Multidisciplinary Evidences that *Synechocystis* PCC6803 Exopolysaccharides Operate in Cell Sedimentation and Protection against Salt and Metal Stresses

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Abstract

Little is known about the production of exopolysaccharides (EPS) in cyanobacteria, and there are no genetic and physiological evidences that EPS are involved in cell protection against the frequently encountered environmental stresses caused by salt and metals. We studied four presumptive EPS production genes, *sll0923*, *sll1581*, *slr1875* and *sll5052*, in the model cyanobacterium *Synechocystis* PCC6803, which produces copious amounts of EPS attached to cells (CPS) and released in the culture medium (RPS) as shown here. We show that *sll0923*, *sll1581*, *slr1875* and *sll5052* are all dispensable to the growth of all corresponding single and double deletion mutants in absence of stress. Furthermore, we report that *sll0923*, *sll1581* and *slr1875* unambiguously operate in the production of both CPS and RPS. Both *sll1581* and *slr1875* are more important than *sll0923* for CPS production, whereas the contrary is true for RPS production. We show that the most EPS-depleted mutant, doubly deleted for *sll1581* and *slr1875*, lacks the EPS mantle that surrounds WT cells and sorbs iron in their vicinity. Using this mutant, we demonstrate for the first time that cyanobacterial EPS directly operate in cell protection against NaCl, CoCl₂, CdSO₄ and Fe-starvation. We believe that our EPS-depleted mutants will be useful tools to investigate the role of EPS in cell-to-cell aggregation, biofilm formation, biomineralization and tolerance to environmental stresses. We also suggest using the fast sedimenting mutants as biotechnological cell factories to facilitate the otherwise expensive harvest of the producer cell biomass and/or its separation from products excreted in the growth media.

Introduction

A wide-range of bacteria synthesize and secrete extracellular polymeric substances, mainly of polysaccharidic nature (exopolysaccharides (EPS)), which are involved in the tolerance to environmental stresses [1]. In addition, EPS have valuable biotechnological applications [2], and are involved in the formation and maintenance of biofilms, which have a high relevance to human health [3], water treatment and agriculture [4]. Two types of EPS can be distinguished: (i) capsular EPS (CPS), which are attached to the cell surface and (ii) EPS that are released into the surrounding environment (RPS). The synthesis of EPS comprises typically four distinct steps occurring in various compartments 1) the activation of monosaccharides and conversion into sugar nucleotides into the cytoplasm; 2) the assembly of the repeat units by sequential addition of sugar on a lipid carrier by glycosyltransferase; 3) the polymerization of the repeat units at the periplasmic face of the plasma membrane; and 4) the export of the polymer to the cell surface. In cyanobacteria, the important photosynthetic prokaryotes, which colonize most waters and soils of our planet and produce a large part of the oxygen [5] and biomass for the food chain [6], EPS are proposed to operate in the protection against environmental stresses [7]. Furthermore, cyanobacterial EPS are also regarded as being involved in the biomineralization of calcium (and/or magnesium) carbonates, sometimes leading to the formation of stromatolites [8]. Cyanobacterial EPS, which harbour six to twelve types of monosaccharides, are more complex than the EPS formed by other bacteria or eukaryotic microalgae, which usually contain less than four monosaccharides [1]. Hence, the complexity of cyanobacterial EPS gives rise to speculations on how many genes may be involved in their production. In addition, cyanobacterial EPS are usually strongly anionic since they contain one or two different uronic acids, as well as sulphate groups, a rare feature among bacteria. The presence of negatively charged EPS surrounding cyanobacterial cells may play an important role in the sequestration of metal cations. On one hand, this can create a microenvironment enriched in those metals that are essential for cell growth but occur in limited amounts in the environment.
On the other hand, the presence of EPS around the cells can also prevent direct contact between the cells and toxic heavy metals such as cadmium (Cd) and cobalt (Co), which are intensively spread out in the environment by human industries [9]. Consequently, EPS-producing cyanobacteria are regarded as promising chelating agents for biosorption and removal of heavy metals from contaminated waters [7]. EPS have also been proposed to contribute to the protection of cyanobacteria against other environmental stresses such as dessication, UV-light, biomineralization, and salt stress. However, direct experimental evidence demonstrating the ecological roles attributed to cyanobacterial EPS are scarce [7]. To our knowledge only one paper reported the analysis of cyanobacterial genes involved in EPS production [10]. This study was carried out in the unicellular model strain Synechocystis PCC6803 (hereafter Synechocystis) that possesses a fully sequenced [http://genome.kazusa.or.jp/ cyanobase/] and easily manipulable genome [11–14], which encodes complex EPS with 12 types of monosaccharides [1]. In their paper, Foster and co-workers showed that the five genes cluster slr1722-slr1726 encoding presumptive glycosyltransferases is involved in the production of EPS and the protection against the light and H2O2 stresses [10]. However, this previous study did not distinguish between CPS and RPS, and did not investigate the influence of EPS on the tolerance to salt and metal stresses, or the formation of biofilm.

