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Brief Report

A microcin processing peptidase-like protein of the cyanobacterium *Synechococcus elongatus* is essential for secretion of biofilm-promoting proteins

Rami Parnasa,[1] Eleonora Sendersky,[1] Ryan Simkovsky,[2] Hiba Waldman Ben-Asher,[1] Susan S. Golden[2] and Rakefet Schwarz[1]*

[1]*The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.*
[2]*Division of Biological Sciences, University of California, San Diego, La Jolla, CA, 92093, USA.*

Summary

Small secreted compounds, e.g. microcins, are characterized by a double-glycine (GG) secretion motif that is cleaved off upon maturation. Genomic analysis suggests that small proteins that possess a GG motif are widespread in cyanobacteria; however, the roles of these proteins are largely unknown. Using a biofilm-proficient mutant of the cyanobacterium *Synechococcus elongatus* PCC 7942 in which the constitutive biofilm self-suppression mechanism is inactivated, we previously demonstrated that four small proteins, Enable biofilm formation with a GG motif (EbfG1-4), each with a GG motif, enable biofilm formation. Furthermore, a peptidase belonging to the C39 family, Peptidase transporter enabling Biofilm (PteB), is required for secretion of these proteins. Here, we show that the microcin processing peptidase-like protein encoded by gene Synpcc7942_1127 is also required for biofilm development – inactivation of this gene in the biofilm-proficient mutant abrogates biofilm development. Additionally, this peptidase-like protein (denoted EbfE – enables biofilm formation peptidase) is required for secretion of the EbfG biofilm-promoting small proteins. Given their protein-domain characteristics, we suggest that PteB and EbfE take part in a maturation-secretion system, with PteB being located to the cell membrane while EbfE is directed to the periplasmic space via its secretion signal.

Received 30 October, 2018; accepted 13 March, 2019. *For correspondence. E-mail rakefet.schwarz@biu.ac.il; Tel. +972 3 531 7648; Fax: +972 3 738 4058.

Introduction

The switch from a planktonic to a sessile lifestyle strongly affects the ability of an organism to acquire nutrients (Stanley and Lazazzera, 2004; Kostakioti *et al.,* 2013; Flemming *et al.,* 2016). Light strongly affects the nutritional status of photosynthetic microorganisms including cyanobacteria, and thus planktonic vs sessile is a fundamental cyanobacterial behavioural decision that drastically alters its energy inputs. For example, under limiting light conditions, self-shading in biofilms may further restrict light availability, and consequently slow down cell growth (Bolhuis *et al.,* 2014). In contrast, cell clustering may serve as a protective mechanism under damaging high-light intensities (Koblizek *et al.,* 2000). The mechanisms that underlie a sessile/floating lifestyle transition, however, only started emerging in recent years. Cyclic-di-GMP, a known second messenger that regulates biofilm development in heterotrophic bacteria, promotes biofilm formation in the cyanobacterium *Synechocystis* sp. PCC6803 (Agostoni *et al.,* 2016). Furthermore, studies of the thermophilic cyanobacterium *Thermosynechococcus vulcanus* identified three cyanobacteriochrome photoreceptors that mediate light-colour input and control cell aggregation via c-di-GMP signalling (Enomoto *et al*., 2015). Further analysis identified the protein Tlr1612, which acts downstream of the cyanobacteriochromes and serves as a repressor of cell aggregation under teal-green illumination (Enomoto *et al.,* 2018).

