Outer Membrane Proteins Derived from Non-cyanobacterial Lineage Cover the Peptidoglycan of Cyanophora paradoxa Cyanelles and Serve as a Cyanelle Diffusion Channel*

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The cyanelle is a primitive chloroplast that contains a peptidoglycan layer between its inner and outer membranes. Despite the fact that the envelope structure of the cyanelle is reminiscent of Gram-negative bacteria, the Cyanophora paradoxa genome appears to lack genes encoding homologs of putative peptidoglycan-associated outer membrane proteins and outer membrane channels. These are key components of Gram-negative bacterial membranes, maintaining structural stability and regulating permeability of outer membrane, respectively. Here, we discovered and characterized two dominant peptidoglycan-associated outer membrane proteins of the cyanelle (∼2 × 10^6 molecules per cyanelle). We named these proteins CppF and CppS (cyanelle peptidoglycan-associated proteins). They are homologous to each other and function as a diffusion channel that allows the permeation of compounds with M_r <1,000 as revealed by permeability measurements using proteoliposomes reconstituted with purified CppS and CppF. Unexpectedly, amino acid sequence analysis revealed no evolutionary linkage to cyanobacteria, showing only a moderate similarity to cell surface proteins of bacteria belonging to Planctomycetes phylum. Our findings suggest that the C. paradoxa cyanelle adopted non-cyanobacterial lineage proteins as its main outer membrane components, providing a physical link with the underlying peptidoglycan layer and functioning as a diffusion route for various small substances across the outer membrane.

The outer membrane of Gram-negative bacteria functions as a permeability barrier that inhibits the entry of toxic compounds into the cell and prevents leakage of various substances essential for proliferation from the cell (1, 2). Gram-negative bacteria require peptidoglycan-associated proteins, such as Lpp and Pal in Escherichia coli, to maintain the structural integrity of their outer membrane (3–6). Most peptidoglycan-associated proteins that play this structural role are abundant (>10^4 molecules per cell) and are involved in the physical link between the outer membrane and peptidoglycan. Absence of these proteins results in disordered cell surface structure and defective outer membrane function, often accompanied by abnormal cell morphology and growth. Our recent studies demonstrated that an anaerobic Gram-negative bacterium, Selenomonas ruminantium, in which the outer membrane-peptidoglycan association had long been questioned because of the absence of Lpp and Pal, exploits an interaction between peptidoglycan-bound polyamines and major outer membrane proteins to anchor its outer membrane to the peptidoglycan (7–9). This finding reinforced the concept that a physical connection between the outer membrane and peptidoglycan is a prerequisite for structural stability of the outer membrane of Gram-negative bacteria.

The cyanelle is a primitive chloroplast with a cyanobacterium-like envelope structure comprising an inner membrane, peptidoglycan, and outer membrane (10, 11). It is widely accepted that the chloroplast has evolved from an endosymbiotic cyanobacterium. Therefore, the following considerations led us to focus on the stability and permeability of the outer membrane of the cyanelle. (i) The barrier property of the outer membrane of endosymbiotic cyanobacterium is expected to be reorganized in the cyanelle because of the requirement for a metabolic link with the host cell, and (ii) the physical connection between the peptidoglycan and the outer membrane appears to be present in the cyanelle because no structural defect of its outer membrane has been observed (12, 13). However, none of the known peptidoglycan-associated proteins or outer membrane channels seem to be conserved in the Cyanophora paradoxa genome, reflecting the consequence of evolution from endosymbiotic cyanobacterium to cyanelle and implying the involvement of a novel factor(s). Here, we isolated and characterized two dominant peptidoglycan-associated outer membrane proteins (∼2 × 10^6 molecules per cyanelle). We named these proteins CppS and CppF (cyanelle peptidoglycan-associated proteins). They are homologous and function as a diffusion channel. Unexpectedly, amino acid sequence analysis revealed no evolutionary linkage to cyanobacteria.