In the present study, we have thoroughly analyzed four Synechocystis genes, slr1875, sll1581, sll0923 and sll5052, which share some sequence homology with the EPS production genes exoD (slr1875), gumB (sll1581) and gumC (sll0923, sll5052) from non-photosynthetic bacteria. We report that these four Synechocystis genes are all dispensable to cell growth under standard laboratory conditions (all single and double-deletion mutants grow as healthy strains). Genes slr1875, gumB and gumC are all dispensable to cell growth under standard laboratory conditions. These findings are the first genetic and physiological evidences demonstrating the ecological roles attributed to cyanobacterial EPS that EPS protect cyanobacteria against salt and metal stresses.

Materials and Methods

Bacterial Strains, Growth and Survival Analyses

Synechocystis PCC6803 was grown under continuous agitation (180 rpm) and white light (2,500 luxes; 31.25 μE m⁻² s⁻¹) at 30°C on mineral medium (MM) corresponding to BG11 medium [15] enriched with 3.78 mM Na₂CO₃ [16]. E. coli TOP10 (Invitrogen) used for gene manipulation was grown on LB at 37°C. Antibiotic selection was performed with kanamycin (Km) 2.5 mg ml⁻¹, streptomycin (Sm) 2.5 μg ml⁻¹ or spectinomycin (Sp) 2.5 μg ml⁻¹ for Synechocystis; and ampicillin (Amp) 100 μg ml⁻¹, Km 50 μg ml⁻¹ or Sp 75 μg ml⁻¹ for E. coli. For NaCl challenges, Synechocystis cultures grown three times up to mid-exponential phase (OD₅₇₀ = 0.5 units, i.e. 2.5×10⁷ cells.ml⁻¹) were inoculated (initial OD₅₇₀ = 0.02) into fresh MM medium with or without NaCl 0.9 M, and OD₅₇₀ was measured at time intervals. For survival analyses, 1 ml aliquots of mid-exponential phase cultures were incubated for 3 h with various concentrations of CoCl₂ or CdSO₄, washed with ultrapure water (UPW, Purelab, Elga), and spread on MM plates after appropriate dilutions with MM. Colonies generated by surviving cells were counted after 5–7 days under standard conditions.

Construction of the DNA Cassette for Targeted Deletion of the sll0923, sll1581, slr1875, and sll5052 Genes

The Synechocystis DNA regions (about 300 bp in length) flanking the protein coding sequence (CS) of the studied genes were independently amplified by PCR, using specific oligonucleotides primers (Table S1). These two DNA regions were joined through standard PCR-driven overlap extension [17] in a single DNA segment harbouring a Smal restriction site in place of the studied CS. After cloning in pGEMT (Promega) the resulting plasmids (Table 1) were opened at the unique Smal where we cloned the Km’ cassette (a HincII fragment of pUC4K) or the Sm’/Sp’ cassette amplified by PCR and digested with EcoRV in the same orientation as the CS it replaced (Table 1). The resulting deletion cassettes were verified by PCR and nucleotide sequencing (Big Dye kit, ABI Perkin Elmer), before and after transformation [18] to Synechocystis.

Extraction and Quantification of Exopolysaccharides (EPS), and ATR-FTIR Spectroscopy

EPS were extracted mostly as described [19]. 30 ml of late log phase cultures (OD₅₇₀ = 0.7) grown for three days under standard conditions were harvested by a 30 min centrifugation (5,000 rpm) at room temperature (RT) to separate the supernatants containing the released EPS (RPS) from the cell pellets containing the capsular EPS (CPS) attached to the cells. RPS were recovered from filtered supernatants (0.45 μm, Pall) after overnight precipitation with 2 volumes of chilled ethanol at −20°C, centrifugation at 13,000 rpm for 30 min at 4°C, washing with 95% ethanol, drying under air, and re-suspension in a 1 ml UPW. CPS were recovered from cell pellets, washed, re-suspended in 1 ml of UPW, boiled for 15 min at 100°C and centrifuged at 13,000 rpm at RT to eliminate cell debris. Total carbohydrate contents of RPS and CPS were measured by the phenol-sulfuric method, and the amount of EPS was calculated as the average ratio of the EPS quantity over total proteins quantified with Bradford assay, using BSA as a standard (Biorad protein assay). For ATR-FTIR spectroscopy, cells were deposited on an ATR crystal of ZnSe in a FTIR Nicolet 570 spectrometer, equipped with a cadmium telluride (MCT) detector. A total of 50 scans at a resolution of 4 cm⁻¹ was averaged for each studied strains, and the spectrum of the MM growth medium alone was subtracted.

Cell Sedimentation and Electrophoretic Mobility Assays

Synechocystis mid-log phase cultures were either stored motionless on the bench for 18 days prior to photographs, or transferred into disposable folded capillary cells with gold covered electrodes (Malvern).

Electrophoretic mobility was measured at the 17° fixed scattering angle with a Zetasizer Nano ZS instrument (Malvern) equipped with a 633 nm laser, using runs performed at a voltage of 150 V and a frequency of 285 Hz. The values were automatically converted to Zeta potentials using the Smoluchows-kı’s equation.

