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Self-suppression of biofilm formation in the cyanobacterium *Synechococcus elongatus*

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Summary

Biofilms are consortia of bacteria that are held together by an extracellular matrix. Cyanobacterial biofilms, which are highly ubiquitous and inhabit diverse niches, are often associated with biological fouling and cause severe economic loss. Information on the molecular mechanisms underlying biofilm formation in cyanobacteria is scarce. We identified a mutant of the cyanobacterium *Synechococcus elongatus*, which unlike the wild type, developed biofilms. This biofilm-forming phenotype is caused by inactivation of homologues of type II secretion /type IV pilus assembly systems and is associated with impairment of protein secretion. The conditioned medium from a wild-type culture represses biofilm formation by the secretion-mutants. This suggested that the planktonic nature of the wild-type strain is a result of a self-suppression mechanism, which depends on the deposition of a factor to the extracellular milieu. We also identified two genes that are essential for biofilm formation. Transcript levels of these genes are elevated in the mutant compared with the wild type, and are initially decreased in mutant cells cultured in conditioned medium of wild-type cells. The particular niche conditions will determine whether the inhibitor will accumulate to effective levels and thus the described mechanism allows switching to a sessile mode of existence.

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Introduction

Biofilms are structured bacterial communities that are enclosed by a self-produced extracellular matrix. Natural cyanobacterial populations often reside in biofilms, which inhabit a wide range of niches (Crispim and Gaylarde, 2005; De Los Ríos *et al.*, 2007; Gorbushina, 2007; Boomer *et al.*, 2009; Gorbushina and Broughton, 2009; Cuzman *et al.*, 2010; Stal *et al.*, 2010; Klatt *et al.*, 2011). Contrary to the wealth of knowledge on mechanisms involved in biofilm formation, maintenance and dispersal in heterotrophs [see Reviews (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004; Stanley and Lazazzera, 2004; Branda *et al.*, 2005; Parsek and Greenberg, 2005; Kolter and Greenberg, 2006; Karatan and Watnick, 2009; Monds and O'Toole, 2009; Landini *et al.*, 2010; McDougald *et al.*, 2011; Elias and Banin, 2012; Rendueles and Ghigo, 2012) and references therein], information on the molecular basis of these processes in cyanobacteria is scarce, despite the industrial implications and environmental prevalence of phototrophic biofilms (Wagner and Loy, 2002; Ivnitsky *et al.*, 2007; Egan *et al.*, 2008; Roeselers *et al.*, 2008). Studies of the filamentous cyanobacterium, *Nostoc commune*, which exhibits extreme resistance to desiccation, UV irradiation and oxidation, established the role of the extracellular matrix protein, WspA in its resistance to harsh environmental conditions (Wright *et al.*, 2005). It has been suggested that this protein, which binds the UV-absorbing pigment, scytonemin, allows this cyanobacterium to cope with an adverse environment. An additional study identified a glycoprotein that mediates cell–cell interactions in *Microcystis aeruginosa* (Zilliges *et al.*, 2008), and provided important insight into the mechanism of cell clustering in this cyanobacterium.

Synechococcus elongatus PCC7942 (hereafter, *S. elongatus*) is a common model unicellular cyanobacterium that exhibits a planktonic phenotype while grown under bubbling or agitation. A single report documented an isolate of this cyanobacterium that exhibits biofilm formation; however, the nature of the molecular modification

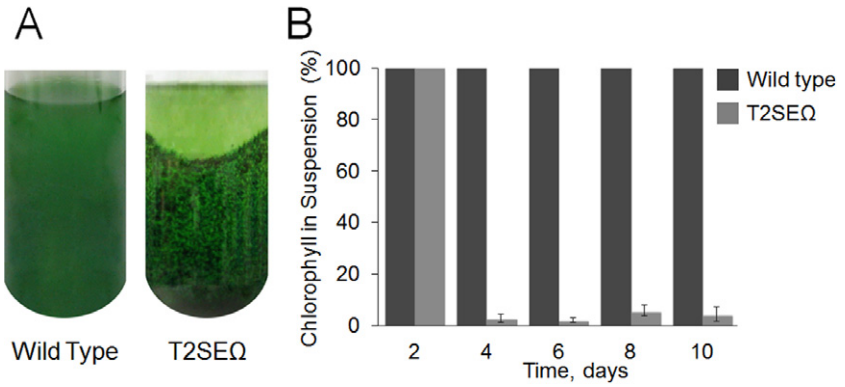

Fig. 1. Impairment of a homologue of subunit E of type II protein secretion systems (T2SE), results in a sessile phenotype.
A. The T2SE-mutant (T2SEΩ) adheres to the growth tube, in contrast to the wild-type strain, which is characterized by planktonic growth. Cultures shown are 7 days old.
B. Percentage of chlorophyll in the suspended cells. Bars indicate average of four independent cultures (± standard deviation).

leading to biofilm development and the mechanism of biofilm formation were not investigated (Jiang and Ohtaguchi, 2006). Here, we report a self-suppression mechanism of biofilm formation in *S. elongatus* and show that components of a type II protein secretion system (T2S)/type IV pilus assembly system are essential for the inhibitory process. Furthermore, we identified two genes, the products of which are required for biofilm formation, and demonstrated that their expression is governed by a substance present in the extracellular milieu of wild-type cells.