Results
Isolation of CppS and CppF. Cyanelle Peptidoglycan-associated Outer Membrane Proteins—Because no detectable homologs of known peptidoglycan-associated proteins are encoded in the C. paradoxa genome, we used a biochemical approach to isolate cyanelle peptidoglycan-associated proteins. We first obtained crude membranes of cyanelles, separated them by...
sucrose density gradient centrifugation, and collected the fraction that co-separated with peptidoglycan, which was found to be located at 1.22 g/cm³ in the gradient (Fig. 1A). To remove proteins that were not associated with the peptidoglycan, we subjected this fraction to detergent treatment, relying on the fact that peptidoglycan-associated proteins are generally resistant to such treatment. Two major proteins remained insoluble after extraction with 2% SDS at 37 °C (Fig. 1B), representing the possible candidate cyanelle peptidoglycan-associated proteins. Practically the same results were obtained after extraction using other detergents, such as 1% N-lauroylsarcosine, 2% octyl glucoside, 1% Triton X-100, and 1% dodecyl maltoside, at 37 °C (data not shown). We further purified the detergent-extracted fraction by sucrose density gradient centrifugation and verified that these two proteins co-fractionated with peptidoglycan (peptidoglycan/protein preparation) (Fig. 1C). We initially attempted to obtain direct evidence of a physical association between these proteins and peptidoglycan, via peptidoglycan binding assay, using purified and solubilized proteins detached from the peptidoglycan. However, we observed that these proteins were highly cross-linked by disulfide bonding, forming a high molecular weight complex that did not enter the SDS-PAGE gel and that was highly resistant to detergent treatment (Fig. 2A). Detachment of these proteins from peptidoglycan required prolonged and denaturing detergent treatment, i.e., incubation at 100 °C in 2% SDS for 1 h. Adding reducing agents to the detergents successfully solubilized these complexes, but the reduced/solubilized form of the proteins had little affinity for purified peptidoglycan (Fig. 2B). To eliminate the effect of disulfide bonds that might have formed during the isolation procedure, we isolated these proteins under the presence of alkylation agent, i.e., 0.5 M iodoacetamide. However, we observed that the alkylated sample was also highly resistant to detergent treatment and was highly cross-linked (Fig. 2C). These results suggest that the majority of the disulfide bonds within these proteins are formed in the C. paradoxa cell. In view of this situation, we attempted a direct observation of the association of these proteins with the peptidoglycan using electron microscopy. We first examined the peptidoglycan/protein preparation and detected a layer-like structure closely attached to the electron-dense peptidoglycan layer (Fig. 3). We verified that this layer disappeared upon removing the proteins from the peptidoglycan preparation. These observations suggest that the two candidate proteins were actually the peptidoglycan-associated proteins of the cyanelle, constructing a layer-like structure that is closely attached to the peptidoglycan. We named these proteins CppF and CppS (cyanelle peptidoglycan-associated outer membrane proteins).
doglycan-associated proteins with fast and slow mobility on SDS-polyacrylamide gel, respectively). The following observations suggested that CppS/F are outer membrane proteins. (i) The CppS/F-peptidoglycan preparation was enriched for zeaxanthin (8.8 nmol/10^6 g of protein) (Fig. 4), the major outer membrane pigment of the cyanelle and of cyanobacteria (11), and (ii) electron micrographs of CppS/F-peptidoglycan preparation were reminiscent of those of peptidoglycan-associated outer membrane proteins of cyanobacteria, detected as a similar layer closely attached to the peptidoglycan (14). Indeed, we verified that CppS/F could be isolated from the outer membrane preparation of the cyanelle (Fig. 5). A roughly estimated number of CppS/F was ~2 × 10^6 molecules per cyanelle, and this quantity appeared sufficient to cover most (≥70%) of the peptidoglycan layer surface area, only if we can assume that CppS/F are equivalent in size to the major outer membrane proteins of similar molecular mass of Gram-negative bacteria (15).

Sequence Analysis of CppS/F Proteins—To identify CppS/F, we degraded CppS/F proteins by cyanogen bromide treatment and determined N-terminal amino acid sequences of the product fragments. CppS degradation produced one major fragment of ~40 kDa, whereas CppF degradation resulted in two major fragments of ~18 and 23 kDa as judged by SDS-PAGE analysis (data not shown). N-terminal sequences of the CppS fragment and smaller CppF (18-kDa) fragment were determined to be APECTVVNSGI and IPIEETIVQTKTYSAKL, respectively. The sequence of the larger (23-kDa) CppF fragment could not be determined. cDNA sequences that encode these amino acid sequences were retrieved from C. paradoxa EST^2 database by BLAST search. Based on these sequences, full-length cDNA sequences of cppS and cppF were determined using the rapid amplification of cDNA ends (RACE) method. Deduced amino acid sequences of CppS/F are shown in Fig. 6A.

The cppS and cppF genes are nuclearly encoded. Each contains five introns and is located at a different chromosomal

