CccS and CccP are Involved in Construction of Cell Surface Components in the Cyanobacterium Synechocystis sp. strain PCC 6803

Hidehisa Yoshimura1,*, Yasuko Kaneko2, Shigeki Ehira3, Shizue Yoshihara4, Masahiko Ikeuchi1 and Masayuki Ohmori3

1Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo. 153-8902 Japan
2Biological Section in the Faculty of Education, Saitama University, 255 Shimo-Ohkubo, Sakura-ku, Saitama-shi, Saitama, 338-8570 Japan
3Department of Biological Science, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyou-ku, Tokyo, 112-8551 Japan
4Research Institute for Advanced Science and Technology, Osaka Prefecture University, 1-2 Gakuen-cho, Sakai-shi, Osaka, 599-8531 Japan

*Corresponding author: E-mail, yoshimura_h@bio.c.u-tokyo.ac.jp; Fax. +81-3-5454-4337
(Received July 28, 2009; Accepted June 2, 2010)

We have previously identified two target genes (slr1667 and slr1668) for transcriptional regulation by a cAMP receptor protein, SYCRP1, in a cAMP-dependent manner. For this study we investigated the localizations of products of slr1667 and slr1668 (designated cccS and cccP, respectively) biochemically and immunocytochemically, and examined the phenotypes of their disruptants. CccS protein was detected in the culture medium and the acid-soluble fraction containing proteins derived from outside the outer membrane. Disruptants of cccS and cccP showed a more or less similar pleiotropic phenotype. Several proteins secreted into the culture medium or retained on the outside of the outer membrane were greatly reduced in both disruptants compared with the wild type. Electron microscopy revealed that the cccS disruptant lacked the thick pili responsible for motility and that the cccP disruptant had almost no discernible thick pili on its cell surface. Both disruptants largely secreted far greater amounts of yellow pigments into the culture medium than did the wild type. Furthermore, the disruptions reduced the amount of UV-absorbing compound(s) extractable from the exopoly-saccharide layer. These results suggest that the cccS and cccP genes are involved in the construction of cell surface components in Synechocystis sp. strain PCC 6803.

Keywords: Cell surface protein • Cyanobacteria • Extracellular protein • Pigments • UV-absorbing compound(s).

Abbreviations: BSA, bovine serum albumin; CRP, cAMP receptor protein; Cya, adenylate cyclase; OD, optical density; PAS, periodic acid–Schiff; PVDF, poly(vinylidene difluoride); S-layer, cell surface layer; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing 0.05% Tween-20.

Introduction

The cAMP signaling pathway plays an important role in biological regulation in prokaryotes and eukaryotes by altering enzyme activities and gene expression (Botsford and Harman 1992, Skalhegg and Tasken 2000). Cyanobacteria, a distinct group of Gram-negative bacteria, are the most ancient organisms in the history of life that perform oxygenic photosynthesis. To date, various findings on cAMP have been documented for several representative species of cyanobacteria. For instance, the cellular cAMP level responds rather rapidly to various environmental changes such as oxic–anoxic, light−dark, low pH–high pH, salt and specific monochromatic lights in the filamentous N2-fixing cyanobacteria, Anabaena cylindrica (Ohmori et al. 1988, Ohmori 1989, Ohmori et al. 2002) and Anabaena sp. PCC 7120 (Okamoto et al. 2004, Imashimizu et al. 2005), and in the unicellular cyanobacterium, Synechocystis sp. PCC 6803 (Terauchi and Ohmori 2004) (Synechocystis herein). cAMP also induces gene expression for regulation of nitrogen–carbon metabolism and for protection against oxidative stress in Anabaena sp. PCC 7120 (Suzuki et al. 2007, Higo et al. 2008), and enhances respiration and cellular gliding motility of a filamentous cyanobacterium, Spirulina platensis (Ohmori et al. 1992), and twitching motility of Synechocystis cells (Terauchi and Ohmori 1999).