Scanning Electron Microscopy and Energy Dispersive X-ray Analyses

Cells from late log phase cultures (OD₅₇₀ = 0.7) were fixed overnight with glutaraldehyde (2.5%) and alcin blue (0.15%), washed twice with UPW and dried in CO₂ critical point dryer (BAL-TEC CPD030) as described [20]. Samples were mounted on aluminium stubs using double-sided carbon tape, coated with carbon, and observed with a Zeiss Ultra 55 FEG SEM microscope, operated at 2.0 kV or 10 kV, at a working distance
of 2.7 mm or 7.5 mm. Images were acquired in secondary electron mode using an Everhart Thornley or the InLens detectors in backscattered electron mode. Energy dispersive x-ray microanalysis was performed with an EDS QUANTAX microanalyzer operated with the Esprit, Hypermap software allowing acquisition of X-ray maps and drift correction.

Results

The Four Synechocystis Genes sll0923, sll1581, slr1875 and sll5052 are Dispensable to the Growth of All Corresponding Single and Double Deletion Mutants

Among other Synechocystis genes that share sequence homology with EPS production genes from non-cyanobacterial prokaryotes (http://genome.kazusa.or.jp/cyanobase) we decided to study the three chromosomal genes sll0923, sll1581 and slr1875, and one plasmidic gene sll5052. sll1583 encodes a presumptive protein homologous to the EPS synthesis ExoD enzyme of Rhizobium meliloti [21]; sll0923 and sll5052 code for proteins resembling the EPS-assembling enzyme EpsB/GumC/Wzc of Methylobacterium [22]; and sll581 encodes a presumptive protein homologous to the EPS exporting outer membrane protein GumB/Wza involved in xanthan production in Xanthomonas axonopodis [23] and biofilm formation in Xylella fastidiosa [24].

To investigate the four genes sll0923, sll1581, slr1875 and sll5052, we used single and double deletion analysis. Therefore, we replaced the full protein-coding sequence of these genes with a transcription terminator-less marker, Km or Sm'/Sp' (Table 1), for antibiotic selection. The resulting deletion cassettes (Δsll0923::Km', Δsll1581::Km', Δslr1875::Km', Δsll5052::Km', Δsll0923::Sm'/Sp', Δsll1581::Sm'/Sp', Δslr1875::Sm'/Sp' and Δsll5052::Sm'/Sp') were independently introduced in Synechocystis by transformation. In each case a few transformant clones were selected and analyzed by PCR (Fig. 1 for the Δsll0923::Km', Δsll1581::Km', Δslr1875::Km' and Δsll5052::Sm'/Sp' cassettes) and DNA sequencing (data not shown). We verified that the Km' or the Sm'/Sp' marker had properly replaced the studied genes in all copies of genome, which is polyploid [18,25]. All eight single mutants were found to grow healthy under standard laboratory conditions (Fig. S1), and they retained no wild-type (WT) allele of the studied genes. The complete absence of WT alleles in each mutant was also verified in cultures subsequently grown for about 100 generations in absence of the selective antibiotics.

We also constructed all six possible double mutants Δsll0923-sll1581, Δsll0923-slr1875, Δsll0923-sll5052, Δslr1875-sll1581, Δsll5052-sll1581 and Δsll5052-slr1875, which were selected on the basis of their resistance to all three antibiotics Km, Sm and Sp. The double mutants Δsll0923-sll1581; Δsll0923-slr1875 and Δsll0923-sll5052 were generated after the introduction of the Δsll1581::Sm'/Sp', Δslr1875::Sm'/Sp' and Δsll5052::Sm'/Sp' cassettes in the Δsll0923::Km' recipient mutant (Fig. S2). The Δslr1875-sll1581 double mutant was obtained after the introduction of the Δsll1581::Sm'/Sp' cassette into the Δslr1875::Km' recipient mutant (Fig. S3). The Δsll5052-sll1581 and Δsll5052-slr1875 double mutants were generated after the introduction of the Δsll1581::Km' and Δslr1875::Km' cassettes into Δsll5052::Sm'/Sp' recipient mutant (Fig. S4). Like all four single-deletion mutants, all six double mutants grew as healthy as the WT strain (Fig. S1), and lacked WT-type alleles of the studied genes, even after a subsequent cultivation in absence of the selective antibiotics.

These data show that the four Synechocystis genes sll0923, sll1581, slr1875, sll5052 are dispensable to the growth of all corresponding single and double deletion mutants under standard laboratory conditions.