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2 The abbreviations used are: EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; SLH, S-layer-homologous; LDS, lithium dodecylsulfate; CBB, Coomassie Brilliant Blue.
locus. No other genes that encoded similar proteins were detected in the *C. paradoxa* genome. Their deduced amino acid sequences were homologous to each other (30% identity) (Fig. 6A). It should be noted that neither protein possessed a conventional N-terminal cyanelle-targeting signal, although this was not unexpected because a previous proteomic analysis of cyanelles revealed that >70% of nuclearly encoded cyanelle proteins possessed no defined targeting signal (16). Interestingly, CppS/F sequences shared no homology with cyanobacterial proteins, suggesting their non-cyanobacterial origin. Instead, both CppS/F share a moderate homology (25% identity; E-value e-6) with YTV protein, a functionally uncharacterized cell surface protein widely distributed among bacteria of the phylum Planctomycetes (17–19). YTV proteins are generally 2–2.5 times bigger in size than CppS/F, and CppS/F show homology to the N-terminal half of YTV proteins. By searching the EST database, we found that similar proteins are expressed in *Glauconystis nostochinearum*, a species closely related to *C. paradoxa*. We detected slight homology (20–25% identity) between CppS/F and several uncharacterized proteins of the plant lineage, such as those from *Oryza barthii*, *Physcomitrella patens*, and *Eutrema salsugineum*, but it is unclear whether these were real CppS/F homologs. Weak homology (~23% identity) was also detected between CppS/F and extensin-like protein or zonadhesin proteins from mammals. No other obvious homologs were identified. We detected no conserved domains/motifs within CppS/F sequences. Although the homology-based database search did not clearly characterize CppS/F sequences, their amino acid sequences itself had several notable features in common. (i) Both proteins possessed abundant negatively charged residues (the calculated pI values were 4.0 for CppS and 4.1 for CppF); (ii) the predicted secondary structures were rich in β-strands, and no putative α-helices were predicted (Fig. 6B); and (iii) an intrinsically disordered region was predicted in both CppS/F (Fig. 6B) (20). It should be noted that CppF (deduced molecular mass of 41,177.86 Da) migrated faster in the SDS-polyacrylamide gel than CppS (37,186.43 Da). However, CppS-specific protein modifications were unlikely as electrophoresis on a gel containing 6 M urea reversed the CppS and CppF mobility pattern (data not shown), a phenomenon also observed for OmpF and OmpC of *E. coli* (21, 22).

**Cyanobacterial Peptidoglycan-associated Proteins Are Not Evolutionarily Conserved in the Cyanelle or Extant Plant Lineages**—Because sequence analysis of CppS/F unexpectedly revealed a lack of an evolutionary link to cyanobacteria, it was pertinent to clarify the phylogenetic distribution of peptidoglycan-associated proteins in cyanobacteria and their evolutionary relationship with extant plant lineages. Although the presence of peptidoglycan-associated proteins in cyanobacteria was demonstrated in the early 1980s (14), the identity of these proteins has not been investigated in a comprehensive manner except that S-layer-homologous (SLH) domain-containing outer membrane channels SomA and SomB were suggested to be associated with peptidoglycan (23, 24). We therefore identified and characterized peptidoglycan-associated proteins from *Synechocystis* sp. PCC 6803 (hereafter referred to as PCC 6803) as a model cyanobacterium. Three peptidoglycan-associated proteins were isolated, and they were observed by electron
microscopy as a layer closely attached to the electron-dense
peptidoglycan layer (Fig. 7A). We identified these proteins as
Slr0042, Slr1841, and Slr1908 (Fig. 7B). All of them are major
outer membrane proteins and are homologous to SomA and
SomB (≈40% identity) (25). Their domains are arranged as fol-
low: N-terminal SLH domain followed by an α-helix region and a C-terminal predicted β-barrel-forming region. D, peptidoglycan binding assay of GST-SLHs. Purified GST-SLHs were mixed with purified peptidoglycan. Proteins remaining in the supernatant were collected by centrifugation at 20,000 × g for 20 min at room temperature and analyzed by SDS-PAGE. Gels were stained with CBB.

FIGURE 7. Peptidoglycan-associated proteins of PCC 6803. A, electron micrographs of a purified peptidoglycan and peptidoglycan co-purified with its
associated proteins. Samples were stained with Ti Blue and 0.4% lead citrate. The boxed region is enlarged in the right panel. B, SDS-PAGE analysis of
peptidoglycan-associated proteins. Gels were stained with CBB. M, molecular mass standards. C, domain arrangement of Slr0042, Slr1841, and Slr1908
comprising an N-terminal SLH domain followed by an α-helix region and a C-terminal predicted β-barrel-forming region.

not detect protein binding to this peptidoglycan preparation.
Thus, we concluded that the SLH domains of Slr0042, Slr1841,
and Slr1908 associate with the peptidoglycan by binding to the
polysaccharide moiety.

The phylogenetic distribution of Slr0042, Slr1841, and
Slr1908 was analyzed using a BLAST search. All 11 genera of
cyanobacteria with sequenced genomes possess high sequence
similarity homologs of these proteins (>30% identity; E-value
< e−10; sequence coverage >60%) (Table 1). Conversely, we
did not identify any homologs of these proteins outside of the
Cyanobacteria phylum, although outer membrane proteins
with similar domains are found in Negativicutes and some spe-
cies in the bacterial phylum Deinococcus-Thermus (9, 26).
Considering that the cyanelle peptidoglycan does not contain a
covalently linked polysaccharide (32), it is clear that the cyano-
bacterium-type mechanism for the interaction of outer mem-
brane with the peptidoglycan is not conserved in the cyanelle or
other extant plant lineages. These findings corroborated our
supposition that CppS/F originate from a non-cyanobacteria
lineage.