We noticed that the genome sequences of many species of cyanobacteria reveal the presence of genes for adenylate cyclase (Cya) and cAMP receptor protein (CRP), except marine Prochlorococcus and Synechococcus species. This strongly supports the idea that the cAMP signaling pathway plays important roles in cyanobacterial life. The Synechocystis genome has at least one gene for active adenylate cyclase (cyaI), which is...
essential for its switching motility (Terauchi and Ohmori 1999). The cellular cAMP level is up-regulated by irradiation with blue–UV-A light (450–380 nm) and the regulation is abolished by inactivation of the cya1 gene. Furthermore, this irradiation enhances cellular motility (Terauchi and Ohmori 2004). The genome also harbors a gene (sycrp1) coding for active CRP that binds cAMP for transcriptional regulation (Yoshimura et al. 2000). This sycrp1 gene is also essential for the twitching motility (Yoshimura et al. 2002a). Round-shaped cells ofSynechocystis have two distinct pilus structures: the thick pili of 5–8 nm in diameter are essential for the twitching motility and natural transformation, whereas the thin pili of approximately 3 nm in diameter have no known function and often occur as bundles (Bhaya et al. 2000, Yoshihara et al. 2001). The non-motile sycrp1 disruptant cells showed a phenotype with much reduced thick pili (Yoshimura et al. 2002a). Independently, we have demonstrated that one molecule of a dimeric form of the transcriptional regulator SYCRP1 tightly binds one molecule of cAMP (Yoshimura et al. 2000). DNA microarray analysis has revealed that the expression levels of 18 genes were repressed by disruption of the sycrp1 gene under normal culture conditions (Yoshimura et al. 2002b). Since the expression levels of slr1667 and slr1668 were the most strongly repressed by disruption of the sycrp1 gene, we surmised that these genes play important roles in the cAMP signaling pathway and cellular motility of Synechocystis.

For this study, we disrupted the open reading frames of slr1667 and slr1668. We also investigated the localization of slr1667 and slr1668 gene products and the influence of disruption of the genes on the cell surface components, including surface pili, biochemically and immunocytochemically. The results suggested that the slr1667 and slr1668 gene products are located in the cell surface and in the cell peripheral region, respectively, and both are involved in construction of cell surface components. We thus designated the target genes of cAMP-SYCRP1 cccS (slr1667) and cccP (slr1668) hereafter.

**Results**

**Influence of cccS and cccP disruption on extracellular proteins**

We disrupted the upstream cccS gene with a readthrough-type kanamycin resistance cassette in the same direction to allow transcription of the downstream cccP gene, while the cccP gene was disrupted with the aadA cassette carrying the transcriptional terminator. The growth of these disruptants was measured by optical density (OD) at 750 nm. Both disruptants grew photosynthetically without appreciable reduction in the growth under normal culture conditions compared with the wild type (data not shown).

Extracellular proteins released into the culture medium were recovered, concentrated, and resolved by SDS–PAGE. We found marked differences in the protein profile between the wild type and the disruptants, but very few between the cccS disruptant and the cccP disruptant. Most prominently, the proteins of apparent molecular mass of 25.1, 22.6 and 19.5 kDa (Fig. 1A, arrowheads a–c, respectively), and those of >105 kDa were practically absent from the extracellular protein fraction of both disruptants, while a 32 kDa protein was reduced there.

Of these, the 25.1 and 22.6 kDa proteins were identified as PilA1 (sll1694) by N-terminal sequencing. An N-terminal region of 21 amino acid residues in the predicted PilA1 was missing in the PilA1-derived proteins. The two PilA1-derived proteins could be stained with periodic acid–Schiff (PAS), suggesting that both proteins were modified (Fig. 1B). The 19.5 kDa protein was identified as CccS by N-terminal sequencing. An N-terminal region of 28 amino acid residues in the predicted CccS was missing in the CccS-derived protein. The CccS-derived protein was not stained by PAS, suggesting that the protein was not modified (Fig. 1B). The 32 kDa protein could not be sequenced, because its N-terminus was blocked.

Since surface pili are a major component of the cell surface and thick pili were lost in the cccS disruptant (Fig. 4, see below), we conjectured that CccS might be a component of thick pili. Surface pili were collected from the culture medium of wild-type cells according to the pili precipitation method (Alm and Mattick 1995). Western blotting analysis using anti-CccS antisera found no CccS in the pilus fraction (data not shown), suggesting that CccS is in fact not a component of pili.

The two protein-derived bands between 15 and 20 kDa observed in the extracellular protein fraction of cccS and cccP disruptants were blue-purple and thus derived from phycocyanin or allophycocyanin (Fig. 1A, lanes 2 and 3); they were missing in the wild-type fraction (Fig. 1A, lane 1).
The difference in the release of phycocyanin or allophycocyanin between the wild type and the two disruptants was reproducible, although it was not particularly large. It is likely that some disruptant cells partly ruptured during centrifugation, suggesting that the disruptants are more fragile than the wild type.