Influence of the sll0923, sll1581, slr1875 and sll5052 Genes on the Buoyant Density of Synechocystis

Bacterial autoaggregation is the process whereby cells physically interact with each other and settle down to the

| Table 1. Characteristics of the plasmids used in this study. |
|---------------------------------|-----------------|----------------|---|
| Plasmids | Relevant features | Reference |
| pgEMT | Amp' AT overhang cloning vector | Promega |
| pUC4K | Source of the Km' marker gene | Promega |
| pFC1 | Source of the Sm'/Sp' marker gene | [12] |
| Δsll0923 | pgEMT with the sll0923 gene and its two 0.5 kb flanking sequences, where the coding sequence (CS; from 18 bp to 2192 bp) was replaced by a SmaI site | This study |
| Δsll1583::Km' | pΔsll0923 with the Km' marker inserted into its unique SmaI site | This study |
| Δsll0923::Sm'/Sp' | pΔsll0923 with the Sm'/Sp' marker inserted into its unique SmaI site | This study |
| Δsll1581 | pgEMT with salr1875 gene and its two 0.5 kb flanking sequences, where the sll1581 CS (from 43 bp to 1419 bp) was replaced by a SmaI site | This study |
| Δslr1581::Km' | pΔsll1581 with the Km' marker inserted into its unique SmaI site | This study |
| Δslr1581::Sm'/Sp' | pΔsll1581 with the Sm'/Sp' marker inserted into its unique SmaI site | This study |
| Δsll1875 | pgEMT with the slr1875 gene and its two 0.3 kb flanking sequences, where the CS (from 16 bp to 568 bp) was replaced by a SmaI site | This study |
| Δsll1875::Km' | pΔsll1875 with the Km' marker inserted into its unique SmaI site | This study |
| Δsll1875::Sm'/Sp' | pΔsll1875 with the Sm'/Sp' marker inserted into its unique SmaI site | This study |
| Δsll5052 | pgEMT with the sll5052 gene and its two 0.3 kb flanking sequences, where the coding sequence (CS; from 100 bp to 2202 bp) was replaced by a SmaI site | This study |
| Δsll5052::Km' | pΔsll5052 with the Km' marker inserted into its unique SmaI site | This study |
| Δsll5052::Sm'/Sp' | pΔsll5052 with the Sm'/Sp' marker inserted into its unique SmaI site | This study |

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Figure 1. Construction of the single deletion mutants of the genes slr0923, slr1581, slr5052 and slr1875. (A) Schematic representation of the studied chromosome loci in the wild-type (WT) strain and the mutants \( \Delta \text{slr0923::Km} \), \( \Delta \text{slr5052::Sp} \), \( \Delta \text{slr1875::Km} \) and \( \Delta \text{slr1581::Km} \) constructed in this study. The studied genes are represented by boxes, which point into the direction of their transcription. The PCR primers used to verify the presence (in the WT strain) and absence (in the deletion mutant) of the studied genes are represented by the small grey triangles. The size of
bottom of culture flask in static liquid cell suspensions. This process is of industrial significance in facilitating the separation of cell biomass from valuable products excreted by the cells in their growth media, such as fatty acids produced by a genetically modified strain of Synechocystis [26]. With this in mind, we noticed with great interest that Synechocystis spontaneously settles down and forms a “slime” like structure at the bottom of liquid-cultures flasks that are stored under light without agitation. This process is slow and takes more than three weeks in the cases of the WT strain and the three mutants \(sll0923\), \(Asl1875\) and \(sll0923-asl1875\). By contrast, the seven mutants \(Asl1581\), \(Asl5052\), \(Asl5052-1581\), \(Asl0923-1581\), \(Asl0923-5052\), \(Asl1875-asl1581\) and \(Asl5052-asl1875\) settled down faster to the bottom of the flask. Their sedimentation was complete in less than 18 days (Fig. 2). Altogether these findings show that the absence of \(sll5052\), \(sll1581\), \(slr1875\) and \(sll0923\) accelerates the spontaneous cell sedimentation.

The Three Genes \(sll0923\), \(sll1581\) and \(slr1875\) Operate in the Production of Exopolysaccharides, While \(sll5052\) Play a Minor Role

To investigate the influence of the \(sll0923\), \(sll1581\), \(slr1875\) and \(sll5052\) genes on EPS production, we measured and compared the abundance of both CPS (capsular polysaccharides attached to cells) and RPS (polysaccharides released in the liquid culture medium) in the WT strain and the 10 studied deletion mutants. As compared to the WT strain (Fig. 3), the quantity of CPS was found to be lower in the three single mutants \(Asl0923\) (2-fold decrease), \(Asl1581\) (4-fold reduction) and \(Asl1875\) (2-fold decrease). Furthermore, the CPS levels were even lower in the three corresponding double mutants \(Asl0923-asl1581\) (5.5-fold decrease as compared to WT), \(Asl0923-asl1875\) (5-fold reduction), and \(Asl1581-asl1875\) (11-fold decrease). By contrast, the abundance of CPS was not affected by the deletion of the \(sll5052\) gene, irrespectively of the strains used for deleting \(sll5052\). Indeed (Fig. 3), the amount of CPS were similar in the following pair-wise strain comparisons (i) \(Asl5052\) with WT cells; (ii) \(Asl5052-sll0923\) with \(Asl0923\); (iii) \(Asl5052-sll1581\) with \(Asl1581\); and (iv) \(Asl5052-asl1875\) with \(Asl1875\). Together, these findings show that the \(sll0923\), \(sll1581\), \(slr1875\) genes operate in the quantitative production of CPS, unlike \(sll5052\).