Cpps/F Function as a Diffusion Channel—The property of
the cyanelle outer membrane is expected to be directly linked to
Cpps/F function. Our primary interest was to clarify whether
Cpps/F function was linked with membrane permeability.
Because the separation of Cpps and CppF proteins required
reducing and solubilizing treatments, we characterized the
properties of intact (peptidoglycan-associated), reduced/alky-
lated, and solubilized forms of CppS/F to verify that the property was not significantly affected by each step.

We first examined channel-forming activity of the peptidoglycan-associated form of CppS/F by reconstituting it to liposomes and using arabinose as a substrate. Permeability of the proteoliposomes was measured by monitoring their swelling caused by influx of the substrate, resulting in the reduction of absorbance ($A_{490}$) of the reaction mixture. A slow, but detectable, swelling rate was observed upon mixing the proteoliposomes with arabinose (Fig. 8A). Zeaxanthin that was carried over with CppS/F (8.8 nmol/10 μg of protein) did not affect the behavior of liposomes (Fig. 8A). We also verified that denatured CppS/F protein did not allow the penetration of arabinose (data not shown). Preliminary analysis revealed that small carbohydrates and amino acids penetrated the channel at rates similar to arabinose (data not shown). However, slow and often unstable permeation rates observed in this assay prevented us from reaching a definitive conclusion.

We treated the CppS/F preparation with dithiothreitol (DTT) or β-mercaptoethanol and blocked the reformation of disulfide bonding by alkylation of thiol groups using iodoacetamide. The reduced/alkylated CppS/F retained its channel activity, and we found that the permeability was enhanced 3–4-fold (Fig. 8A). We examined the channel activity per unit amounts of CppS/F proteins using arabinose as a substrate and observed that it was almost the same as for the OmpA channel of E. coli (33) (Fig. 8B). The permeability was not linearly dependent on the amount of reconstituted protein but showed a concave upward curvature, suggesting that CppS/F formed channels by assembling into larger structures, i.e., the oligomeric form of CppS/F. Substrate specificity was re-examined using reduced/alkylated CppS/F (Table 2). We observed that small carbohydrates and amino acids penetrated CppS/F channels at similar rates. It is possible, however, that we failed to register the “true” substrate and observed a nonspecific “leak” of permeation. Nonetheless, it was apparent that substrate specificity of the CppS/F channel was not particularly high. The channel exclusion limit was determined by increasing the size of carbohydrate substrates, and we observed that substrates with $M_r$ >1,000 did not penetrate the channel at detectable rates. The low diffusion coefficient of dextran cannot itself solely explain its undetectable permeation in this assay because the relative diffusion coefficients of carbohydrates with $M_r$ ~1,000 in aqueous solution are about one-half that of glucose (34), but the observed difference in permeation rates between them was >10-fold.

Permeability of the solubilized form of CppS/F was similar to that of reduced/alkylated CppS/F (Table 2). We separated CppS and CppF proteins by gel filtration chromatography in which CppF eluted slightly faster (by ~30 s) than CppS (Fig. 8C). Judging from band intensity on the SDS-polyacrylamide gel, the purity of CppS and CppF proteins was around 95%. The apparent molecular mass of CppS/F was ~160 kDa. Taking into account the amounts of CppS- and CppF-bound lithium dodecylsulfate (LDS), which were 0.91 ± 0.1 and 0.95 ± 0.06 μg/1 μg of protein, respectively, the estimated molecular masses of CppS/F were ~80 kDa. This value closely corresponds to that of the CppS/F dimer. The permeability of CppS or CppF was similar to that of solubilized CppS/F, suggesting that CppS and CppF are able to form a channel independently of each other. Based on these observations, we concluded that CppS and CppF function as a nonspecific diffusion channel that is permeable to substrates with $M_r$ less than ~1,000.

**Discussion**

This study showed that CppS/F are able to fulfill two functions as peptidoglycan-associated outer membrane proteins that form a physical link between the outer membrane and peptidoglycan of the cyanelle and as an outer membrane diffusion channel that allows permeation of compounds smaller than $M_r$ ~1,000. Considering the fact that CppS/F are the dominant outer membrane protein of cyanelle, CppS/F are expected to act as a main pathway through which small compounds diffuse across the cyanelle outer membrane.

Our unexpected finding was that CppS/F showed no homology to cyanobacterial proteins and that cyanobacterial peptidoglycan-associated proteins are not evolutionarily conserved in the cyanelle or extant plant lineages. Recent studies revealed
that a large number of genes of Archaeplastida (e.g. ~5% of nuclearly encoded genes of red alga Porphyridium purpureum) are derived from non-cyanobacterial prokaryote (35, 36). Plastid proteome studies also showed that 7–15% of plastid proteins are originated from non-cyanobacterial lineages (37). Clearly, CppS/F are categorized into these “foreign” proteins. Amino acid sequence analysis revealed similarity between Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats. Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats. Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats. Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats. Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats. Planctomycetes YTV proteins and CppS/F. Although the extent of sequence homology itself was moderate (25% identity), the following shared characteristics support their similarity. (i) Their amino acid compositions are quite similar except for a higher content of acidic residues in CppS/F (Table 3), and (ii) both of them are highly cross-linked by disulfide bonding and are resistant to detergent treatment (18, 19). Bacteria belonging to Planctomycetes phylum are ubiquitous in the natural environment, including fresh water, seawater, and soil. Although Planctomycetes and cyanobacteria are phylogenetically remote, in natural niche they often share their habitats.
Cyanelle Peptidoglycan-associated Outer Membrane Proteins