**Influence of cccS and cccP disruption on proteins outside the outer membrane**

We examined proteins from the outside of the outer membrane in wild-type, cccS and cccP disruptant cells, which were extracted from these cells by acidic solution. This method has been established to extract cell surface layer (S-layer) proteins associated with the outer membrane in Gram-negative bacteria (Walker et al. 1992, Nitta et al. 1997). The acid-soluble fraction from the wild type contained several proteins of molecular mass >60 kDa. Again, the acid-soluble fractions from the two disruptants, being similar to each other in protein profile, were greatly depleted of at least two proteins of molecular mass between approximately 110 and 160 kDa, which were abundant in wild-type fractions (Fig. 2A). We tried to identify these high molecular mass proteins by N-terminal sequencing but we were not successful. It is also evident that a minor protein of 23.6 kDa found in the wild type was missing in both cccS and cccP disruptants (Fig. 2A). Western blotting analysis using anti-CccS antiserum detected two bands at 23.6 and 19.5 kDa in the fraction derived from the wild type (Fig. 2B, arrows a and b), which were not present in either disruptant.

**Localization of CccS and CccP**

In conventional electron microscopy, the ultrastructure of cccS and cccP disruptant cells (Fig. 3B, C) did not look significantly different from that of the wild type (Fig. 3A).
Immunocytochemical analysis revealed the localization of CccS and CccP in wild-type cells. We raised antisera against the synthetic peptides and affinity purified them to avoid nonspecific reactions. As a result, the detection frequency of gold particles was low, but labeling was reliable and reproducible: no gold particles at all were detected in controls where the respective antibodies were used on the cccS or cccP disruptant cells. As it was rather difficult to locate the particles precisely in the fine structure (as shown in Fig. 3A–C), we divided technically the area where particles were observed into two regions: (i) the cell surface region outside the outer membrane consisting of the S-layer (Sleytr and Beveridge 1999) and the exopolysaccharide layer (Kumar et al. 2007); and (ii) the cell peripheral region including the cytoplasmic membrane, the periplasm and the outer membrane. Remarkably, no gold particles were detected in the interior of the cells. Gold particles for CccS were mainly localized at the cell surface region (Fig. 3D1–4 for CccS) and particles for CccP were mainly localized in the cell peripheral region (Fig. 3E1–4 for CccP). Statistically, we found that for CccS 69% of gold particles were located at the cell surface region and 31% in the cell peripheral region (39 particles in total), while the distribution for CccP was approximately the reverse: 32% at the cell surface region and 68% in the cell peripheral region (47 particles in total).

Electron microscopy of pili
The cccS and cccP disruptant cells formed dome-shaped colonies, while wild-type cells formed flat sheet-like colonies (data not shown). This indicates that both types of disruptant cells were non-motile, in agreement with the non-motile phenotype of scrp1 disruptant cells (Yoshimura et al. 2002a). It is established that the unicellular Synechocystis employs surface pili to drive cellular motility. We examined the pilus structure on the cell surface by electron microscopy. The presence of pili on wild-type cells (Fig. 4A) was consistent with the earlier observations (Bhaya et al. 2000, Yoshihara et al. 2001). Thick pili and bundles of thin pili were clearly visualized, while thin pili were not clearly visualized by negative staining. It is remarkable that the non-motile cccS disruptant cells lacked thick pili (Fig. 4B), while the non-motile cccP disruptant cells retained some thick pili although the number was much reduced compared with wild-type cells (Fig. 4C). On the other hand, the quantity of thin pili was not affected by disruption of cccS or cccP.

Yellow pigments and UV-absorbing compound(s)
Yellow pigments (absorption maxima at 397 and 365 nm) were accumulated far more abundantly in the culture media of cccS and cccP disruptants (Fig. 5A, traces b and c), than in wild-type culture media (Fig. 5A, trace a). On the other hand, it has been reported that UV-absorbing compounds are secreted into the exopolysaccharide layer of a terrestrial cyanobacterium Nostoc commune (Böhm et al. 1995). We examined such compounds in 20% methanol extract from cells according to the method of Böhm et al. (1995), which allows release of small compounds without cell rupture. Both the wild type and disruptants secreted similar UV-absorbing compound(s) peaking at approximately 332 nm, but the amount was significantly lower in the disruptants than in the wild type (Fig. 5B). We also examined the amount of exopolysaccharide of the wild type and the disruptants according to the method of Bertocchi et al. (1990). There was no significant difference among them (data not shown).

