Two Menkes-type ATPases Supply Copper for Photosynthesis in Synechocystis PCC 6803*

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Synechocystis PCC 6803 contains four genes encoding polypeptides with sequence features of CPx-type ATPases, two of which are now designated pacS and ctaA. We show that CtaA and PacS (but not the related transporters, ZiaA or CoaT) facilitate switching to the use of copper (in plastocyanin) as an alternative to iron (in cytochrome c6) for the carriage of electrons within the thylakoid lumen. Disruption of pacS reduced copper tolerance but enhanced silver tolerance, and pacS-mediated restoration of copper tolerance was used to select transformants. Disruption of ctaA caused no change in copper tolerance but reduced the amount of copper cell−1. In cultures supplemented with 0.2 μM copper, photooxidation of cytochrome c6 (PetJ) was depressed in wild-type cells but remained elevated in both Synechocystis PCC 6803(ctaA) and Synechocystis PCC 6803(pacS). Conversely, plastocyanin transcripts (petE) were less abundant in both mutants at this [copper]. Synechocystis PCC 6803(ctaA) and Synechocystis PCC 6803(pacS) showed increased iron dependence with impaired growth in deferoxamine mesylate (iron chelator)-containing media. Double mutants also deficient in cytochrome c6, Synechocystis PCC 6803(petJ,ctaA) and Synechocystis PCC 6803(petJ,pacS), were viable, but the former had increased copper dependence with severely impaired growth in the presence of bathocuproinedisulfonic acid (copper chelator). Analogous transporters are likely to supply copper to plastocyanin in chloroplasts.

Cyanobacteria contain internal thylakoid membranes where oxygen-evolving photosynthetic electron transport occurs. Respiratory electron transport occurs in both thylakoid and plasma membranes (1). Thylakoid membranes contain two protein complexes that include photosystems II and I. Within photosynthetically active cells, mobile soluble carriers shuttle electrons between these two complexes. Some cyanobacteria and green algae (see Refs. 2 and 3 and references therein) adapt to copper deficiency by exploiting alternative carriers. In copper-sufficient Synechocystis PCC 6803, electrons transfer between the complexes via copper in plastocyanin (PetE) whereas under copper deficiency heme iron in cytochrome c6 (PetJ) is used (4). Both PetE and PetJ are located “inside” the thylakoid lumen.

One subset of proteins is imported into cyanobacterial (and plant chloroplast) thylakoids via the Sec system, whereas others are imported via a P-type-dependent pathway (28). The latter transports folded proteins, and its substrates tend to be proteins that require complex cofactors, thereby avoiding separate thylakoid import of the cofactors. Plastocyanin is imported using Sec indicating that a copper delivery system into this compartment is required when Synechocystis PCC 6803 switches from PetJ to PetE. Higher plant chloroplasts rely exclusively on plastocyanin for electron transport between the two photosystems (2), and therefore thylakoid copper import is predicted to be especially important in higher plants.

Copper can impair cell function by associating with adventitious sites or engaging in “elicit” redox chemistry. In recent years, it has become apparent that there are efficient systems to deliver intracellular copper while avoiding adverse interactions en route (5). These include transporters and copper chaperones that target specific intracellular compartments and/or apoproteins (5). How is copper supplied to plastocyanin?

The human copper transporters MNK and WND, aberrant in Menkes and Wilson diseases, respectively, are two (prominent) members of a subgroup of P-type ATPases (6) often termed P1-7 or CPx-type (8). P1-type ATPases transport larger metal ions, with the founder, CadA from Staphylococcus aureus, exporting Cd2+ (9). Known representatives include the bacterial copper transporters CopA and CopB; CCC2 that transports copper in yeast; ZntA from Escherichia coli that exports zinc but also lead and cadmium; ZiaA, zinc; and CoaT, cobalt (reviewed in Refs. 10 and 11). At present, the metal ion transported and the direction of transport cannot be predicted from the sequence of a CPx-type ATPase. We have previously characterized two of the four ORFs encoding deduced CPx-type ATPases in the genome of Synechocystis PCC 6803 (12), ZiaA (slr0798) (13) and CoaT (slr0797) (14), but ORFs slr1920 and slr1950 remained uncharacterized.