### Table 3
Amino acid compositions of CppS/F and YTV proteins

| Property | Residues     | CppS | CppF | RB850* | RB7455* |
|----------|--------------|------|------|--------|---------|
| Small    | Ala, Cys, Gly, Ser, Thr, Asp, Asn, Pro, Val | 57.9 | 56.5 | 63.5   | 63.0    |
| Aliphatic| Ala, Ile, Leu, Val                           | 27.8 | 28.5 | 24.2   | 21.7    |
| Aromatic | Phe, His, Trp, Tyr                           | 8.1  | 7.7  | 5.8    | 7.5     |
| Non-polar| Ala, Cys, Phe, Gly, Ile, Leu, Met, Pro, Val, Trp, Tyr | 47.5 | 48.5 | 49.0   | 47.4    |
| Polar    | Asp, Glu, His, Lys, Asn, Gln, Arg, Ser, Thr  | 52.5 | 51.5 | 51.0   | 52.6    |
| Charged  | Asp, Glu, His, Lys, Arg                      | 26.6 | 26.4 | 22.0   | 17.5    |
| Basic    | His, Lys, Arg                                | 8.7  | 9.3  | 11.3   | 9.2     |
| Acidic   | Asp, Glu                                     | 17.9 | 17.1 | 10.7   | 8.4     |

* YTV protein of *R. baltica* SH1.

Cyanelles is one of the dominant bacterial groups constituting the microbial community in marine stromatolites and photosynthetic biofilms (38–40). Therefore, it is not unreasonable to assume that the similarity between CppS/F and YTV proteins is derived from ancient horizontal gene transfer between an endosymbiotic cyanobacterium (or a host cell) and Planctomycetes, although further biochemical/phylogenetic analyses are needed to elucidate their relationship.

CppS/F could not be categorized into any known class of peptidoglycan-associated proteins or outer membrane diffusion channels because their sequences and experimentally determined functions do not share any common features with functionally characterized proteins. Except for the fact that disulfide cross-linking is required for the maintenance of association between CppS/F and peptidoglycan, their peptidoglycan binding mechanism(s) remains elusive. Disulfide bonding itself, however, is not required for the channel-forming activity of CppS/F. It is noteworthy, however, that reducing and alkylation treatment of CppS/F preparation enhanced the channel activity by 3–4-fold (Fig. 8A). This enhancement could be interpreted in one of the following ways. (i) The reducing treatment enhanced the reconstitution efficiency of CppS/F into liposomes, (ii) the channel activity was regulated by disulfide bonding, or (iii) the less dispersive nature of intact CppS/F (because of the pronounced cross-linking) caused uneven distribution of CppS/F upon reconstitution into liposomes, resulting in lower numbers of CppS/F-containing proteoliposomes, thereby reducing the responsiveness of the assay. We consider that (i) and (ii) are unlikely because we confirmed that (i) the efficiency of reconstitution was ~70% for both non-reduced and reduced/alkylated CppS/F and (ii) reducing reagent, such as 10 mM DTT, did not increase the non-reduced CppS/F permeability (data not shown). By comparing with the outer membrane diffusion channels (porin channels) of Gram-negative bacteria, the following three features become apparent as the main characteristics of the CppS/F channel. First, CppS/F allow the penetration of relatively large substrates, such as stachyose, in contrast with the general porin channels that are less permeable to substrates larger than Mw ~600 because of the size limitation of the channel (33, 41, 42). Second, the permeability of the CppS/F channel is low. The permeation rate of arabinose produced by a unit amount of protein was ~50-fold lower than that of general porin channels OmpF and OmpC (41) and as low as that of OmpA, one of the least permeable porin channels (33). Third, the rates of substrate penetration through the CppS/F channel were not substrate size (molecular weight)-dependent as evidenced by the fact that arabinose penetrated the channel at a rate similar to that of stachyose. Strict dependence of the permeation rates on substrate size was observed in porin channels, which form a rigid β-barrel pore structure, with the relationship fitting well the Renkin equation that rationalizes the effect of substrate size on the penetration rates through the pore (43). Accordingly, despite allowing penetration of a variety of substrates, the CppS/F channel cannot be approximated as a simple “pore.” Although more studies are obviously needed to explain this intriguing property, one possible assumption is that CppS/F form a highly flexible structure, allowing the adaptation of channel structure during physical interaction with the penetrating substrates. In this regard, it is worth noting that CppS/F are predicted to possess an intrinsically disordered region, which is unfolded and flexible (20). This disordered region may confer flexibility to the channel, allowing the penetration of a wide variety of substrates.