Discussion

Architecture and localization of CccS and CccP
The predicted CccS protein shows no clear homology to known proteins. However, closer inspection with PFAM Search shows that the C-terminal region from residue 154 to 174 is weakly related to an SCPU domain in the C-terminal region of spore coat protein U (E value 0.08, Supplementary Fig. S1). The SCPU domain has been found in bacterial spore coat proteins and type-I pilin proteins which are secreted across the outer membrane in Gram-negative bacteria, and is also found in genes within a conserved operon that encodes a recently discovered chaperone–usher pilus assembly system (Tomaras et al. 2003). In the csu operon of Acinetobacter Baumannii, the products...
of csuA/B and csuB contain the SCPU domain and the products of the downstream csuC and csuD are related to pili assembly chaperones and ushers of Gram-negative bacteria, respectively.

The predicted CccP protein harbors a region from residue 70 to 154 homologous to the N-terminal region of pili assembly chaperones of Gram-negative bacteria such as PapD and FimC according to PFAM Search (E value 2.7e-6). PapD and FimC targeted into the periplasm mediate proper folding and transfer of outer surface proteins such as pilin subunits of type-I pili or P pili (Hung et al. 1996, Sauer et al. 2000). Pili assembly chaperones harbor 10 residues essential for the assembly of pili. However, while four functional residues in the N-terminal region of PapD and FimC are conserved in that of CccP, the other six functional residues in the C-terminal region of PapD and FimC are not conserved in that of CccP (Supplementary Fig. S2). It is thus not clear whether CccP interacts with pilin subunits or not. On the other hand, thick pili were reduced in the cccP disruptant. Chaperone-like CccP may interact with other factors which are involved in the assembly of the thick pili.

CccS has not been detected in proteomic analysis of protein extract from isolated cytoplasmic membrane, periplasm or outer membrane (Fulda et al. 2000, Huang et al. 2002, Huang et al. 2004). On the other hand, our results suggested that CccS is predominantly located in the cell surface region (Fig. 3D), although some degraded CccS was released into the culture medium (Fig. 2B). CccP has been detected in the periplasm fraction prepared from Synechocystis cells (Fulda et al. 2000). We confirmed this localization by immunocytochemistry (Fig. 3E).

We propose a model depicting the architecture and localization of CccS and CccP (Fig. 6). The model can explain why the accumulation of CccS in the cell surface region was abolished in the cccP disruptant (Fig. 2). The abolition indicates that intracellular CccS is not able to be secreted across the outer membrane without the support of CccP. Indeed, immunocytochemical analysis suggests that CccS is mainly located in the interior of the cccP disruptant cells by immunocytochemical analysis (see below). CccP may play an important role for proper folding and targeting of CccS to the outer surface of the outer membrane. Direct interaction of CccP and CccS was suggested by yeast two-hybrid screening in both sets of bait and prey (Sato et al. 2007).

Construction of cell surface components via CccS and CccP

We have summarized the construction of various cell surface components via CccS and CccP in Fig. 6, and would like to note three points.

First, thick pili were completely lost in the cccS disruptant, while thick pili were much reduced but not absent in the cccP disruptant (Fig. 4). Immunocytochemical analysis of CccS in wild-type and cccP disruptant cells showed that there were consistently more gold particles in the interior of the cccP disruptant cells than in the interior of wild-type cells, while significantly fewer gold particles were found on the cell surface or peripheral regions of the cccP disruptant cells than in these regions of wild-type cells (data not shown). These results imply that the presence of CccS in the interior of the cells is not sufficient to ensure the assembly of thick pili. The PilQ channel in the outer membrane is essential for the assembly and protrusion of the thick pili filaments and for motility (Yoshihara et al. 2001, Nudleman and Kaiser 2004). CccS and/or CccP may support targeting or stable assembly of the PilQ channel. Notably, the thin pili on the cell surface appeared unaffected in the cccS and cccP disruptants. The pilus phenotypes of the disruptants were similar to the non-motile pilQ disruptant pilus phenotype: the thick pili absent but the thin pili present (Yoshihara et al. 2001). The thin pili are not involved in cellular motility. Although nothing is known about assembly of the thin pili, our results suggest that CccS and CccP are not involved in the assembly or targeting of the thin pili to the cell surface.
Environmental changes

plasma membrane

periplasm

outer membrane

cell-surface proteins

extracellular proteins

thick pili

yellow pigments

UV-absorbing compound(s)