Results

Inactivation of the Synpcc7942_2071 gene results in biofilm formation

We isolated a biofilm-forming mutant of *S. elongatus* in the course of a screen for strains resistant to oxidative stress. Molecular analysis indicated that the biofilm-forming phenotype results from inactivation of the gene, pcc7942_2071 (*Experimental procedures*), which encodes a homologue of protein E of type II secretion systems (T2SE) or PilB of type IV pilus assembly apparati [components of these complexes are homologous (Peabody *et al*., 2003; Filloux, 2004; Cianciotto, 2005; Johnson *et al*., 2006)]. Analysis of conserved domains revealed that the protein encoded by this gene possesses a P-loop NTPase domain, which is found in diverse proteins including T2SE and PilB. Insertional inactivation of pcc7942_2071 impaired protein secretion (see below) and the gene is referred to as *t2sE*.

The T2SE null mutant (T2SEΩ) showed a planktonic phenotype at the exponential stage of growth; however, once reaching the stationary phase, the cells adhered to the glass tube, in contrast to the wild type, which remained planktonic under the same conditions (Fig. 1A and B). Some of the mutant cells (5–20%) remained planktonic in stationary phase (Fig. 1B). This is not due to heritable change, as the progeny of these planktonic cells exhibited the typical phenotype of a T2SEΩ culture (Fig. S1A). The mechanism underlying this phenotype of T2SEΩ is, as yet, unknown. Inactivation of T2SE did not affect biomass accumulation as measured by total chlorophyll content (Fig. S1B).

Cryo-scanning electron microscopy (cryo-SEM) revealed that T2SEΩ cells were encased in filamentous, multi-layered networks (Fig. 2A and B), which in their hydrated state, assumed a gelatinous appearance, as indicated by environmental SEM (Fig. S2).

T2SE and PilC of S. elongatus *are involved in protein secretion*

As noted above, the gene impaired in T2SEΩ, pcc7942_2071, encodes a homologue of subunit E of the

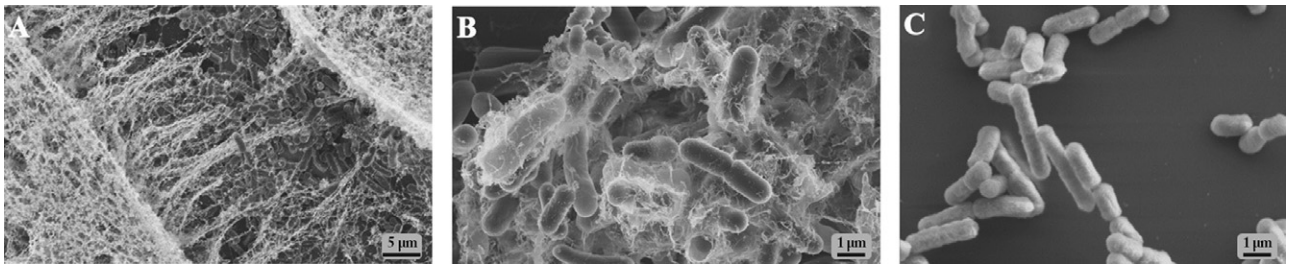

Fig. 2. Cryo-SEM reveals biofilms of T2SEΩ.
A and B. Biofilms of the T2SE-mutant of *Synechococcus elongatus*.
C. Wild-type cells. Samples shown in B and C were chemically fixed prior to cryo-fixation (see *Experimental procedures*).

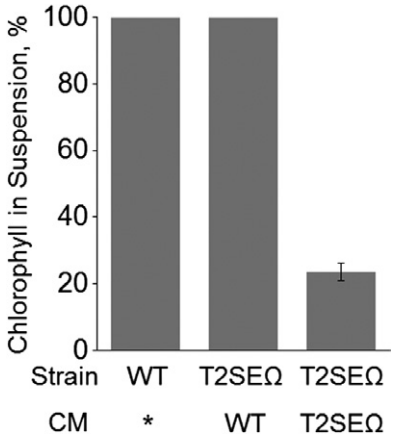

Fig. 3. Conditioned medium of wild-type culture inhibits biofilm formation. Chlorophyll in suspended cells following 14 days of growth of wild-type (WT) or T2SEΩ inoculated into fresh medium or into cell-free conditioned medium from 8-day-old cultures (CM). Conditioned medium was supplemented with nutrients. Asterisk indicates that the medium was not replaced.

bacterial type II protein secretion system. We therefore examined the conditioned medium of the wild-type and T2SEΩ strains for the presence of extracellular proteins.

The results shown in Fig. S3A indicate that T2SE is indeed involved in protein secretion. The two genes present immediately downstream of *t2sE* (Fig. S3C) encode homologues of components of type II secretion or type IV pilus assembly systems (as noted, subunits of these complexes exhibit high similarity). The protein encoded by pcc7942_2070 is annotated as PilT and the product of pcc7942_2069 exhibits motif of T2SF and is annotated as PilC. In order to examine possible involvement of these genes in protein secretion and biofilm formation we constructed and characterized the relevant mutants. Inactivation of pcc7942_2069 resulted in a sessile phenotype and impaired protein secretion, similar to inactivation of *t2sE* (Fig. S3A and B; PilCΩ). In contrast, inactivation of pcc7942_2070 affected neither protein secretion nor the planktonic nature of the culture (Fig. S3A and B, PilTΩ). This may be explained by functional redundancy, since *S. elongatus* possesses two additional genes encoding homologues of PilT (pcc7942_0847 and pcc7942_2349).

The planktonic nature of the *pilT*-mutant and in contrary, biofilm formation obtained by inactivation of the downstream gene *pilC*, negate the possibility of polar effect of inactivation of *pilT* on *pilC*. Thus, it is inconceivable that insertional inactivation of the further upstream gene, *t2sE*, has a polar effect on *pilC* (see physical map in Fig. S3C).

Interestingly, inactivation of *t2sE* rendered the mutant non-transformable, contrary to the parental strain that naturally takes up externally applied DNA. This phenotype of the mutant may be explained in light of the similarity between DNA uptake systems and type II secretion/type IV pilus assembly systems (Chen and Dubnau, 2004).