During the course of evolution from an endosymbiotic cyanobacterium to the chloroplast, the function of the outer membrane changed from permeability barrier to an interface that connects two metabolic entities, the chloroplast and host cell. The existence of the cyanelle indicates that the functional alteration of the outer membrane preceded the disappearance of the peptidoglycan layer, which is not present in chloroplasts of higher plants (34). Therefore, the task of renovating the outer membrane function while maintaining its stability by ensuring the physical connection with the underlying peptidoglycan appears necessary for generating cyanelles. Adopting CppS/F in place of cyanobacterial major outer membrane proteins might have had a profound effect in the context of evolution from an endosymbiotic cyanobacterium to the cyanelle. Chloroplasts of photosynthetic *Paulinella* contain peptidoglycan (44). In addition, the presence of peptidoglycan in the chloroplasts was suggested for several species in the plant lineage, although it has not yet been verified by biochemical evidence (45–48). Analysis of peptidoglycan-associated proteins in these species may provide further insights into the outer membrane renovation process during the cyanobacterium-chloroplast conversion.

### Experimental Procedures

**Strains and Culture Conditions**—*C. paradoxa* NIES-547 was grown in C medium (49) at 24 °C under 14-h/10-h light/dark conditions. PCC 6803 was grown in BG11 medium with continuous light at 30 °C.

**Cyanelle Isolation**—Cyanelles were isolated according to a method of Koike et al. (11).
Isolation of CppS/F—Cyanelles were disrupted by sonication (30-s pulse and 30-s rest, six times), and unbroken cyanelles were removed by centrifugation at 2,000 \( \times g \) for 3 min at 4 °C. Crude membranes were sedimented by centrifugation at 100,000 \( \times g \) for 1 h at 4 °C. The membranes were then loaded onto a discontinuous sucrose gradient (60, 55, 50, 45, 40% (w/v) sucrose in 20 mM Tris-HCl, pH 7.5) and centrifuged at 20,000 rpm for 4 h at 4 °C. The solution was then divided into 10 fractions following the density gradient. Each fraction was diluted 10-fold with 20 mM Tris-HCl at pH 7.5 (Tris buffer), and envelope membranes were suspended in Tris buffer containing 2% SDS and incubated at 37 °C for 20 min. Insoluble material was collected by centrifugation at 100,000 \( \times g \) for 30 min at room temperature. The insoluble fraction was repurified by discontinuous sucrose gradient centrifugation as above. The purified preparation was collected from the fraction at 1.22 g/cm\(^3\). This fraction was diluted 10-fold as above, and peptidoglycan-associated CppS/F were collected by centrifugation at 100,000 \( \times g \) for 30 min at room temperature (CppS/F-peptidoglycan preparation).

Detection of Cyanelle Peptidoglycan—Cyanelle envelopes separated by sucrose density gradient centrifugation were incubated in Tris buffer containing 2% SDS and 0.1% \( \beta \)-mercaptoethanol at 100 °C for 20 min. Insoluble material was collected by centrifugation at 100,000 \( \times g \) for 20 min at room temperature. This procedure was repeated once. The insoluble material was washed twice with distilled \( \text{H}_2\text{O} \), suspended in 0.2 ml of 6 M HCl, and hydrolyzed at 110 °C for 20 h. Because cyanelle peptidoglycan contains covalently linked N-acetylputrescine (32), the hydrolysates were subjected to polyamine analysis according to the method of Koski et al. (50) for putrescine detection. Putrescine was detected in hydrolysates obtained from a 1.22 g/cm\(^3\) fraction of sucrose density gradient centrifugation. This fraction was further analyzed for peptidoglycan by verifying the presence of amino acids constituting the peptide moiety of the peptidoglycan. Amino acids in the hydrolysate were dabsylated according to Koski et al. (50) and analyzed by high performance liquid chromatography (HPLC) using TSKgel ODS-80Ts (Tosoh, Tokyo, Japan) at a flow rate of 1.0 ml/min. Dabsylated amino acids were eluted with a gradient of solvent A (20 mM sodium acetate, pH 6.0) and solvent B (100% acetonitrile). The timetable for making the gradient, designated as percentage of solvent A, was as follows: 0 min, 22%; 25 min, 30%; 40 min, 60%; 45 min, 80%; 50 min, 100% (continued until 55 min). Solvent A content was linearly increased between each step. Dabsylated amino acids were detected by monitoring at 364 nm. Alanine, glutamic acid, diaminopimelic acid, and putrescine were detected at a ratio of 1.5:1.0:0.9:0.35, which was in good agreement with the reported amino acid composition of the peptidoglycan peptide moiety (10, 32).