Additional studies revealed conditions and uncovered components that promote cell aggregation and surface attachment in some species. For example, *Acaryochloris marina*, a cyanobacterium that contains the far-red light absorbing pigment chlorophyll *d* exhibits increased aggregation and surface attachment under far-red light (Hernandez-Prieto *et al.,* 2018). Genetic studies implicated the polyamine spermidine in the regulation of aggregation in *Synechocystis,* in which inactivation of two arginine decarboxylases resulted in reduced spermidine content and enhanced aggregation (Kera *et al.,* 2018). Other studies of this cyanobacterium support involvement of

extracellular polysaccharides in surface adhesion (Fisher *et al.,* 2013) and cell sedimentation (Jittawuttipoka *et al.,* 2013). Cellulose accumulation is responsible for cell aggregation in *T. vulcanus* RKN (Kawano *et al.,* 2011). In the thermophilic cyanobacterium *Synechococcus elongatus* Näg strain Kovrov, light-induced cell aggregation is mediated by components of the photosynthetic electron transport chain downstream of photosystem I (Koblizek *et al.,* 2000). [Note that cyanobacterial nomenclature has been revised and this organism is not in the same genus as species currently designated as *S. elongatus* (Herdman *et al.,* 2001).]

Cell–cell interactions are crucial for the development of sessile biofilms, and for the formation of cell clusters that are not attached to a substratum. In some cases, proteins involved in the physical interaction between cyanobacterial cells have been identified. The exoprotein HesF of *Anabaena* sp. PCC 7120 is required for filament adhesion and aggregation (Oliveira *et al.,* 2015), and the surface glycoprotein MrpC is implicated in cell–cell attachment in *Microcystis aeruginosa* PCC 7806 (Zilliges *et al.,* 2008).

Previously, we demonstrated that *S. elongatus* PCC 7942 possesses an autoinhibitory mechanism that actively suppresses biofilm formation by constitutively depositing one or more inhibitors of biofilm formation to the extracellular milieu (Schatz *et al.,* 2013; Nagar and Schwarz, 2015). Inactivation of Synpcc7942_2071, encoding a protein homologous to the ATPase subunit of type II secretion systems (T2SE) and to PilB, the assembly ATPase of the type IV pilus assembly system, abrogates the inhibitory process and enables the mutant to form biofilms as observed by scanning electron microscopy (SEM), environmental SEM and confocal fluorescence microscopy (Schatz *et al.,* 2013; Parnasa *et al.,* 2016). Furthermore, inactivation of Synpcc7942_2071 impairs protein secretion and prevents the formation of cell pili (Schatz *et al.,* 2013; Nagar *et al.,* 2017). In addition, we demonstrated the involvement of small secreted proteins characterized by double-glycine (GG) secretion motifs in biofilm development (Parnasa *et al.,* 2016).

GG-motifs are N-terminal secretion signals that allow secretion of multiple families of natural products including microcins (van Belkum *et al.,* 1997; Riley and Wertz, 2002; Dirix *et al.,* 2004; Arnison *et al.,* 2013; Yang *et al.,* 2014; Chikindas *et al.,* 2018). Data mining indicated that genes encoding small proteins with GG-motifs and their putative related transporters are prevalent in cyanobacteria (Haft *et al.,* 2010; Wang *et al.,* 2011; Micallef *et al.,* 2015); however, the roles of these cyanobacterial components are largely unknown. We demonstrated that four small proteins with GG-motifs similar to those of microcins enable biofilm development in a biofilm-proficient mutant of *S. elongatus* (T2SEΩ) (Parnasa *et al.,* 2016). These proteins were designated EbfG1-4 (for Enable biofilm formation with a GG motif).

Studies of heterotrophic bacteria established a model in which the GG-motif leader peptide is cleaved off during transport by the N-terminal domain of a transporter component, which belongs to the Peptidase C39 protein family (Michiels *et al.,* 2001; Bobeica *et al.,* 2019). Mutation of the conserved cysteine of the peptidase domain of the protein encoded by Synpcc7942_1133 of *S. elongatus* (denoted PteB for Peptidase transporter enabling Biofilm), which belongs to the C39 family, indicated its requirement for biofilm development and for proper secretion of the EbfG small proteins (Parnasa *et al.,* 2016).

The gene Synpcc7942_1127 encodes a protein annotated as a 'microcin-processing peptidase'. This annotation guided investigation of possible involvement of its product in the secretion of the EbfG small proteins that have GG-motifs similar to those of microcins, in addition to PteB. Here, we demonstrate that the peptidase-like protein encoded by Synpcc7942_1127 is required for adequate secretion of EbfG proteins, and impairment of its function results in substantial changes to the exoproteome.