An inhibitory factor for biofilm formation is present in the extracellular milieu of the wild type

As shown thus far, the genetic programme that underlies biofilm formation is not manifested in the wild-type strain under standard growth conditions. Since the biofilm-forming mutants of *S. elongatus* are impaired in protein secretion, we hypothesized that the wild-type strain secretes a substance that inhibits biofilm formation. To test this, we inoculated T2SEΩ into cell-free conditioned medium from an 8-day-old wild-type culture (hereafter conditioned medium); under these conditions the mutant did not form biofilms (Fig. 3). We also inoculated T2SEΩ cells into 8-day-old cell-free conditioned medium from a T2SEΩ culture to test if the manipulation of the T2SEΩ culture (centrifugation and resuspension) interferes with the ability of this strain to form biofilms. Such conditioned medium did not prevent biofilm formation (Figs 3 and S4A), supporting the conclusion that at this stage of growth, conditioned medium from the wild-type strain contains an inhibitory compound not secreted by the mutant. This substance is effective in prevention of biofilm formation, but does not disperse a preformed biofilm (Fig. S4B).

Conditioned medium of older cultures of T2SEΩ (14 days) inhibited biofilm formation (Fig. S4A). This suggests that T2SEΩ is capable of synthesizing the inhibitory compound at sufficiently high levels for inhibition of biofilm; inactivation of *t2sE* either retards, but does not entirely block, the secretion of the negative factor. Alternatively, cell lysis in late stationary phase may allow for the release of internal stores of this inhibitor.

Initial characterization indicated that the biofilm-inhibiting substance is a heat stable compound, which is resistant to protease treatment (Table 1). Furthermore, this factor is of low molecular weight (< 3 kDa), thus, the

Table 1. Effect of various treatments of conditioned medium on biofilm formation.

Growth conditions	Treatment	Biofilm formation
Fresh medium	None	+
Conditioned medium	None	–
	Autoclave (120°C, 45 min)	–
	Protease mixture	–
	3 kDa-filtrate	–

Protease-treatment was performed using Pronase E from Sigma-Aldrich (0.5%, 20 h, 37°C) following autoclave treatment for inactivation of the proteases. Minus symbol (–) indicates lack of biofilm formation.

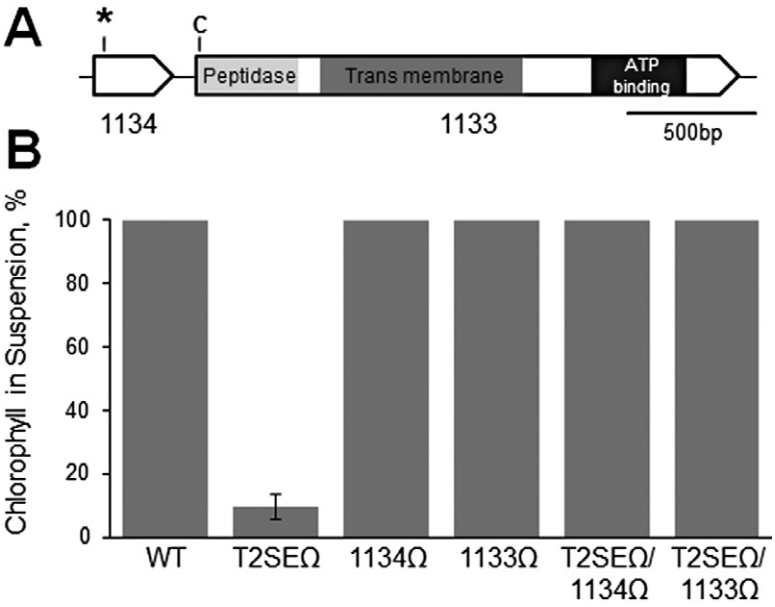

Fig. 4. The genes pcc7942_1134 and pcc7942_1133 are essential for biofilm formation.
A. Physical map of the genomic region of pcc7942_1134 and pcc7942_1133. The peptidase, transmembrane and ATP binding domains of the pcc7942_1133 protein are indicated. Asterisk denotes the putative cleavage site of the pcc7942_1134 protein, whereas 'C' designates the position of the cysteine residue typically involved in processing of a double-glycine containing substrate.
B. Chlorophyll in suspended cells following 14 days of growth in wild-type culture (WT), and cultures of the mutants T2SEΩ, 1134Ω, 1133Ω, and the double mutants, T2SEΩ/1134Ω and T2SEΩ/1133Ω.

proteins detected in the extracellular milieu of the wild type, which are absent in the case of the mutant (Fig. S3, days 2 and 4), do not correspond to this inhibitor. Taken together, it may be suggested that the inhibitor is a non-peptide compound or a modified short peptide that escaped the protease treatment.

Biofilm formation in S. elongatus *requires the genes pcc7942_1134 and pcc7942_1133*

Thus far, we presented evidence for self-suppression of biofilm development in *S. elongatus*. In addition, we attempted to identify genes essential for the process of biofilm formation once it is derepressed. A bioinformatics approach aimed at identification of secreted peptides revealed that the product of pcc7942_1134 is characterized by an N-terminus 'double-glycine motif' (Fig. S5). Such a motif, which occurs just upstream of the cleavage site of precursor peptides, was initially characterized in secreted peptides of Gram-positive bacteria, but was later shown to be present in Gram-negative bacteria as well (Dirix *et al*., 2004). Importantly, a recent comprehensive genome mining approach indicated the widespread occurrence of such a secretion motif in cyanobacteria (Wang *et al*., 2011). The putative precursor product of the gene pcc7942_1134 is of 102 amino acids and cleavage after the glycine motif should yield a short protein of 60 amino acids; however, further processing yielding a much smaller active compound should not be excluded. The gene immediately downstream of pcc7942_1134 encodes a putative component of transporters, which process their designated substrates by cleaving off the N-terminal glycine-motif. The product of pcc7942_1133 possesses an N-terminal peptidase C-39 domain exhibiting the putative catalytic cysteine residue followed by a transmembrane binding domain and an ATP binding domain (Fig. 4A).