CppS/F Peptidoglycan Binding Assay—The CppS/F-peptidoglycan preparation was suspended in 2% SDS in Tris buffer containing 0.1% \( \beta \)-mercaptoethanol and incubated for 20 min at 37 °C. This suspension was centrifuged at 20,000 \( \times g \) for 30 min at room temperature, and solubilized CppS/F were collected from the supernatant. Solubilized CppS/F were dialyzed against Tris buffer overnight at room temperature and diluted 10-fold with Tris buffer. This CppS/F-containing solution was mixed with purified cyanelle peptidoglycan and incubated for 15 min at room temperature. The suspension was then centrifuged at 100,000 \( \times g \) for 20 min at room temperature to separate the peptidoglycan (pellet) and supernatant. The presence of CppS/F was examined by SDS-PAGE.

Electron Microscopy—For ultrathin sectioning, samples were first fixed in 2% glutaraldehyde for 1.5 h at room temperature. They were then pelleted by centrifugation at 20,000 \( \times g \) for 20 min at room temperature and embedded in 2% agar. Next, the samples were treated with 1% osmium tetroxide, dehydrated, and embedded in Quetol651 (Nissin EM, Tokyo, Japan) according to Kwata et al. (51). They were then ultrathin sectioned (60 nm) using an ultramicrotome and stained with Ti Blue (Nissin EM; diluted 10-fold) and 0.4% lead citrate. The samples were examined under a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan) at an accelerating voltage of 100 kV.

To examine samples without thin sectioning, they were mounted onto a 400-mesh copper grid and stained with 10-fold diluted Ti Blue. Observation was performed as above.

Preparation of Cyanelle Peptidoglycan—Crude cyanelle membranes were suspended in Tris buffer containing 2% SDS and 0.1% \( \beta \)-mercaptoethanol and incubated at 100 °C for 20 min. The peptidoglycan was pelleted by centrifugation at 100,000 \( \times g \) for 20 min at room temperature. This procedure was repeated twice. Finally, the peptidoglycan was washed at least three times with distilled \( \text{H}_2\text{O} \).

Pigment Analysis—Pigment was extracted using 100% methanol. Extracted pigments (20 μl) were analyzed by HPLC on TSKgel ODS-80Ts at a flow rate of 1.0 ml/min. Pigments were eluted with a gradient of solvent A (80% (v/v) methanol with 20 mM ammonium acetate) and solvent B (30:70 solution (v/v) of ethyl acetate:methanol). The gradient started at 0% solvent B, and the timetable was set as follows. For the first 4 min, the solvent Bsolvent A ratio increased linearly to 25% and then gradually increased to 100% over the next 26 min. The flow of solvent B (100%) was maintained for the next 20 min to complete the elution of hydrophobic pigments. Pigments were detected by monitoring absorbance at 450 nm (A\(_{450}\)).

Isolation of CppS/F from Cyanelle Outer Membrane Preparation—Outer membranes were isolated according to the method of Koike et al. (11). The outer membrane preparation was suspended in Tris buffer containing 2% SDS and incubated for 30 min at 37 °C. Isolated CppS/F were obtained from the insoluble fraction after centrifugation at 100,000 \( \times g \) for 20 min at room temperature.

Rough Estimation of the Number of CppS/F Molecules per Cyanelle—Crude cyanelle membranes were suspended in Tris buffer containing 2% SDS and incubated at 75 °C for 15 min. Because of the resistance of CppS/F to detergent treatment, this procedure solubilized proteins other than CppS/F. No detectable amounts of CppS/F were detected in the solubilized fraction as judged by SDS-PAGE analysis. Isolated CppS/F were obtained from the insoluble fraction after centrifugation at 100,000 \( \times g \) for 20 min at room temperature.
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10,000 × g for 20 min. CppS/F proteins were quantified, and the number of CppS/F molecules per cyanelle was calculated.

Identification of cpps/F Genes—CppS/F were solubilized by incubation in Tris buffer containing 2% SDS and 0.1% β-mercaptoethanol for 15 min at 100 °C. The insoluble debris was removed by centrifugation at 20,000 × g for 30 min at room temperature. Proteins in the supernatant were precipitated by adding ice-cold acetone to a final concentration of 80% and incubated on ice for 4 h. Precipitated proteins were collected by centrifugation at 20,000 × g for 20 min, washed once with 90% acetone, and dried. Cyanogen bromide degradation was performed by suspending the proteins in 100 μl of cyanogen bromide (10 mg/ml) in 70% formic acid followed by incubation at room temperature for 16 h in the dark. After the incubation, 1 ml of distilled H₂O was added, and the solvent was evaporated. The degraded fragments of CppS/F were separated by SDS-PAGE. These fragments were blotted onto a polyvinylidene fluoride (PVDF) membrane, and their N-terminal sequences were analyzed using a gas-phase protein sequencer (PPSQ-10, Shimadzu, Kyoto, Japan). The determined sequences were used to BLAST search the EST database, and 16 sequences that encode APECTVVNSGI (N-terminal sequence of CppS fragment) were retrieved. Multiple alignments of these hit sequences matched the reported genomic sequence (100% match). Peptide mass fingerprinting (see below) of CppS and CppF verified that the peptide mass spectra matched the predicted cDNA-encoded sequences.