CccS

cccS  cccP

Ccp

Motility

The pilus phenotypes of cccS and cccP disruptants are also very similar to that of the sycrep1 mutant (Yoshimura et al. 2002a). It is likely that the pilus phenotype of the sycrep1 mutant was caused by the reduced expression of the cccS and cccP genes. PilA1 is the major structural subunit of thick pili (Bhya et al. 2000). PilA1 proteins with different molecular mass without the N- or C-terminal truncation of mature PilA1 have previously been detected and were found to be modified by glycosylation (Kim et al. 2004, Kim et al. 2009). We also detected PilA1 proteins of different molecular mass (Fig. 1). Evidently, PilA1 proteins with different glycosylation patterns are produced in Synechocystis, though there may be other modifications to PilA1 besides glycosylation.

Secondly, accumulation of UV-absorbing compound(s) in the exopolysaccharide layer was reduced in cccS and cccP disruptants. Release of yellow pigments into the culture medium was significantly enhanced in the disruptants. These yellow pigments are also UV-absorbing compounds (absorption maxima at 397 and 365 nm). A similar release of yellow pigments was observed in Synechocystis with mutated sigF, a gene essential for the biogenesis of pili (Bhya et al. 1999). The reduction in UV-absorbing compound(s) and the release of the yellow pigments in cccS and cccP disruptants may be a consequence of the absence or reduction of cell surface components including thick pili. In cyanobacteria, UV-absorbing compounds and pigments are associated with the cell surface (Böhm et al. 1995) and have been reported to serve as a screen for protection against UV-induced photodamage (Sinha et al. 1998, Holzinger and Lütz 2006). It seems likely that the UV-absorbing compound(s) and the yellow pigments on the cell surface are involved in UV tolerance.

Thirdly, extracellular proteins larger than 105 kDa in the culture medium and accumulation of proteins of molecular mass from 110 to 160 kDa on the cell surface were reduced in the cccS and cccP disruptants. It is not clear whether these extracellular proteins were actively secreted into the culture medium or not. The extracellular proteins may have been originally located on the cell surface. The extracellular and the cell surface proteins may be involved in the assembly of thick pili or accumulation of UV-absorbing compounds. At the moment, we do not know the identity of these proteins.

The adenylate cyclase Cya1 is a key factor for accumulation of cAMP. The expression of cccS and cccP is strictly regulated by cAMP and SYCRP1 (Yoshimura et al. 2002b), and our results suggest that CccS and CccP are involved in the construction of cell surface components. Similarly, cAMP is known to be involved in various cell surface or cell peripheral aspects in many bacteria: induction of the P pilus (pap) operon in uropathogenic Escherichia coli (van der Woude et al. 1996), induction of cellular motility, production of extracellular...
protein and biofilm formation in the soil bacterium *Pseudomonas aeruginosa* (West et al. 1994, Beatson et al. 2002, Whitchurch et al. 2005), production of yellow pigment in *Erwinia herbicola* (Perry et al. 1986), and so on. The cAMP regulation of various cell surface aspects is thus found in many bacteria, including cyanobacteria. We hypothesize that the regulation of cell surface components via the cAMP signaling pathway responds to environmental changes. Recently, it has been reported that some environmental changes affect expression of many genes including *cccS* and *cccP*. These are transitions of CO₂ concentration, pH, dark to light and low to high light (Eisenhut et al. 2007, Summerfield and Sherman 2007, Singh et al. 2008, Summerfield and Sherman 2008). Further analysis is necessary to fill in the details of the mechanisms that control the response of CccS and CccP to environmental changes.

### Materials and Methods

**Bacterial strains and culture conditions**

Wild-type and disruptant cells of *Synechocystis* sp. PCC 6803 were grown in BG11 medium (Stanier et al. 1971) at 30°C under continuous illumination provided by fluorescent lamps at 25–30 µmol photons m⁻² s⁻¹. Liquid cultures were bubbled with air containing 1% CO₂.