Insertional inactivation was used to examine possible involvement of the products of pcc7942_1134 and pcc7942_1133 in biofilm formation. Inactivation on a wild-type genetic background did not change the planktonic nature of growth of the parental strain; however, biofilm formation was abolished in the double T2SEΩ/1134Ω mutant, as well as in T2SEΩ/1133Ω (Fig. 4B). To test for a possible polar effect of the insertional inactivation of pcc7942_1134 on the downstream gene (pcc7942_1133), we constructed a T2SEΩ/1134Ω mutant possessing pcc7942_1134 *in trans*. This strain formed a biofilm (Fig. S6), demonstrating that both pcc7942_1134 and pcc7942_1133 are required for biofilm formation.

Conditioned medium affects the transcript levels of pcc7942_1134 and pcc7942_1133

RT-PCR analysis was used to follow changes in transcript level from the genes pcc7942_1134 and pcc7942_1133 over time. Examination of pcc7942_1134 revealed that T2SEΩ cells, at a growth stage prior to biofilm formation, exhibited a ~2.5-fold increase in transcript level compared with wild type (Fig 5, day 1, Fresh Medium). High transcript abundance from this gene (four- to fivefold increase) was observed in both the suspended and the sessile cells of T2SEΩ (Fig 5).

Conditioned medium prevented biofilm formation (Fig 3); thus, we tested its effect on transcript abundance of these biofilm-essential genes. Initially, T2SEΩ cultured

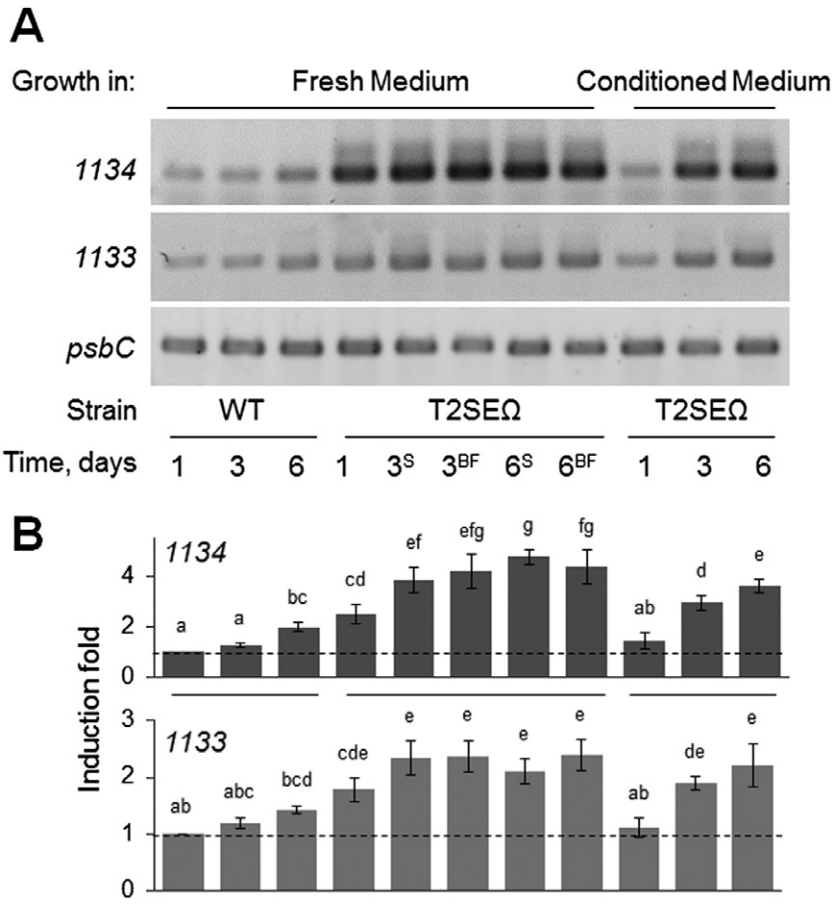

Fig. 5. Transcript level of pcc7942_1134 and pcc7942_1133 as affected by fresh or conditioned-medium. Wild-type (WT) and T2SEΩ cultures were analysed. Transcript level of *psbC*, encoding CP43, a chlorophyll binding protein of photosystem II, served to normalize the data. 'Induction fold' indicates transcript abundance relative to the level in wild-type cells on the first day of growth in fresh medium (depicted by the dashed horizontal line). In cases where biofilm was formed, the transcript level is shown for suspended cells (S) and cells in the biofilm (BF).
A. Gel image of PCR products of a representative experiment.
B. Averages ± standard deviations of data from three independent biological experiments. In each bar-graph, different letters assign statistical significance [One-way ANOVA multiple comparisons (Duncan's test) was employed. For 1134: $P < 0.001$. For 1133: $P < 0.00001$].

in conditioned medium exhibited transcript levels comparable to the wild type (Fig 5, day 1). Growth in conditioned medium for a longer time resulted in increased transcript abundance (compare days 3 and 6 with day 1), although these culture conditions did not support biofilm formation (Figs 3 and 5). Similar trends of changes in transcripts abundance were observed for pcc7942_1133 (Fig 5).