Similarity of the deduced products of slr1920 and slr1950 with PacS (15) and CtaA (16), respectively, from Synechococcus PCC 7942, encouraged a prediction that these polypeptides contribute to copper homeostasis. We report that this is correct and designate the Synechocystis genes pacS and ctaA. However, disruption of pacS or ctaA in Synechocystis PCC 6803 confers different phenotypes (in part) to those observed in Synechococcus PCC 7942. PacS is located in thylakoid membranes in Synechococcus PCC 7942 (15), but the direction of copper transport by PacS is unknown. Synechococcus PCC 7942, unlike Synechocystis PCC 6803, does not show copper-dependent switching between cytochrome c6 and plastocyanin (see Ref. 2).

Here we describe experiments showing that the action of

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¶The abbreviations used are: ORF(s), open reading frame(s); kb, kilobase.
both pacS and ctaA in Synechocystis PCC 6803 is negative with respect to the photooxidation of cytochrome $c_6$ and positive toward the accumulation of plastocyanin, petE, transcripts in copper-containing medium. Both of these transporters contribute toward the substitution of copper (in place of iron) for photosynthetic electron transport in the thylakoid, consistent with inward copper transport by both. Deletion of pacS impairs cellular copper accumulation. Deletion of pacS confers copper sensitivity but silver resistance, which is interpreted in the context of metal ion sequestration within thylakoids. Structural features that confer metal specificity are considered.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, DNA Manipulations, and Southern Analyses—Synechocystis PCC 6803 was grown either in liquid BG-11 medium or on medium C plates (17) using previously described conditions (14).**

BG-11 contains 0.5 $\mu$M copper. A variant BG-11 (BG-11-C), lacked copper as a microelement but was supplemented with defined copper. Growth under iron limitation was analyzed using medium lacking normal iron and containing 0.2 $\mu$M copper (BG-11-FC). Cells were transformed to antibiotic resistance as described by Hagemann and Zuther (18). $E. coli$ strains JM101 or SURE (Stratagene) were grown in Luria-Bertani medium (19). DNA manipulations were performed as described by Sambrook et al. (19). Genomic DNA was isolated from Synechocystis PCC 6803 using a protocol described previously for the isolation of DNA from plant cell cultures but excluding CsCl gradients (20). Aliquots (10 $\mu$g) of DNA were digested with restriction endonucleases, resolved by agarose gel electrophoresis, transferred to nylon filters, and washed (after probing) to a stringency of 0.5 x SSC, 0.1% w/v SDS at 65 °C (19).