The abundance of the RPS polysaccharides was decreased about 2 fold in the \(Asl0923\) mutant, whereas it was unaffected in \(Asl1581\), and even slightly increased in the \(Asl1875\) and \(Asl5052\) mutants, as compared to the WT strain (Fig. 3). These results suggest that the three genes \(sll1581\), \(slr1875\) and \(sll5052\) are dispensable to RPS production. However, this interpretation is challenged by the findings that most of the corresponding double deletion mutants exhibit less RPS than the WT strain and their parental single deletion mutants. For instance, the RPS abundance in the double mutant \(Asl1581-asl1875\) is at least two-fold lower than that in the WT strain and the two single mutants \(Asl1581\) and \(Asl1875\). Similarly, the RPS abundance of the mutant \(Asl0923\) is decreased upon the secondary deletion of any of the \(sll1581\), \(slr1875\) and \(sll5052\) genes.

Collectively these findings show that the \(sll0923\) gene operates in the production of both CPS and RPS, whereas \(sll1581\) and \(slr1875\) operate in the production of mainly CPS and moderately RPS, and \(sll5052\) is possibly involved in the production of RPS, likely not of CPS.

The Abundance of Synechocystis EPS Influences the Zeta Potential of the Cell

Bacterial surface charge, which influences cell interactions with the medium and the other cells (cell-to-cell aggregation and biofilm formation), can be assessed by measuring the zeta potential that can be deduced from electrophoretic mobility measurements. Thus, we determined and compared the zeta potential of the WT strain (\(\approx -33\) mV, as we shown [20]) with the values observed for the ten presently-studied mutants in order to assess the influence of EPS on the cell surface of Synechocystis (Fig. 4). The single mutants \(Asl1875\) and \(Asl5052\) and the corresponding double mutant \(Asl5052-asl1875\) displayed zeta potentials (\(\approx -34.6\) mV, \(-33.3\) mV and \(-33.3\) mV, respectively) similar to WT cells (Fig. 4). By contrast, less-negative values were observed for the other mutants \(Asl0923\), \(Asl1581\), \(Asl0923-asl1581\), \(Asl0923-sll5052\), \(Asl1875-asl1581\)

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**Figure 2. Influence of the \(sll0923\), \(sll1581\), \(sll5052\) and \(slr1875\) genes on the spontaneous cell sedimentation of Synechocystis.** Typical photographs of cultures of wild-type and mutant strains harbouring either a single or a double deletion of the genes \(sll0923\), \(sll1581\), \(sll5052\) and \(slr1875\), as indicated. The suspensions were kept static on the bench for 18 days prior to imaging. These experiments were performed at least three times.

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and Δsll5052-sll1581 (between -20 mV and -25 mV; Fig. 4 panel A), which produce only about half or less of the WT amount of EPS (Fig. 4 panel B). Collectively, these findings indicate that the zeta potential is correlated with the total amount of EPS and the resulting density of ionic surface charges produced by the cells (Fig. 4, compare panels A and B). In turn, our data suggest that zeta potential assays should be used in the future for facile screening of EPS-depleted mutants.

The Strong Reduction in EPS Content Caused by the Double Deletion of slr1875 and sll1581 Decreases the Tolerance of Synechocystis to Salt and Heavy Metals Stresses

To study the influence of EPS in the protection against the frequently encountered salt and heavy metal stresses we used the Δslr1875-sll1581 double mutant, which possesses the lowest EPS content (Fig. 3 and Fig. 4). First, we confirmed the difference in EPS content between WT cells and Δslr1875-sll1581 cells through (i) SEM microscopy, which showed that these mutant cells lack the
copious EPS mantle that wraps the WT cells (Fig. 5 panel A) and (ii) FTIR absorption analysis, which confirmed the difference in cell surface between EPS-replete WT cells and EPS-depleted Δslr1875-sll1581 cells (Fig. 5 panel B). Then, we showed that Δslr1875-sll1581 cells, which grow as healthy as WT cells in absence of stress, displayed an increased susceptibility to NaCl (Fig. 5 panel C), cadmium (CdSO₄) and cobalt (CoCl₂) (Fig. 5 panel D).

**Figure 4. The abundance of total EPS influences the zeta potential of Synechocystis.** (A) Zeta values for the WT and mutant cells harbouring either a single or a double deletion of the genes sll0923, sll5052, str1875 and sll1581, as indicated. (B) Histogram plots of the total amounts of EPS (CPS+RPS) of each strain. All results are expressed as means ± standard deviation of the data obtained after three biological repetitions of every assay.