Results and discussion

Gene Synpcc7942_1127 is necessary for biofilm development

Four small proteins, each characterized by a bacteriocin or microcin secretion motif (EbfG1-4), is involved in biofilm development (Parnasa *et al.,* 2016). The gene Synpcc 7942_1127, which is located in the vicinity of the genes encoding the EbfG proteins (Fig. 1A), is predicted to encode a PmbA/TldD-like protein. In *Escherichia coli*, the PmbA/TldD protein is required for the processing, maturation and secretion of the microcin peptide antibiotic MccB17 (Rodriguez-Sainz *et al.,* 1990; Allali *et al.,* 2002). The combination of genomic context and predicted function encouraged us to investigate possible involvement of the putative microc in-processing peptidase encoded by Synpcc7942_1127 in biofilm development and secretion of EbfG proteins.

Insertional inactivation of *Synpcc7942_1127* in wild-type (WT) cells did not change its planktonic nature (Fig. 1B and C, strain 1127Ω). However, in contrast to the robust biofilm formation of T2SEΩ, the double mutant in which *t2sE* and Synpcc7942_1127 were both inactivated (T2SEΩ/1127Ω) grew planktonically (Fig. 1B and C). Introduction of a copy of the Synpcc7942_1127 gene into the double mutant, T2SEΩ/1127Ω, restored biofilm formation (Fig. 1B and C, T2SEΩ/1127Ω/RP42, see Table S1 for details). Taken together, these data indicate that Synpcc7942_1127 encodes a component required for biofilm development.

Transcript abundance of Synpcc7942_1127 is similar in WT and T2SEΩ

Previous studies revealed elevated transcript abundances from genes that encode components involved in

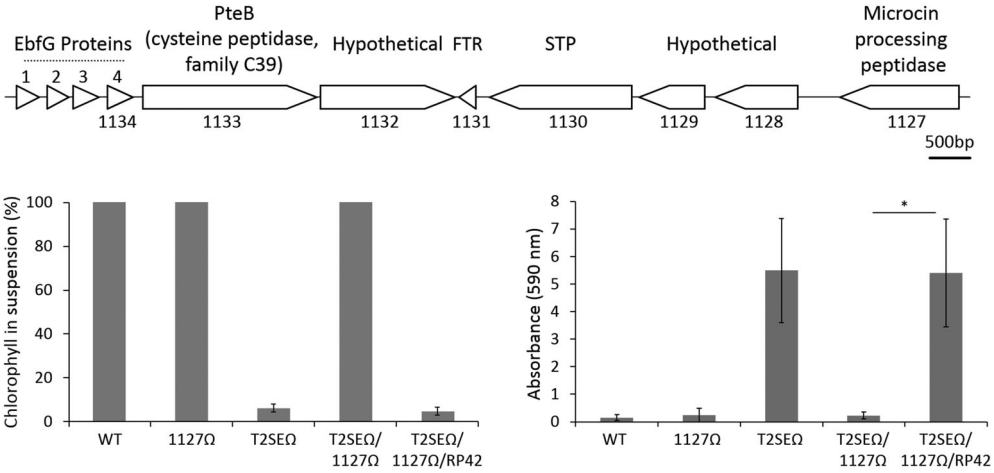


Fig. 1. The putative microcin processing peptidase encoded by Synpcc7942_1127 is required for biofilm development. A. Genomic region of the *ebfG* operon and Synpcc7942_1127. FTR – ferredoxin-thioredoxin reductase variable subunit; STP – serine/threonine phosphatase (protein designations are according to cyanobase). Numbers indicate Synpcc7942 gene designations. EbfG1-3 were missed during annotation, and thus do not have Synpcc7942 identification. B. Assessment of biofilm as percentage of total chlorophyll in suspended cells (average of three independent biological repeats ± standard deviation). C. Crystal violet staining of biofilms formed in 96-well plates (average of four independent biological repeats ± standard deviation). Asterisk indicates significant difference (*t* test $P < 0.002$). Strains analysed: WT; inactivation of Synpcc7942_1127 in WT (1127Ω); inactivation of Synpcc7942_2071 (T2SEΩ); inactivation of Synpcc7942_1127 in combination with inactivation of T2SEΩ (T2SEΩ/1127Ω) and double mutant T2SEΩ/1127Ω with Synpcc7942_1127 replaced in a shuttle vector (T2SEΩ/1127Ω/RP42).

