 2 U ml$^{-1}$, and the reaction mixture was incubated overnight at 37°C with gentle shaking. The insoluble material remaining was collected by centrifugation. Glucoamylase treatments were repeated three times to remove all glycogen completely. The final insoluble materials were then digested with cellulase (final concentration, 200 U ml$^{-1}$) in 1 ml of acetate buffer at 37°C with gentle shaking. Aliquots of the reaction mixture were centrifuged at 20,400 $\times$ g at 4°C for 10 min, and 40 μl of the supernatant was used for the assay of liberated glucose. The glucose concentration was determined by a glucose assay kit (Glucose CII-test, Wako), which includes glucose oxidase.

Putative cellulose synthases and gene disruption
Based on the homologous genes of closely related T. elongatus, the putative cellulose synthase genes of T. vulcanus (Tvtll0007, Tvtlr1795 and Tvtlr1930-33) were cloned by PCR and sequenced. After elimination of PCR errors, the consensus sequences were deposited in the database (see footnote). For disruption of Tvtlr1795 and Tvtlr1930-33, DNA fragments were PCR amplified from genomic DNA of T. vulcanus. Tvtlr1930-33 was cloned into the Novagen pT7Blue T-vector (Merck) and an internal fragment between two MscI sites was replaced with a kanamycin resistance cassette (Fig. 5A). Tvtlr1795 was cloned using the PCR-Script Amp cloning kit (Stratagene) and interrupted at a BbsI site by insertion of the kanamycin resistance cassette for single gene disruption, and a spectinomycin/streptomycin resistance cassette for triple gene disruption. For disruption of Tvtll0007, a DNA fragment of tll0007 was PCR amplified from genomic DNA of T. elongatus, cloned into pT7Blue, and interrupted at a HindIII site with a chloramphenicol resistance cassette. Primers are listed in Supplementary Table S1. Thermosynechococcus vulcanus cells were transformed with the plasmid DNAs according to the method described for T. elongatus (Iwai et al. 2004). Because tll0007 is highly conserved between T. vulcanus and T. elongatus, the disruption construct derived from T. elongatus was efficiently introduced into T. vulcanus. Antibiotics used for screening and maintaining mutants were 3.4 μg ml$^{-1}$ chloramphenicol, 80 μg ml$^{-1}$ kanamycin, 10 μg ml$^{-1}$ streptomycin and 5 μg ml$^{-1}$ spectinomycin. For growth and aggregation experiments, cells were grown in the absence of antibiotics. Complete gene disruption, with full segregation in multiple copies of genomes, was confirmed by PCR using the primers listed in Supplementary Table S1.
Sequence analysis

We used the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) for analyzing domain architectures, and ClustalX for sequence alignment and cluster analysis, using the Neighbor-Joining method (Thompson et al. 1997). For cluster analysis (Supplementary Fig. S2), we obtained sequences from the Microbial Genome Database (MBGD; http://mbgd.genome.ad.jp/) as Cluster ID O312 (release number: mbgd 2010-01). From the constructed tree, we extracted the sequences of a clan including bacterial cellulose synthases, and further manually chose the representative organisms. We also obtained additional sequences from the protein database (http://www.ncbi.nlm.nih.gov/protein), and the final members are listed in Supplementary Table S2.

RNA analysis

The culture conditions were the same as for aggregation measurements. Cells were harvested by centrifugation at 9,400 × g for 3 min, washed, frozen in liquid N2 and stored at −20°C. Cells were disrupted five times by homogenization with zirconia beads (0.1 mm diameter) in a micro homogenizing system (Micro Smash MS-100, Tomy) at 5,000 r.p.m. for 40 s at 0°C. RNA was extracted using an RNeasy Mini kit (Qiagen), and further treated with RNase-free DNase I (TAKARA). For cDNA preparation, RNA was reverse transcribed (Reverse Transcriptase M-MLV, TAKARA) using random primers (TAKARA). Real-time PCR was performed using SYBR Premix Ex Taq (TAKARA) and the 7300 Real-Time PCR System (Applied Biosystems). The expression level at each time point was normalized to that of 16S rRNA (Tvrrn16S, internal control). The primers used are listed in Supplementary Table S1.

Supplementary data

Supplementary data are available at PCP online.

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