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Exopolysaccharides Sorb Iron and Protect Cells from Iron Starvation

Our evidence that EPS play a direct role in the protection against both Cd and Co (Fig. 5), which disturb iron (Fe) homeostasis [27,28], which is especially important in cyanobacteria [29], prompted us to test whether EPS might operate in the sorption of Fe. Therefore, we analyzed WT and EPS-depleted mutant Δslr1875-sll1581 cells by SEM (in secondary and backscattered electron modes) and energy-dispersive x-ray
Figure 5. Influence of *Synechocystis* exopolysaccharides on cell shape and tolerance to salt and heavy metal stresses. (A) Typical SEM (scanning electron microscopy) images of WT cells and Δslr1875-sll1581 double mutant cells. (B) Typical FTIR absorption spectra of WT cells (solid line) and EPS-depleted Δslr1875-sll1581 cells (dashed lines). (C) Typical growth of the WT strain (dark symbols) and EPS-depleted Δslr1875-sll1581 double mutant (open symbols) incubated for the indicated durations in standard liquid mineral medium (MM) without or with NaCl 0.9 M. (D) Typical...
spectroscopy (EDXS) analyses of the same cells. We observed that similarly to EPS, Fe is highly abundant in the vicinity of WT, not Δslr1875-sll1581, cells (Fig. 6A), like EPS. This co-localization indicates that Fe is trapped by the abundant EPS surrounding WT cells. In comparison, nitrogen (N), which is mostly associated with intracellular molecules (amino-acids, proteins and nucleic acids), displayed a similar intracellular localization in both WT and mutant cells. In addition, phosphorus (P) was observed both inside the cells and as EPS-chelated materials (Fig. 6A). Then, we showed that the WT strain can grow for some time in absence of iron, unlike strain the EPS-depleted Δslr1875-sll1581 mutant (Fig. 6B). Altogether, these findings show that EPS play a role in Fe-sorption, which protects *Synechocystis* against iron starvation.

**Discussion**

Little is known in cyanobacteria about the production of exopolysaccharides (EPS), in spite of their proposed involvement in the protection against environmental stresses [7]. Even in the best-characterized unicellular cyanobacterium, *Synechocystis PCC6803* (*Synechocystis*), only a few genes, *sll1722* to *sll1726*, have been identified as operating in EPS production [10]. Furthermore, there is no strong evidence in the literature that EPS play a direct role in the protection of cyanobacteria against salt and metal stresses, which they frequently encounter in nature. This scarcity of information prompted us to study four *Synechocystis* genes, *sll0923, sll1581, slr1875* and *sll5052* that share sequence homology with EPS production genes from other bacteria (http://genome.kazusa.or.jp/cyanobase/). These four *Synechocystis* genes appeared...
to be dispensable to cell growth under standard laboratory conditions, of all corresponding single and double deletion mutants. Both sll1581 and slr0923 play a role in the buoyant density of Synechocystis, unlike both sll0293 and slr1875. Indeed, all single or double deletion mutants of sll1581 and slr0923 displayed an accelerated sedimentation of the cells down to the bottom of the flask in static suspensions (Fig. 2). Such fast sedimenting mutants might be useful cell factories for the future production of biotechnologically interesting products, in the sense that they should facilitate the otherwise expensive harvest of the producer biomass and/or the separation of products excreted in the growth medium. We also show that the sll0293, slr1875 and slr1875 genes unambiguously operate in the quantitative production of both CPS (capsular) and RPS (released) EPS, unlike slr0923 (Fig. 3). Both slr1875 and slr1875 are more important than sll0293 for CPS production, whereas the contrary was true for RPS production. Our findings that EPS-depleted Synechocystis mutants can form biofilms, even faster than the WT strain (Fig. 2), are consistent with previous EPS studies in many other bacteria including E. coli, P. putida, S. haemolyticus, S. pneumoniae, etc [4,30]. These investigations identified several EPS that inhibit biofilm formation in acting as surfactants, rather than bactericidal agents. As these antibiofilm EPS destabilize biofilm matrix without affecting bacterial fitness, unlike antibiotics, they should be less prone than antibiotics to develop resistance. Hence, these antibiofilm EPS should have interesting potentials in industry and medicine [30].