biofilm formation (Synpcc7942_1133 and *ebfG1-4)* in T2SEΩ as compared with WT (Schatz *et al.,* 2013; Parnasa *et al.,* 2016). This finding is consistent with a hypothesis that expression of these genes is inhibited by a specific factor(s) that is secreted into the medium by WT but not by the T2SEΩ mutant. In contrast, qRT-PCR assays showed similar transcript levels in T2SEΩ and WT cells for Synpcc7942_1127 throughout the time course for biofilm formation (Fig. S2 in Supporting Information), suggesting that the putative microcin processing peptidase does not share this pathway of regulated transcription.

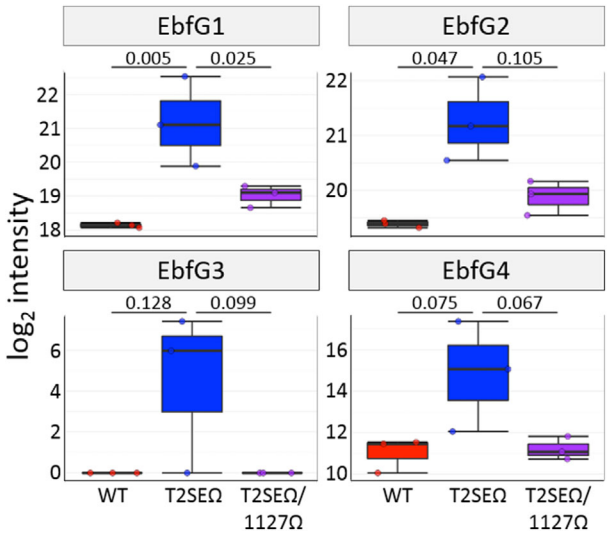


Fig. 2. The amount of EbfG proteins in extracellular fluids in different strains. Intensity value correlates with the amount of peptides detected by MS. Strains analysed: WT (red), T2SEΩ (blue) and T2SEΩ/1127Ω (purple). Numbers over horizontal lines indicate false discovery rate (FDR) values.

Inactivation of Synpcc7942_1127 in T2SEΩ reverses the elevation of extracellular levels of EbfG proteins seen in the T2SEΩ background

A mutational approach demonstrated that the GG-motifs characterizing the EbfG proteins of *S. elongatus* are required for adequate secretion and biofilm development (Parnasa *et al.,* 2016). Processing of small proteins possessing such secretion motifs, e.g. microcins, often precedes their secretion (Michiels *et al.,* 2001; Bobeica *et al.,* 2019) and, therefore, we examined whether the putative microcin-processing peptidase encoded by Synpcc7942_1127 affects extracellular levels of these proteins. Exoproteome analysis revealed higher levels of each of the EbfG proteins in extracellular culture fluids of T2SEΩ compared with WT (Nagar *et al.,* 2017). In contrast, inactivation of Synpcc7942_1127 in combination with T2SEΩ significantly reduced the extracellular levels of EbfG proteins (Fig. 2, compare T2SEΩ and T2SEΩ/1127Ω). These data indicate that activity of the putative microcin-processing peptidase, which we dub EbfE (enable biofilm formation enzyme), is required for proper secretion of EbfG proteins. Furthermore, these data support the suggestion that the inability of strain T2SEΩ/1127Ω to form biofilms stems, at least in part, from impaired secretion of EbfG proteins.