**Disruption of *cccS* and *cccP***

The DNA fragment containing the *cccS* and *cccP* genes of *Synechocystis* was amplified by PCR with the genomic DNA. The primers used were designed on the basis of sequence information from Cyanobase. The two primer sets for *cccS* disruption were as follows: 1-forward, 5′-TGGGGAAGCAATAGAC-3′; 1-reverse, 5′-CCCAGTTGACTTTGAC-3′; 2-forward, 5′-CTGGCTATGCG-3′; and 2-reverse, 5′-TAATGGCCAGCTTGGG-3′. Smal sequences (underlined) attached to the 5′ end of the 1-reverse and 2-forward primers created a Smal restriction site within the open reading frame of the *cccS* gene. The respective PCR products (CCCS-1 and CCCS-2) were cloned into pGEM-T Easy (Promega) according to the manufacturer’s instructions. Cloning of the DNA fragments was verified by sequencing. The two plasmids (pCCCS-1 and pCCCS-2) were digested with Smal and ScaI (a restriction site on the pGEM-T Easy vector), and CCCS-2 was ligated into pCCCS-1. A Tn5-derived kanamycin resistance cassette (readthrough type) was inserted into the upstream slr1667 gene at the Smal site in the same direction as slr1667/slr1668 to allow expression of the downstream slr1668 gene in the resulting plasmid. It is established that the kanamycin resistance gene and the downstream bleomycin resistance gene are co-transcribed in the transposon Tn5 in bacteria (Mazodier et al. 1985). The 1.3 kb cassette includes the kanamycin resistance gene and a C-terminally truncated bleomycin gene, and has been used for disruption of genes in operons without polar effect. The primers for *cccP* disruption were as follows: forward, 5′-ATTCAACACCGCTGTTGTC-3′; reverse, 5′-TCATGGGGCAAACCTAAGC-3′. The PCR products were cloned into the pGEM-T Easy and sequenced. The resultant plasmid was digested with AccI in the downstream *cccP* gene, and then a 2.0 kb streptomycin/spectinomycin resistance cassette (aadA gene) derived from plasmid pRL453 was inserted (Elhai and Wolk 1988). This cassette is known to possess a transcriptional terminator. Wild-type cells were transformed according to the Williams method (Williams 1988). Transformants were selected on BG11 agar containing 20 µg mL⁻¹ kanamycin or spectinomycin. Full segregation of these mutants was confirmed by PCR (data not shown).

**Collection of extracellular proteins in culture media, PAS staining and N-terminal sequencing**

Cells (OD₇₅₀ 3.5–4.0) in 60 ml of BG11 medium in the light were sedimented by centrifugation at 17,700 × g for 5 min at 4°C. The centrifugation was repeated to remove all of the cells. Proteins in the supernatant were precipitated by adding trichloroacetic acid to a final concentration of 10%, incubating on ice for 1 h, and then centrifuging for 1 h at 17,700 × g at 4°C. The protein pellet was washed twice with ice-cold 90% acetone, air-dried and resuspended in 250 µl of 1× SDS loading buffer. A 20 µl aliquot of solubilized sample was analyzed by 12% SDS–PAGE (Laemmli 1970). The gel was stained with Coomassie Brilliant Blue R-250 or by PAS for detection of glycoprotein. The PAS staining was performed with a GelCode Glycoprotein Staining kit (Pierce Biotechnology, Inc.). The N-terminal sequences of proteins blotted to a poly(vinylidene difluoride) (PVDF) membrane (GE Healthcare Bioscience, Tokyo, Japan) were determined by a protein sequencer (Shimadzu Corporation, Kyoto, Japan).

**Extraction of proteins outside the outer membrane**

Extraction of proteins outside the outer membrane was performed basically as described by Nitta et al. (1997). S-layer proteins in Gram-negative bacteria are not anchored to the cell surface via a covalent interaction but interact with the sugar moiety of the component of the outer membrane (Sleytr and Beveridge 1999). A low pH solution that can break ionic bonds between S-layer proteins and the component is effective in extracting S-layer proteins from whole cells (Walker et al. 1992). Cells (OD₇₅₀ 3.5) from 130 ml of liquid culture were harvested by centrifugation at 17,700 × g for 5 min at 4°C. The harvested cells were washed with BG11 medium and then lyophilized. The lyophilized cells were incubated in 10 mM HEPES, pH 2.5 for 2 h at 37°C. The cells were removed by centrifugation at 17,700 × g for 5 min at 4°C. This removal step was repeated three times. The supernatant was adjusted to pH 7.5 with NaOH and insoluble material was sedimented at 17,700 × g for 30 min. The final supernatant was lyophilized and was dissolved in 500 µl of distilled water. Protein concentration was determined by the Lowry method (Lowry et al. 1951). A 5 µg aliquot of each sample was analyzed by 12% SDS–PAGE. Proteins in the gel were stained with SYPRO® Orange.
Western blotting analysis