We also studied the influence of EPS on the cell surface charge of Synechocystis, estimated as the zeta potential value. We found that the mutants, Asl10923, Asl11581, Asl0923-111581, Asl10923-sll1581 and Asl5052-sll1581, which produce about half of the WT amount of EPS, displayed higher zeta potentials than the WT strain (Fig. 4). These data indicate that the zeta potential of cells is correlated with the total amount of EPS and the associated density of ionic surface charges. Thus, we suggest that zeta potential assays should be systematically used in the future for facile screening of EPS production mutants. Then, we thoroughly analyzed the most EPS-depleted mutant, Asl10923-111581 (17-fold lower EPS abundance than the WT strain) and the WT strain with various microscopy techniques. We show that Asl10923-111581 cells lack the copious EPS mantle that surrounds WT cells (Fig. 5) and massively stores Fe in their vicinity (Fig. 6). Consistently, we found that the EPS protect cells against the starvation of Fe (Fig. 6), which is crucial to cyanobacteria, and often limited in their natural environments [29]. Hence we propose that similarly to siderophores, which are not produced by Synechocystis [29], EPS operate in Fe homeostasis in sorbing Fe, which can be subsequently released and taken up by the cells when required. Finally, we show (Fig. 5) that the EPS protect cells from the toxicity of NaCl, CdSO₄ and CoCl₂, which are frequently encountered by cyanobacteria in nature [9,31]. Our results support the notion that EPS, in surrounding cells, behave as a physico-chemical barrier preventing direct contacts between cells and toxics. Furthermore, our data are consistent with the previous findings that the toxicity of both Cd and Co disturb Fe homeostasis, and can be decreased by increasing Fe availability [27,28]. Collectively, our results support the proposed utilization of EPS-producing cyanobacteria as chelating agents for biosorption processes aiming at removing toxic heavy metals from contaminated waters [7]. In addition, we believe that our mutants defective in EPS formation will be of help to better understand the role of cyanobacterial EPS in autoaggregation, protection against environmental stresses, and the biomineralization of the carbon dioxide gas by calcium and/or magnesium carbonates precipitation. This latter objective has both a basic research interest and an interesting biotechnological potential for carbon capture and storage [8]. We also suggest to investigate the biotechnological potentials offered by fast sedimenting EPS mutants, such as those presented here, for the characterization of potential surfactants, and to serve as cell factories that can be easily harvested and/or separated from products excreted in the growth media.

Supporting Information

Figure S1 Typical growth curves of the WT strain and the ten EPS-depleted mutants. (TIF)

Figure S2 PCR analysis of the double deletion mutants Asl0923-sll1581, Asl0923-slr1875 and Asl0923-sll5052. These double mutants were generated after the introduction of the Asl1581::Smr/Spr, Asl1875::Smr/Spr and Asl5052::Smr/Spr cassettes in the Asl0923::Kmr recipient mutant. Left panel : Schematic representation of the studied chromosome loci in the wild-type (WT) strain and the corresponding deletion mutants. The studied genes are represented by boxes pointing into the direction of their transcription. The size of the PCR products (double arrows) generated by the primers (small triangles) are indicated in kb. Right panels Typical UV-light images of the agarose gels showing the PCR products corresponding to the WT and mutant chromosomes. These data show that all studied clones of the deletion mutants harbour no WT copy of the chromosome. M₁ and M₂₀ respectively indicate the 1 kb DNA ladder and 50 bp DNA ladder (Invitrogen). (TIF)

Figure S3 Construction of the double deletion mutant Asl1875-sll1581. The Asl1875-sll1581 double mutant was obtained after introduction of Asl1581::Smr/Spr cassette into the Asl1875::Kmr recipient mutant. Left panel : Schematic representation of the studied chromosome loci in the wild-type (WT) strain and the corresponding deletion mutant. The studied genes are represented by boxes pointing into the direction of their transcription. The size of the PCR products (double arrows) generated by the primers (small triangles) are indicated in kb. Right panels Typical UV-light images of the agarose gels showing the PCR products corresponding to the WT and mutant chromosomes. These data show that all studied clones of the deletion mutant harbour no WT copy of the chromosome. M₁ and M₂₀ respectively indicate the 1 kb DNA ladder and 50 bp DNA ladder (Invitrogen). (TIF)

Figure S4 PCR analysis of the double deletion mutant Asl5052-sll1581 and Asl5052-slr1875. These double mutants were generated after the introduction of the Asl1581::Kmr and Asl1875::Kmr cassettes into the Asl5052::Smr/Spr recipient mutant. Left panels : Schematic representation of the studied chromosome loci in the wild-type (WT) strain and the corresponding deletion mutants. The studied genes are represented by boxes pointing into the direction of their transcription. The size of the PCR products (double arrows) generated by the primers (small triangles) are indicated in kb. Right panels Typical UV-light images of the agarose gels showing the PCR products corresponding to the WT and mutant chromosomes. These data show that all studied clones of the deletion mutants harbour no WT copy of the chromosome. M₁ and M₂₀ respectively indicate the 1 kb DNA ladder and 50 bp DNA ladder (Invitrogen). (TIF)
Table S1  Sequence of the PCR primers used in this study.

(PDF)

References

1. Pereira S, Zille A, Micheletti E, Moradas-Ferreira P, De Philippis R, et al. (2009) Complex anatomy of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS Microbiol Rev 33: 917–941.

2. Freitas F, Alves VD, Rein MA (2011) Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends Biotechnol 29: 388–390.

3. Consowr MS, Redfearn CJ, Ganguly T, Sukumar N, Sloan G, et al. (2012) BpR modulates Borrellella biofilm formation by negatively regulating the expression of the Bps polysaccharide. J Bacteriol 194: 233–242.

4. Nilsson M, Chiang WC, Fadz M, Gjerme A, Givokov M, et al. (2011) Influence of putative exopolysaccharide genes on Pseudomonas putida KT2440 biofilm stability. Environ Microbiol 13: 1357–1369.

5. Mulinaiazan AY, Kosunen PV, Makarova KS, Sorokin A, et al. (2006) The cyanobacterial genome core and the origin of photosynthesis. Proc Natl Acad Sci U S A 103: 13126–13131.