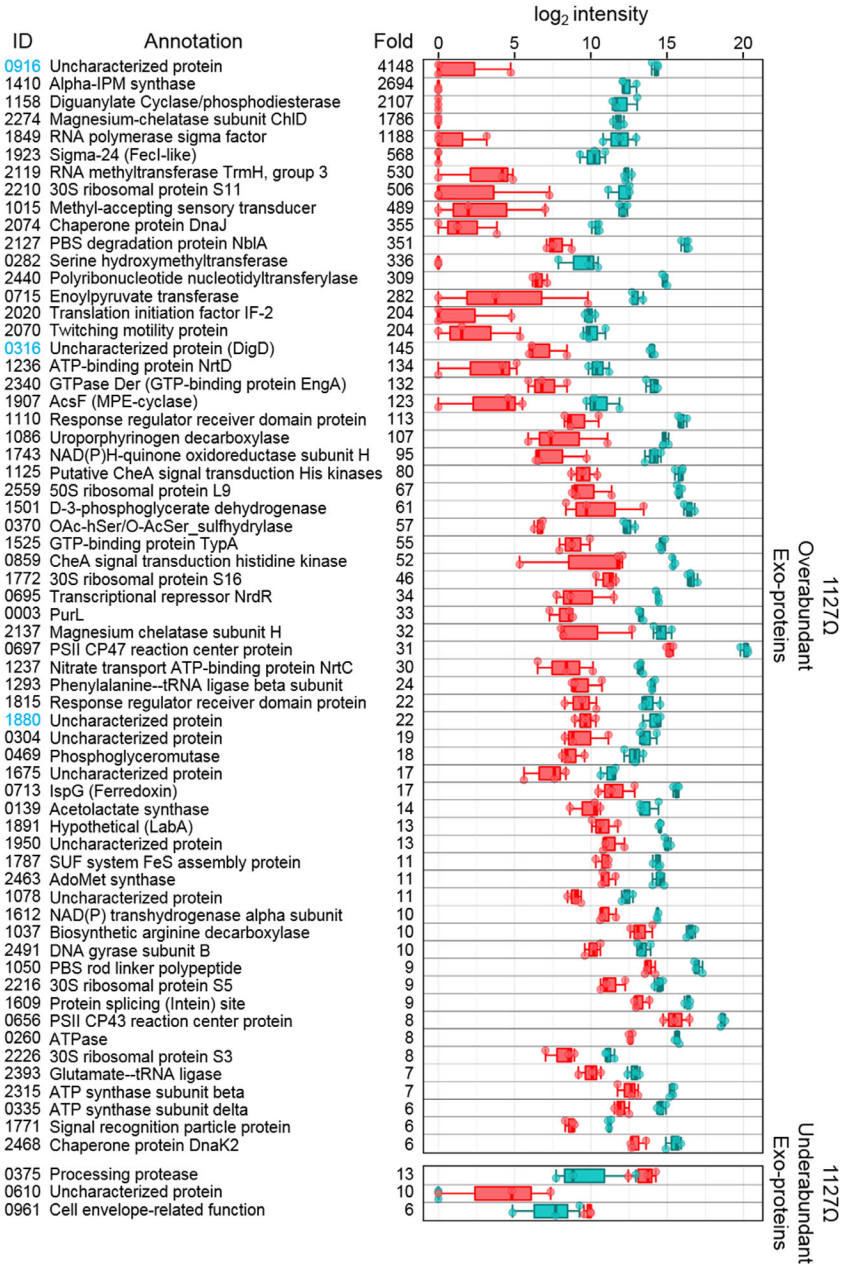
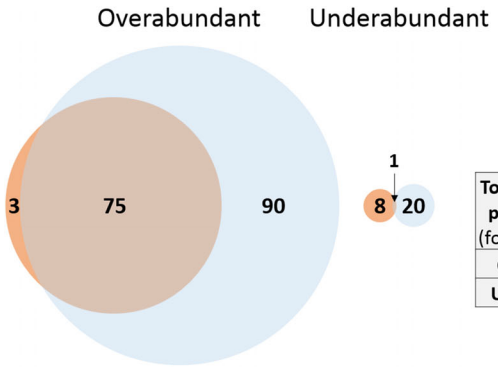