Taken together, these data suggest that an increase in transcript level of the genes, pcc7942_1134 and pcc7942_1133, is required but not sufficient for biofilm formation. Conditioned medium of wild-type cells interferes with the initial accumulation of transcripts of these essential genes, and thus prevents the formation of a biofilm (Figs 3 and 5). Though accumulation of transcripts of these genes is required to promote biofilm formation, their presence may not be sufficient, as indicated by the high transcript level in the subpopulation of T2SEΩ that grow in suspension.

Discussion

This study demonstrates a process of self-suppression of biofilm development, which depends on an extracellular biofilm inhibiting factor, thereby suggesting intercellular communication as part of this mechanism. Possibly, the inhibitory pathway of *S. elongatus* evolved to prevent aggregation and thereby to maximize light absorption for photosynthesis. Such a 'community escape response' was reported for the photosynthetic bacterium *Rhodobacter sphaeroides* (Puskas *et al*., 1997). On the other hand, biofilm formation may at times be advantageous (e.g. cells in biofilms can withstand harsh conditions, and metabolic cooperation within multispecies consortia is beneficial). Thus, diverse cyanobacteria, including various *Synechococcus* species, are found in microbial mats in their natural habitats (Miller *et al*., 1998; Becker *et al*., 2004; Kilian *et al*., 2007; Gorbushina and Broughton, 2009; Becraft *et al*., 2011). Possibly, in such open growth niches the inhibitor may diffuse and thus may not reach an effective concentration for prevention of biofilm development. Additionally, the possibility that laboratory strains of *S. elongatus* were selected for elevated secretion of the inhibitor should not be excluded. Taken together, cyanobacteria exhibit a mechanism enabling them to control their mode of existence, either in a sessile or in a planktonic form of growth.

We suggest that a type II system, in which T2SE and PilC participate, is involved in secretion of an inhibitory factor. As noted above, this substance is of low molecular weight (< 3 kDa) and therefore, it may be suggested that the T2S system directly secretes a small peptide that prevents biofilm development, though typically, T2S apparati secrete proteins. An additional possibility involves the transport of an enzyme that is anchored to the outer cell surface, which is required for the formation of the inhibitory substance. Support for the latter scenario is gained from a study implicating the second type II secretion system (stt) of the phytopathogen *Dickeya dadantii*, in deposition of pectin lyase on the cell surface (Ferrandez and Condemine, 2008).

The possibility that T2SE and PilC take part in pilus assembly should not be excluded. Impairment of pili formation or pilus retraction may promote biofilm formation by hampering motility, thereby interfering with cell detachment after the initial adherence to the surface (Burrows, 2012).

The extracellular milieu of certain bacteria was shown to contain compounds that inhibit biofilm formation by other, often distantly related bacteria (Kolodkin-Gal *et al.*, 2010; 2012; Rendueles and Ghigo, 2012). Some of these compounds also cause self-inhibition of biofilm development, specifically, capsular polysaccharides of particular uropathogenic strains of *Escherichia coli* (Valle *et al.*, 2006), and D-amino acids (Kolodkin-Gal *et al.*, 2010) and norspermidine (Kolodkin-Gal *et al.*, 2012) produced by *Bacillus subtilis*. The biofilm inhibitors of *B. subtilis* also lead to dispersal of an existing biofilm, in contrast to the inhibitor of *S. elongatus* that was active only in preventing biofilm development (Figs S4 and 3).

We also identified two genes, pcc7942_1133 and pcc7942_1134, the products of which are essential for biofilm formation (Fig. 4). The product of pcc7942_1134 possesses a glycine-motif shared by secreted small proteins (Fig. S5), whereas, as suggested by its homology (Fig. 4A), the product of pcc7942_1133 may take part in secretion and processing of the pcc7942_1134 product. Transcript abundance of both of these genes is increased in the T2SEΩ mutant (Fig. 5), whereas culturing the mutant in wild-type conditioned medium initially decreased expression of these transcripts, although their increase at later time points apparently does not allow biofilm formation (Figs 3 and 5).

Our results are consistent with a mechanism of self-suppression of biofilm development by an extracellular substance that affects expression of genes essential for biofilm formation. Conceivably, a T2S system secretes a factor, which eventually leads to reduction in the transcript level of the genes essential for biofilm development, pcc7942_1134 and pcc7942_1133. Under these inhibitory conditions, the gene products are not present at sufficient levels to support biofilm development, and thus the wild-type remains planktonic. Inactivation of the T2S system releases the cell from the suppression imposed by the extracellular inhibitor. Accordingly, elevated expression of the genes pcc7942_1134 and pcc7942_1133 allows the production, secretion and concomitant processing of the pcc7942_1134 protein, yielding a product that is required for biofilm development.

Experimental procedures

Strains, culture conditions and initial isolation of the t2sE mutant

Synechococcus elongatus PCC7942 and all derived strains were cultured in Pyrex tubes containing BG11 medium (Stanier *et al.*, 1971) buffered to pH 8.0 with 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) under bubbling with 5% CO_2 in air. Incandescent light producing a flux of 20 μmole photons m^{-2} s^{-1} was used as a light source. The initial biofilm forming strain was isolated in the course of a screen for mutants resistant to oxidative stress. This mutant did not reproducibly exhibit the elevated resistance to H_2O_2 that was initially detected; thus, we did not continue characterization of this aspect of the phenotype. The library of mutants used for screening was raised by random transposon insertion, as previously described (Sendersky *et al.*, 2005). Identification of the transposon insertion site in the biofilm forming mutant was performed as follows: Genomic DNA was digested with *Pst*I and ligated to pBluescript KS. Transformants of *E. coli* (DH5α) were selected in the presence of kanamycin; only clones containing the insert were resistant to the antibiotic due to *nptII* included in the transposon. The sequence of the genomic regions neighbouring the transposon was determined using the transposon-specific primers provided with the EZ::TNTM insertion kit. The transposon was found to be inserted 1072 nucleotides after the beginning of the open reading frame (ORF) of T2SE.