6. Zeh J (2011) Nitrogen fixation by marine cyanobacteria. Trends Microbiol 19: 162–173.

7. De Philippis R, Colica G, Micheletti E (2011) Exopolysaccharide-producing cyanobacteria in heavy metal removal from water: molecular basis and practical applicability of the biosorption process. Appl Microbiol Biotechnol 92: 697–708.

8. Janssens C, Norther T (2010) Calcifying cyanobacteria—the potential of biomimetic calcification for carbon capture and storage. Curr Opin Biotechnol 21: 365–371.

9. Tchounouw PB, Yedjou CG, Patlolla AK, Sutton DJ (2012) Heavy metal toxicity and the environment. EXS 101: 133–164.

10. Foster JS, Havemann SA, Singh AK, Sherman LA (2009) Role of mrgA in peroxide and light stress in the cyanobacterium Synechocystis sp. PCC 6803. FEMS Microbiol Lett 295: 296–304.

11. Grigorieva G, Shestakov S (1982) Transformation in the cyanobacterium Synechocystis sp. PCC 6803. J Bacteriol 150: 485–487.

12. Poncelet M, Cassier-Chauvat C, Leschelle X, Bottin H, Chauvat F (1998) Role of Synechocystis sp. PCC 6803, under the control of the Slr1738 regulator. BMC Microbiol 8: 350.

13. Zeyons O, Thill A, Chauvat F, Menguy N, Cassier-Chauvat C, et al. (2009) Direct and indirect CoO2 nanoparticles toxicity for Escherichia coli and Synechocystis. Nanotoxicology 3: 284–295.

14. Reed JW, Walker GC (1991) The exuD gene of Rhizobium meliloti encodes a novel function needed for alfalfa nodule invasion. J Bacteriol 173: 664–667.

15. Doksa T, Ayabe Y, Yasunaga M, Usami Y, Habe H, et al. (2003) Genes involved in the synthesis of the exopolysaccharide methanolan by the obligate methylotroph Methylobacillus sp. strain 128. Microbiology 149: 431–444.

16. Domon F, Houl C, Chauvat F, Cassier-Chauvat C (2004) Function and regulation of the cyanobacterial genes lexD, nclA and ruvB. LexD is critical to the survival of cells facing inorganic carbon starvation. Mol Microbiol 53: 63–80.

17. Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2: 924–932.

18. Labarre J, Chauvat F, Thuriaux P (1989) Insertional mutagenesis by random cloning of antibiotic resistance genes into the genome of the cyanobacterium Synechocystis strain PCC 6803. J Bacteriol 171: 3449–3457.

19. Cerantola S, Bounery J, Segonds C, Marty N, Montrozier H (2000) Exopolysaccharide production by mucoid and non-mucoid strains of Burkholderia cepacia. FEMS Microbiol Lett 185: 243–246.

20. Zouaou LC, Wulff NA, Gauravud P, Mariano AG, Virgilio AC, et al. (2006) Disruption of Xylella fastidiosa CVC gumB and gumF genes affects biofilm formation without a detectable influence on exopolysaccharide production. FEMS Microbiol Lett 257: 236–242.

21. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.

22. Liu X, Sheng J, Curtis R, S Redd (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci U S A 108: 6899–6904.

23. Houl C, Stuloff M, Bart C, Michau M, Picciotto A, et al. (2007) Biofilm formation, epiphytic fitness, and cancer development in Xanthomonas campestris pv. citri. Mol Plant Microbe Interact 20: 1222–1230.

24. Palma LG, Wulff NA, Gauravud P, Mariano AG, Virgilio AC, et al. (2006) Disruption of Xylella fastidiosa CVC gumB and gumF genes affects biofilm formation without a detectable influence on exopolysaccharide production. FEMS Microbiol Lett 257: 236–242.

25. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.

26. Liu X, Sheng J, Curtis R, S Redd (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci U S A 108: 6899–6904.

27. Houl C, Stuloff M, Bart C, Michau M, Picciotto A, et al. (2007) Biofilm formation, epiphytic fitness, and cancer development in Xanthomonas campestris pv. citri. Mol Plant Microbe Interact 20: 1222–1230.

28. Palma LG, Wulff NA, Gauravud P, Mariano AG, Virgilio AC, et al. (2006) Disruption of Xylella fastidiosa CVC gumB and gumF genes affects biofilm formation without a detectable influence on exopolysaccharide production. FEMS Microbiol Lett 257: 236–242.

29. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.

30. Liu X, Sheng J, Curtis R, S Redd (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci U S A 108: 6899–6904.

31. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.

32. Liu X, Sheng J, Curtis R, S Redd (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci U S A 108: 6899–6904.

33. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.

34. Liu X, Sheng J, Curtis R, S Redd (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci U S A 108: 6899–6904.

35. Giess M, Lange C, Soppa J (2011) P3012 in cyanobacteria. FEMS Microbiol Lett 323: 124–131.