Amino acid residues 65–79 of CccS were used as an antigenic epitope. The oligopeptide TLTSIGNVNVKSNYS was synthesized. Peptide synthesis and immunization of rabbit were performed by Qiagen. After SDS–PAGE, proteins were transferred to a PVDF membrane. The membrane was blocked by incubation with 10% non-fat dry milk in 50 mM Tris-buffered saline, pH 8.0 (TBS) for 1 h, washed with TBS containing 0.05% Tween-20 (TBST), and reacted with a 1:1,000 dilution of the rabbit antisera to the synthetic peptide of CccS with shaking for 1 h. After washing with TBST, the membrane was reacted with a 1:5,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA) for 1 h, followed by washing and subsequent color development with 3′,3′-diaminobenzidine (Sigma-Aldrich, Tokyo, Japan) and H2O2 as substrates.

Affinity purification of anti-CccS and anti-CccP antibodies

Peptides 11–24, EMETLTWSPSKETE, and 98–111, GVGDMETP, of CccS were synthesized as antigenic epitopes. Amino acid residues 144–157, EGAEPSTTEDGSQK, and 195–208, KGVGENILAGNKR, of CccP were synthesized as antigenic epitopes. These peptides were predicted as regions arranged on the surface of CccS and CccP structures by 3D structure prediction programs from Sigma-Aldrich. Peptide synthesis and immunization of rabbit were performed by Sigma-Aldrich (Tokyo, Japan). For affinity purifications, the whole-cell extract including CccS and CccP was resolved by SDS–PAGE, and transferred to a PVDF membrane. The membrane was blocked by incubation with 10% non-fat dry milk in 50 mM Tris-buffered saline, pH 8.0 (TBS) for 1 h, washed with TBS containing 0.05% Tween-20 (TBST), and reacted with a 1:10 dilution of the antisera to the synthetic peptide of CccS with shaking for 1 h. After washing with TBST, the membrane was reacted with a 1:1,000 dilution of the rabbit antisera to the synthetic peptide of CccS with shaking for 1 h. After washing with TBST, the membrane was reacted with a 1:5,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA) for 1 h, followed by washing and subsequent color development with 3′,3′-diaminobenzidine (Sigma-Aldrich, Tokyo, Japan) and H2O2 as substrates.

Conventional electron microscopy

For ultrastructural studies, cells were fixed in 2% glutaraldehyde in 50 mM potassium phosphate buffer (pH 7.0) for 2 h at room temperature and in a refrigerator overnight. After rinsing in buffer, the cells were post-fixed in 2% OsO4 in buffer for 2 h at room temperature. They were then dehydrated in an acetone series and embedded in Spurr’s resin. Ultrathin sections (silver–gold) were cut with a diamond knife on a Sorvall MT2-B ultramicrotome. After staining with uranyl acetate and lead citrate, the sections were observed with a Hitachi H-7500 electron microscope at an accelerating voltage of 100 kV.

Detection of pili by electron microscopy

Negative staining of pili on the cell surface was examined as described by Yoshihara et al. (2001). Cells of each strain growing on agar plates were gently suspended in BG11 medium, stained with 0.8% (w/v) phosphotungstic acid (pH 7.0), and examined with a transmission electron microscope (model 1200EX, JEOL, Tokyo, Japan).

Extraction of UV-absorbing compound(s)

The extraction was carried out based on the method of Böhm et al. (1995). Cells of liquid cultures were harvested by centrifugation for 5 min at room temperature. The cells were washed with fresh culture medium three times by centrifugation. UV-absorbing compound(s) from the exopolysaccharide layer were extracted from the harvested cells by submersion in 20% (v/v) aqueous methanol for 1 h at 45°C.
Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Science, Sports and Culture of Japan (Grant-in-aid for General Scientific Research (12206002, 15370019)); Japan Space Forum.

Acknowledgments

We have benefited from the excellent support of Dr. Shinobu Okamoto.

References