Fig. 3. Inactivation of Synpcc7942_1127 affects the exoproteome. MS analysis of the entire exoproteome of two-day-old cultures. Shown are proteins with intensity fold change between the strains ≥5.0 and false discovery rate (FDR) ≤0.1. Numbers in the column 'Fold' indicate the fold change of the respective protein (upper panel – 1127Ω versus WT; lower panel – WT versus 1127Ω). Numbers in the 'ID' column refer to specific four-digit gene identities derived from Synpcc7942_xxxx gene designations. The indicated intensity (log2 scale) is proportional to the amount of the peptide(s) detected. The intensity value of a peptide identified with multiple charge states is based on the highest signal among the detected species. Individual data points from three biological repeats are plotted as circles (WT = red, 1127Ω = green) overlaid on top of box plots, with the box representing the second and third quartiles, the bold line across the box indicating the median, and the whisker bars representing the maximum and minimum values. Blue gene ID numbers indicate small proteins annotated as 'conserved hypothetical'.

A study of the marine cyanobacterium *Synechococcus* sp. WH8102 revealed a role of a microcin-C-like biosynthetic gene cluster in allelopathic interactions (Paz-Yepes *et al.,* 2013). Genes in this cluster encode a core peptide and several enzymes whose activity results in a cyclic modified peptide. It is unlikely that EbfE and other proteins encoded in the genomic vicinity of Synpcc7942_1127 modify the EbfG proteins in a manner similar to the *Synechococcus* microcin because of a lack of sequence similarity between the protein products of these gene clusters.



	1127Ω vs WT	T2SEΩ/1127Ω vs T2SEΩ
Total differentially present proteins (fold > 2, FDR <0.1)	87 (3+75+8+1)	186 (90+75+20+1)
Overabundant	78	165
Underabundant	9	21

Fig. 4. Venn diagrams summarizing changes in exoproteomes. Orange circles represent differentially present proteins (fold change ≥2.0 and false discovery rate (FDR) ≤0.1) in 1127Ω compared with WT. Blue circles represent differentially present proteins in T2SEΩ/1127Ω compared with T2SEΩ. The number of proteins depicted is proportional to circle size.

Inactivation of Synpcc7942_1127 in WT and T2SEΩ impacts the exoproteome

The activity of microcin-processing peptidases is required for maturation and secretion of processed small compounds (Michiels *et al.,* 2001; Bobeica *et al.,* 2019). We therefore examined the effect of inactivation of Synpcc7942_1127 on the exoproteome. Comparative mass spectrometry-based proteomics analysis revealed substantial augmentation of the mutant's exoproteome; numerous extracellular proteins were present at increased levels in 1127Ω culture fluids compared with WT (Figs. 3 and 4 and Supporting Information File S1). Of the 87 extracellular proteins that were present in significantly different levels between the two strains (fold >2, false discovery rate (FDR) <0.1), 78 are more prevalent in 1127Ω than in WT (Fig. 4) while only nine are underabundant in 1127Ω compared with WT (Fig. 4). [Figure 3 shows the level of 65 differential proteins (fold >5, FDR <0.1).]

Analysis by a variety of secretion-signal prediction methods revealed that the total exoproteome (extracellular proteins in WT and 1127Ω) was significantly enriched with proteins predicted to carry a secretion signal, compared with the entire potential proteome (Fig. S3 in Supporting Information). No such enrichment was observed for the set of proteins (fold >2, FDR <0.1) that exhibited differential abundance in WT compared with 1127Ω exoproteome (Fig. S3 in Supporting Information). These data imply that the 'differential exo-proteins' are transported by secretion mechanisms that do not recognize the secretion signal sequences commonly used by motif prediction algorithms. The use of unique secretion signals by *S. elongatus* was previously suggested based on the analysis of differential proteins between WT and T2SEΩ exoproteomes (Nagar *et al.,* 2017). It is also possible that outer membrane vesicles (OMVs), which have been reported in other cyanobacteria (Biller *et al.,* 2014; Oliveira *et al.,* 2016), are involved in protein deposition to the extracellular milieu.