Molecular manipulations of cyanobacterial cells

Gene disruption by insertional inactivation was performed by PCR-amplification of the relevant fragment, cloning into pGEM®T Easy vector (Promega), and insertion of an antibiotic-resistance cassette. The resulting construct was transformed into *S. elongatus*, and clones resistant to the particular antibiotic were selected. PCR on genomic DNA confirmed double homologous recombination and complete chromosome segregation. Additional cloning information is provided in Table S1 (see legend for details).

Inactivation of *t2SE* impaired the natural DNA competence characterizing *S. elongatus*; thus, to obtain T2SEΩ strains with additional genetic changes, we initially introduced the other molecular modifications and subsequently inactivated *t2sE*. For example, to obtain the double mutant T2SEΩ/1134Ω we transformed the 1134Ω strain with a plasmid for inactivation of *t2sE*. PCR was employed at each stage to verify the desired genotype.

Analysis of secreted proteins, collection of conditioned medium and chlorophyll measurement for biofilm quantification

Media from 30 ml cultures was collected after centrifugation, desiccated, and resuspended in 300 μl TE buffer supplemented with a protease inhibitor cocktail (Sigma). Samples equivalent to 3 ml of the original media were boiled for 5 min, separated on a Tris-Tricine gel (Schägger *et al*., 1988) and detected using silver staining.

For collection of conditioned medium, cultures were centrifuged (5000 *g*, 10 min) at room temperature, and the supernatant was removed and passed through 0.22 μm filter. This conditioned medium was supplemented with nutrients by addition of medium stock solutions as in the preparation of fresh growth medium.

For assessment of biofilm formation, cultures were initiated at optical density (750 nm) of 0.5. The suspended fraction was sampled for chlorophyll determination by extraction in 80% acetone (final concentration). Determination of chlorophyll in the biofilm was performed following removal of the suspended fraction and addition of 80% acetone to the sessile cells. Chlorophyll in the suspended cells as percent of total chlorophyll served as a measure for biofilm formation.

RNA preparation and RT-PCR

RNA was prepared as previously described (Tu *et al*., 2004) and treated with DNase (TURBO DNase, Ambion). Random hexamers (Promega) were used to prime cDNA using 1.5 μg RNA and reverse transcriptase (RevertAid, Fermentas). PCR amplification was performed using specific primers (as indicated in Table S1) and DNA polymerase (DreamTaq, Fermentas). Gel images were quantified using ImageJ. PCR samples were diluted to meet the linear range of the signal.

Electron microscopy

For cryo-SEM analyses of the T2SEΩ, Aclar slides were placed into growth tubes containing the T2SEΩ cultures (for 6 days) so that biofilms were formed on the slide. The slides were then immersed into fixation buffer (3% paraformaldehyde, 2% gluteraldehyde, 1% sucrose, 5 mM $CaCl_2$, 0.1 M CaCO buffer), shaken for 1 h at room temperature and then placed overnight at 4°C. Alternatively, biofilms of T2SEΩ on Aclar discs were cryo-fixed without prior chemical fixation.

Wild-type cells, which grow planktonically, could not be grown directly on the slide; hence, the culture was mixed with an equal volume of 2× fixation buffer and incubated as described for T2SEΩ. The cells were then concentrated 10-fold and adhered to slides that were pretreated with poly-lysine.

All samples were washed in double-distilled water and were plunged into liquid ethane using a custom made spring-loaded plunger. The frozen samples were mounted on a holder and transferred to a BAF 60 freeze fracture device (Bal-Tec) using a VCT 100 Vacuum Cryo Transfer device (Bal-Tec). Samples were freeze-dried at a temperature of −100°C for 30 min and coated with 3 nm Pt/C by double axis rotary shadowing. An additional backing layer of 5 nm carbon was evaporated perpendicularly. Samples were transferred to an Ultra 55 SEM (Zeiss, Germany), using a VCT 100 and were observed using a secondary electron in-lens detector at 2–5 kV at a temperature of −120°C.

For analysis by environmental SEM, 8 mm diameter round cover glass slides were placed into growth tubes containing the T2SEΩ cultures so that biofilms were formed on the slides. The slides were then immersed into fixation buffer (3% paraformaldehyde, 2% gluteraldehyde, 1% sucrose, 5 mM CaCl2, 0.1 M CaCO buffer), shaken for 1 h at room temp then placed overnight at 4°C. The specimen, immersed in water, was placed in a holder and kept at 5°C on the Peltier stage of the SEM chamber. The chamber was flooded three times at pressures between 9.4 and 6 Torr, and then the excess water was slowly evaporated near the dew point. After the Wet Mode was completed, the slide was left overnight on the bench for drying, then sputtered with gold to form a 15 nm thick cover and observed again using the SEM.

Identification of a double glycine motif in ORF 1134

Multiple Expectation Maximization for motif Elicitation was used to define a Position Specific Scoring Matrix based on previously reported bacterial double-glycine motifs. The matrix obtained was used to search the *S. elongatus* nucleotide database translated in six frames, using Motif Alignment and Search Tool.

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References

Becker, S., Singh, A.K., Postius, C., Böger, P., and Ernst, A. (2004) Genetic diversity and distribution of periphytic *Synechococcus* spp. in biofilms and picoplankton of Lake Constance. *FEMS Microbiol Ecol* **49:** 181–190.