Peptide Mass Fingerprinting—Protein bands separated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) were cut out and destained in 30% acetonitrile containing 25 mM ammonium bicarbonate. Gels were dried and subjected to alklylation as follows. Gels were suspended in 100 μl of 10 mM DTT solution containing 25 mM ammonium bicarbonate and incubated for 1 h at 56 °C. They were then washed with 25 mM ammonium bicarbonate, suspended in an alklylation solution of 55 mM 2-iodoacetamide containing 25 mM ammonium bicarbonate, and incubated at room temperature for 45 min in the dark. Gels were washed with 25 mM ammonium acetate and then dehydrated by suspending in 200 μl of 50% (v/v) acetonitrile containing 25 mM ammonium acetate. Gels were dried and suspended in 20 μl of 10 μg/ml trypsin solution with 50 mM ammonium bicarbonate and incubated overnight at 37 °C for in-gel trypsin digestion. The trypsin solution was then removed, and digested protein fragments were extracted into 75 μl of 50% (v/v) acetonitrile with 5% (v/v) trifluoroacetic acid. Digested fragments were purified using ZipTip pipette tips (Millipore, Billerica, MA) and analyzed by an AB SCIEX TOF/TOF 5800 MALDI-TOF MS system (Applied Biosystems, Wallingford, MA). Proteins were identified based on peptide mass spectra using the MS-Fit program.

Sequence Analyses—A BLAST homology search of UniProt and NCBI databases was performed. A domain/motif search was performed using Pfam (52). Secondary structure prediction was performed with PSIPRED (53). Intrinsically disordered domains were predicted by PONDR (54) and DISOPRED programs equipped with PSIPRED. Amino acid compositions were analyzed by Pepstats, and amino acid sequence alignment was created in Clustal Omega on the European Molecular Biology Laboratory-European Bioinformatics Institute website.

Isolation and Identification of Peptidoglycan-associated PCC 6803 Proteins—Peptidoglycan-associated proteins were isolated as described by Weckesser and Jurgens (31) and identified by peptide mass fingerprinting.

Preparation of Recombinant SLH Domains—SLH domains of Srl0042 (amino acid residues 1–108), Srl1841 (residues 1–108), and Srl1908 (residues 1–126) were expressed in E. coli BL21(DE3) as fusion proteins with GST (GST-SLH) according to the method of Kojima et al. (7). Purification of GST-SLHs and the peptidoglycan binding assay were performed as described by Kojima et al. (7).

Purification of PCC 6803 Peptidoglycan—PCC 6803 peptidoglycan and polysaccharide-devoid peptidoglycan were prepared according to the method of Weckesser and Jurgens (31).

Preparation of Reduced and Alkylated CppS/F—CppS/F were incubated with 75 mM DTT or 0.1% β-mercaptoethanol at room temperature for 15 min. Next, 0.5 M (final concentration) iodoacetamide was added, and the suspension was incubated for a further 15 min. Reduced/alkylated CppS/F were collected by centrifugation at 20,000 × g for 20 min at room temperature and washed once with Tris buffer.

Solubilization and Purification of CppS/F—CppS/F-peptidoglycan preparations were incubated in Tris buffer containing 2% LDS and 75 mM DTT (or 0.1% β-mercaptoethanol) at 37 °C for 20 min. Supernatant was collected by centrifugation at 100,000 × g for 20 min at room temperature. Supernatant was then mixed with 0.5 M (final concentration) iodoacetamide and incubated for 15 min at room temperature. CppS and CppF were separated by gel filtration HPLC using a Superdex 200 Increase 10/300 GL column (GE Healthcare). HPLC conditions were as follows: eluent, 0.1% LDS in 10 mM Tris-HCl, pH 7.5, containing 0.4 M LiCl; flow rate, 0.5 ml/min. Quantification of CppS/F-bound LDS—LDS was quantified according to the method of Hayashi (55).

Liposome Swelling Assay—Before reconstitution into liposomes, excess salt in the samples was removed by dialysis against Tris buffer overnight at 4 °C. Liposome swelling assays were performed as described by Nikiado et al. (56). Reconstitution efficiency of CppS/F proteins into liposomes was checked by the method of Yoshimura et al. (42). Denaturation of CppS/F was performed by incubating in 2% SDS containing 0.1% β-mercaptoethanol at 100 °C for 15 min. Denatured CppS/F were alkylated by incubating with 0.5 M iodoacetamide, and the proteins were precipitated by adding ice-cold acetone to a final
concentration of 80% and incubating on ice for 4 h. Precipitated proteins were collected by centrifugation at 20,000 × g for 20 min, washed once with 90% acetone, and dried.

**Author Contributions**—S. K. designed the research and performed all experiments. K. M. provided technical assistance and contributed to the identification of cppS/F genes. S. K. and T. K. analyzed the data, and S. K. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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