Clusters of orthologous groups [COGs (Galperin *et al.,* 2015)] enrichment analysis of differentially abundant exoproteins compared with the total exoproteome detected for WT and 1127Ω demonstrated enrichment of three functional categories: (i) cell motility, (ii) coenzyme transport and metabolism and (iii) translation, ribosomal structure and biogenesis. These categories were identified using functional categories designations by CyanoBase (Fujisawa *et al.,* 2017) (Fig. S4 in Supporting Information). (Similar results were obtained using COG definitions provided by the Joint Genome Institute's Integrated Microbial Genomes database (Chen *et al.,* 2017). For these three categories, the differentially abundant exoproteins are all more prevalent in the exoproteome of 1127Ω than in that of WT. The relative high extracellular abundance in 1127Ω of proteins predicted or known to be cytoplasmically located suggests an increased incidence of lysis in this mutant or enhanced secretion of cytoplasmic content through other mechanisms, such as OMV production.

Of note are small extracellular proteins, annotated as 'conserved hypothetical', that are highly overabundant in 1127Ω compared with the WT exoproteome [Fig. 3: products of Synpcc7942_0916–42 amino acids (aa), 4148 fold enriched; Synpcc7942_0316–63 aa, 145 fold enriched; and Synpcc7942_1880–68 aa; 22 fold enriched; gene ID indicated in blue colour]. The function of these small proteins is as yet unknown; however, sequence conservation among diverse cyanobacteria suggests their involvement in cellular processes that are shared among the different genera. Synpcc7942_0316 encodes a dark-induced protein (DigD) that is controlled by the circadian clock (Hosokawa *et al.,* 2011).

The T2SEΩ/1127Ω double mutant is characterized by considerable alterations to the exoproteome compared with T2SEΩ (Fig. 4; Supporting Information Fig. S5 and File S1). About 45% of the significant changes are attributable to loss of Synpcc7942_1127 alone, because they are seen when the Synpcc7942_1127 gene is inactivated

in a WT background; however, 90 additional changes specific to the double mutant are also observed (Fig. 4). Aside from reduced extracellular levels of EbfG proteins, the relationship of these changes to biofilm development is, as yet, unclear.

To summarize, we previously revealed that PteB, a C39 family peptidase encoded by Synpcc7942_1133, is required for biofilm development in *S. elongatus* and for adequate secretion of EbfG proteins. Here, we demonstrated that EbfE, the microcin-processing peptidase-like protein encoded by Synpcc7942_1127, is also required for these functions. PteB is characterized by transmembrane regions, whereas EbfE lacks such domains but has an N-terminal secretion sequence. It is possible that these two proteins take part in a maturation–secretion apparatus, whereby PteB is localized to the cell membrane and EbfE is localized to the periplasmic space via its secretion signal. Together, this study uncovers a novel subunit of a system that secretes proteins with microcin-associated GG-motifs. Additionally, comparison of the exoproteomes of WT and the *ebfE*-mutant revealed substantial differences, indicating that the role of the transport system in which EbfE takes part is not manifested only in T2SEΩ. Additionally, inactivation of *ebfE* in a strain in which the biofilm suppression mechanism is abrogated (T2SEΩ) allowed assigning a function to EbfE in biofilm development in *S. elongatus.*

Experimental procedures

Strains, culture conditions, biofilm quantification and RT-qPCR

Growth of *S. elongatus* PCC 7942 and all derived strains, as well as assessment of biofilms by quantification of percentage of chlorophyll in suspension, was as described previously (Sendersky *et al.,* 2017). For quantification of biofilms under static conditions, cultures at the exponential phase of growth were diluted to optical density (750 nm) of 0.5 and 200 μl samples were inoculated into 96-well plates (10 wells for each strain). Plates were incubated at 23 °C under 6 μmol photons·m^2 sec^{-1}. Biofilm quantification was performed following 6 days essentially as described (Merritt *et al.,* 2005) except for crystal violet extraction that was performed in 95% ethanol and not in 30% acetic acid. RT-qPCR, gene inactivation and additional molecular analyses are described in Supporting Information Table S1. RT-qPCR was performed as described earlier (Parnasa *et al.,* 2016).