Becraft, E.D., Cohan, F.M., Kühl, M., Jensen, S.I., and Ward, D.M. (2011) Fine-scale distribution patterns of *Synechococcus* ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. *Appl Environ Microbiol* **77:** 7689–7697.

Boomer, S.M., Noll, K.L., Geesey, G.G., and Dutton, B.E. (2009) Formation of multilayered photosynthetic biofilms in an alkaline thermal spring in Yellowstone National Park, Wyoming. *Appl Environ Microbiol* **75:** 2464–2475.

Branda, S.S., Vik, S., Friedman, L., and Kolter, R. (2005) Biofilms: the matrix revisited. *Trends Microbiol* **13:** 20–26.

Burrows, L.L. (2012) *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol* **66:** 493–520.

Chen, I., and Dubnau, D. (2004) DNA uptake during bacterial transformation. *Nat Rev Microbiol* **2:** 241–249.

Cianciotto, N.P. (2005) Type II secretion: a protein secretion system for all seasons. *Trends Microbiol* **13:** 581–588.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappinscott, H.M. (1995) Microbial biofilms. *Annu Rev Microbiol* **49:** 711–745.

Crispim, C., and Gaylarde, C. (2005) Cyanobacteria and biodeterioration of cultural heritage: a review. *Microb Ecol* **49:** 1–9.

Cuzman, O.A., Ventura, S., Sili, C., Mascalchi, C., Turchetti, T., D'Acqui, L.P., *et al*. (2010) Biodiversity of phototrophic biofilms dwelling on monumental fountains. *Microb Ecol* **60:** 81–95.

De Los Ríos, A., Grube, M., Sancho, L.G., and Ascaso, C. (2007) Ultrastructural and genetic characteristics of endolithic cyanobacterial biofilms colonizing Antarctic granite rocks. *FEMS Microbiol Ecol* **59:** 386–395.

Dirix, G., Monsieurs, P., Dombrecht, B., Daniels, R., Marchal, K., Vanderleyden, J., *et al*. (2004) Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides* **25:** 1425–1440.

Egan, S., Thomas, T., and Kjelleberg, S. (2008) Unlocking the diversity and biotechnological potential of marine surface associated microbial communities. *Curr Opin Microbiol* **11:** 219–225.

Elias, S., and Banin, E. (2012) Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev* **36:** 990–1004.

Ferrandez, Y., and Condemine, G. (2008) Novel mechanism of outer membrane targeting of proteins in Gram-negative bacteria. *Mol Microbiol* **69:** 1349–1357.

Filloux, A. (2004) The underlying mechanisms of type II protein secretion. *Biochim Biophys Acta* **1694:** 163–179.

Gorbushina, A.A. (2007) Life on the rocks. *Environ Microbiol* **9:** 1613–1631.

Gorbushina, A.A., and Broughton, W.J. (2009) Microbiology of the atmosphere-rock interface: how biological interactions and physical stresses modulate a sophisticated microbial ecosystem. *Annu Rev Microbiol* **63:** 431–450.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2:** 95–108.

Ivnitsky, H., Katz, I., Minz, D., Volvovic, G., Shimoni, E., Kesselman, E., *et al*. (2007) Bacterial community composition and structure of biofilms developing on nanofiltration membranes applied to wastewater treatment. *Water Res* **41:** 3924–3935.

Jiang, G., and Ohtaguchi, K. (2006) Kinetics of biofilm formation of a cyanobacterium on bioreactor surfaces. *J Chem Eng Jpn* **39:** 453–460.

Johnson, T.L., Abendroth, J., Hol, W.G., and Sandkvist, M. (2006) Type II secretion: from structure to function. *FEMS Microbiol Lett* **255:** 175–186.

Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73:** 310–347.

Kilian, O., Steunou, A.S., Fazeli, F., Bailey, S., Bhaya, D., and Grossman, A.R. (2007) Responses of a thermophilic *Synechococcus* isolate from the microbial mat of Octopus Spring to light. *Appl Environ Microbiol* **73:** 4268–4278.

Klatt, C.G., Wood, J.M., Rusch, D.B., Bateson, M.M., Hamamura, N., Heidelberg, J.F., *et al*. (2011) Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. *ISME J* **5:** 1262–1278.

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010) D-amino acids trigger biofilm disassembly. *Science* **328:** 627–629.

Kolodkin-Gal, I., Cao, S., Chai, L., Bottcher, T., Kolter, R., Clardy, J., *et al*. (2012) A self-produced trigger for biofilm disassembly that targets exopolysaccharide. *Cell* **149:** 684–692.

Kolter, R., and Greenberg, E.P. (2006) Microbial sciences: the superficial life of microbes. *Nature* **441:** 300–302.

Landini, P., Antoniani, D., Burgess, J.G., and Nijland, R. (2010) Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol* **86:** 813–823.

McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D., and Kjelleberg, S. (2011) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* **10:** 39–50.

Miller, S.R., Wingard, C.E., and Castenholz, R.W. (1998) Effects of visible light and UV radiation on photosynthesis in a population of a hot spring Cyanobacterium, a *Synechococcus* sp., subjected to high-temperature stress. *Appl Environ Microbiol* **64:** 3893–3899.

Monds, R.D., and O'Toole, G.A. (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol* **17:** 73–87.

Parsek, M.R., and Greenberg, E.P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13:** 27–33.

Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P., and Saier, M.H., Jr. (2003) Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology* **149:** 3051–3072.

Puskas, A., Greenberg, E.P., Kaplan, S., and Schaefer, A.L. (1997) A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J Bacteriol* **179:** 7530–7537.

Rendueles, O., and Ghigo, J.M. (2012) Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiol Rev* **36:** 972–989.

Roeselers, G., Loosdrecht, M.C., and Muyzer, G. (2008) Phototrophic biofilms and their potential applications. *J Appl Phycol* **20:** 227–235.

Schägger, H., Aquila, H., and Von Jagow, G. (1988) Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. *Anal Biochem* **173:** 201–205.

Sendersky, E., Lahmi, R., Shaltiel, J., Perelman, A., and Schwarz, R. (2005) NblC, a novel component required for pigment degradation during starvation in *Synechococcus* PCC 7942. *Mol Microbiol* **58:** 659–668.

Stal, L.J., Severin, I., and Bolhuis, H. (2010) The ecology of nitrogen fixation in cyanobacterial mats. *Recent Advances in Phototrophic Prokaryotes* **675:** 31–45.

Stanier, R.Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. (1971) Purification and properties of unicellular

blue-green algae (order Chroococcales). *Bacteriol Rev* **35:** 171–205.
Stanley, N.R., and Lazazzera, B.A. (2004) Environmental signals and regulatory pathways that influence biofilm formation. *Mol Microbiol* **52:** 917–924.
Tu, C.J., Shrager, J., Burnap, R.L., Postier, B.L., and Grossman, A.R. (2004) Consequences of a deletion in *dspA* on transcript accumulation in *Synechocystis* sp. strain PCC6803. *J Bacteriol* **186:** 3889–3902.
Valle, J., Da Re, S., Henry, N., Fontaine, T., Balestrino, D., Latour-Lambert, P., *et al*. (2006) Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. *Proc Natl Acad Sci USA* **103:** 12558–12563.
Wagner, M., and Loy, A. (2002) Bacterial community composition and function in sewage treatment systems. *Curr Opin Biotechnol* **13:** 218–227.
Wang, H., Fewer, D.P., and Sivonen, K. (2011) Genome mining demonstrates the widespread occurrence of gene clusters encoding bacteriocins in cyanobacteria. *PLoS ONE* **6:** e22384.
Wright, D.J., Smith, S.C., Joardar, V., Scherer, S., Jervis, J., Warren, A., *et al*. (2005) UV irradiation and desiccation modulate the three-dimensional extracellular matrix of *Nostoc commune* (Cyanobacteria). *J Biol Chem* **280:** 40271–40281.
Zilliges, Y., Kehr, J.C., Mikkat, S., Bouchier, C., de Marsac, N.T., Borner, T., *et al*. (2008) An extracellular glycoprotein is implicated in cell-cell contacts in the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806. *J Bacteriol* **190:** 2871–2879.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A. Progeny of the planktonic cells of T2SEΩ form biofilms once re-inoculated into fresh medium. Chlorophyll content in suspended cells following 14 days of growth of wild-type (WT) or T2SEΩ culture inoculated from suspended cells.
B. Inactivation of T2SE does not affect biomass accumulation as measured by total chlorophyll.
Fig. S2. Environmental SEM of T2SEΩ. Hydrated sample showing the gelatinous nature of the extracellular matrix surrounding the cells (A), and sample following air drying overnight (B).
Fig. S3. Impairment of protein secretion correlates with the sessile phenotype.
A. Conditioned media from wild-type (WT) and T2SEΩ, PilTΩ and PilCΩ cultures at different stages of growth, analysed by gel electrophoresis followed by silver staining. Arrows indicate molecular weight in kDa.
B. Percentage of chlorophyll in the suspended cells in WT, T2SEΩ, PilTΩ and PilCΩ.
C. Physical map of pcc7942_2071 (encoding T2SE) and its downstream genes (pcc7942_2070 and pcc7942_2069, annotated as type II secretion/type IV pilus assembly system components, PilT and PilC). *BamHI* and *HinDIII* were used for insertional inactivation of T2SE and PilC respectively. For inactivation of PilT, the fragment between the *DraII* sites was deleted, and replaced with a kanamycin resistance cassette.
Fig. S4. A. Biofilm inhibition by conditioned medium of the T2SE-mutant (T2SEΩ). Conditioned medium from 8-day-old T2SEΩ inhibits biofilm formation in contrast to conditioned medium from 14-day-old mutant culture.
B. Conditioned medium from wild-type culture does not cause dispersal of a preformed biofilm of T2SEΩ. Biofilms of T2SEΩ (at the ninth day of growth) were re-suspended in either fresh medium (FM) or conditioned medium of 14-day-old wild-type culture (CM). The latter was confirmed to be active in prevention of biofilm formation.
Fig. S5. Sequence of the double-glycine motif of the product of pcc7942_1134 and selected secreted peptides. Cerein7B of *Bacillus cereus*, EnterocinA and EnterocinB of *Enterococcus faceum*, MicrocinE492 of *Klebsiella pneumonia*, and Microcin24, MicrocinH47, and ColicinV of *E. coli*. Black shading indicates the double-glycine or glycine-alanine present just prior to the peptide cleavage site (arrow). Positions typically occupied by hydrophobic or hydrophilic amino acids are indicated by a circle and a reversed triangle respectively.
Fig. S6. Biofilm formation in *S. elongatus* requires the genes pcc7942_1134 and pcc7942_1133.
A. The genomic region of pcc7942_1134 and fragments used for complementation of the double inactivated mutant, T2SEΩ/1134Ω.
B. Biofilm formation by the different genotypes. Inactivation of syn_PCC1134 in the T2SEΩ mutant abolishes biofilm formation. A DNA fragment bearing pcc7942_1134 (EN2) complements the phenotype of the double mutant T2SEΩ/1134Ω and restores capacity to form a biofilm. Deletion of most of the coding sequence of pcc7942_1134 (EN4) yields a fragment that does not support complementation of the T2SEΩ/1134Ω strain.
Table S1. Summary of molecular manipulations.