Analysis of extracellular fluids

Examination of the entire exoproteome of two-day-old cultures by mass spectrometry was done at the de Botton Institute for Protein Profiling at The Nancy and Stephen Grand Israel National Center for Personalized Medicine (Weizmann Institute of Science) as previously described (Nagar *et al.,* 2017). Protein intensities were adjusted to have a minimum value of 2. Statistical significance (*t* test with FDR adjustment of p-values) and average intensity values for determining fold change were calculated using log2 transformed protein intensities. Differentially abundant exoproteins were defined as any protein with at least a two-fold change in protein intensity and an FDR p-value <0.1. Enrichment analysis of functional categories and signal peptide predictions were performed as described previously (Nagar *et al.,* 2017), with a p-value <0.05 as the threshold for significance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1: Summary of molecular manipulations.

Fig. S1: Sequence alignment of the EbfG proteins. Black and grey shading indicate amino acid identity in four or three proteins, respectively. N-termini of the proteins are not presented; shown is the region that shares homology with GG-secretion motifs of microcins (Parnasa *et al.,* 2016). Asterisks denote the conserved GG or GA just prior to protein cleavage site. Positions typically occupied by hydrophobic or hydrophilic amino acids are indicated by a circle or a triangle, respectively.

Fig. S2: qRT-PCR analysis of Synpcc7942_1127 in WT and T2SEΩ. Transcript abundance was followed in suspended (S) and biofilmed (BF) cells, when present in the culture. Bar graphs represent averages of three independent biological repeats (± standard deviation). All comparisons are not significantly different from each other (p-value <0.05).

Fig. S3: Enrichment analysis for predicted secretion signals. Secretion signal predictions were made using the indicated algorithms. Predictions that are significantly enriched or depleted (p-value <0.05) are shown. Dark grey bars – analysis of proteins detected in the total exo-proteome (WT and 1127Ω exo-proteins) versus the entire potential proteome. Light grey bars – analysis of 87 exo-proteins present at different levels in WT versus 1127Ω (fold change ≥2.0 and FDR ≤0.1) vs the exo-proteome.

Fig. S4: Enrichment analysis for protein functions. Functional categories from Cyanobase that are significantly enriched or depleted (p-value <0.05). PTM – Posttranslational modification. Dark grey bars – analysis of proteins detected in the exo-proteome (WT and 1127Ω exo-proteins) compared to the entire potential proteome. Light grey bars – analysis of 87 exo-proteins present at different levels in WT versus 1127Ω (fold change ≥2.0 and FDR ≤0.1) compared to the exo-proteome.

Fig. S5: Effect of inactivation of Synpcc7942_1127 in T2SEΩ on the exo-proteome.
MS analysis of the entire exo-proteome of two-day old cultures. Shown are proteins with intensity fold change between the strains ≥5.0 and FDR ≤0.1. Numbers in the column 'Fold' indicate the fold change of the respective protein (upper panel - T2SEΩ/1127Ω versus T2SEΩ; lower panel - T2SEΩ versus T2SEΩ/1127Ω). Numbers in the 'ID' column refer to specific four-digit gene identities derived from Synpcc7942_xxxx gene designations. The indicated intensity (log2 scale) is proportional to the amount of the peptide(s) detected. The intensity value of a peptide identified with multiple charge states is based on the highest signal among the detected species. *ebfG3* is a previously un-annotated gene that enable biofilm formation and do not have a Synpcc number (see text, Parnasa *et al.,* 2016). Individual data points from three biological repeats are plotted as circles (T2SEΩ = blue, T2SEΩ/1127Ω = purple) overlaid on top of box plots, with the box representing the second and third quartiles, the bold line across the box indicating the median, and the whisker bars representing the maximum and minimum values.

Appendix S1: Supplementary for MS analysis