**Insertional Inactivation of pacS—**Synechocystis PCC 6803 genomic DNA was used as template for polymerase chain reaction with primers 5' -GAAGATTC(A)TTAATACGACTCACTATAG-3' and 5' -GAAGATTCCTCGAGAGGATATTAG-3' and 5' -GAAGATTCCTGGCCGGATACCATGC-3'. The pacS amplification product (2.36 kb) was labeled with PGM-End (Promega) to create pSTn2. A 1.26-kb BamHI fragment of pUK4 (Amersham Pharmacia Biotech) containing a kanamycin resistance gene was incubated with the Klenow fragment of $E. coli$ DNA polymerase I and the "blunt-ended" fragment ligated to a unique MseI site (within sll1920) of pSTn2, creating pIN-PACS. Synechocystis PCC 6803 was transformed to kanamycin resistance following incubation with pIN-PACS, transformants selected on solid medium containing 25 $\mu$g ml$^{-1}$ kanamycin prior to growth in liquid medium containing 50 $\mu$g ml$^{-1}$ kanamycin. Logarithmically growing cultures containing 50 $\mu$g ml$^{-1}$ kanamycin were centrifuged for 1 min at 5,000 g. An analogous procedure (to that described for pacS) was used with primers 5' -GAAGATTC(A)TTAATACGACTCACTATAG-3' and 5' -GAAGATTCCTCGAGAGGATATTAG-3' and 5' -GAAGATTCCTGGCCGGATACCATGC-3'. The pacS amplification product (2.36 kb) was labeled with PGM-End (Promega) to create pSTn2. A 1.26-kb BamHI fragment of pUK4 (Amersham Pharmacia Biotech) containing a kanamycin resistance gene was incubated with the Klenow fragment of $E. coli$ DNA polymerase I and the "blunt-ended" fragment ligated to a unique MseI site (within sll1920) of pSTn2, creating pIN-PACS. Synechocystis PCC 6803 was transformed to kanamycin resistance following incubation with pIN-PACS, transformants selected on solid medium containing 25 $\mu$g ml$^{-1}$ kanamycin prior to growth in liquid medium containing 50 $\mu$g ml$^{-1}$ kanamycin. Logarithmically growing cultures containing 50 $\mu$g ml$^{-1}$ kanamycin were centrifuged for 1 min at 5,000 g.

**Insertional Inactivation of ctaA—**An analogous procedure (to that described for pacS) was used with primers 5' -GAAGATTC(A)TTAATACGACTCACTATAG-3' and 5' -GAAGATTCCTCGAGAGGATATTAG-3' and 5' -GAAGATTCCTGGCCGGATACCATGC-3'. The pacS amplification product (2.36 kb) was labeled with PGM-End (Promega) to create pSTn2. A 1.26-kb BamHI fragment of pUK4 (Amersham Pharmacia Biotech) containing a kanamycin resistance gene was incubated with the Klenow fragment of $E. coli$ DNA polymerase I and the "blunt-ended" fragment ligated to a unique MseI site (within sll1920) of pSTn2, creating pIN-PACS. Synechocystis PCC 6803 was transformed to kanamycin resistance following incubation with pIN-PACS, transformants selected on solid medium containing 25 $\mu$g ml$^{-1}$ kanamycin prior to growth in liquid medium containing 50 $\mu$g ml$^{-1}$ kanamycin. Logarithmically growing cultures containing 50 $\mu$g ml$^{-1}$ kanamycin were centrifuged for 1 min at 5,000 g. 

**Analyses of Metal Tolerance and Accumulation—**Logarithmically growing cultures were subcultured on alternate days (to $\sim 1 \times 10^6$ cells ml$^{-1}$) for a period of 7 days (to standardize growth rates). Growth of cultures in metal-supplemented media was examined as previously described (14). Growth under copper limitation was analyzed by adding 300 $\mu$M bathocuproinedisulfonic acid, 48 h after inoculation with cells derived from cultures of standardized growth rates that had been maintained (at least 7 days) in BG-11-C. To examine effects of iron deprivation, cells were passed twice through BG-11-FC supplemented with 5 mg ml$^{-1}$ ferric ammonium citrate and once in BG-11-FC before inoculation into either BG-11-FC or BG-11-FC supplemented with 10 $\mu$g deferoxamine mesylate.

To examine metal accumulation, aliquots (30 ml) of logarithmically growing cultures in BG-11-C of standardized optical density (A$$_{660}$) were exposed (2 h) to 0.2, 1, and 2 $\mu$g copper, and cells were harvested, washed in BG-11-C, and finally resuspended in 1.25 ml of 70% v/v HNO$_3$. Metal contents were determined by atomic absorption spectrophotometry. Analyses of cobalt and zinc accumulation used BG-11.

**Single Turnover Cytochrome Kinetics—**Measurements of cytochromes (f plus c$_6$) and of plastocyanin were made by analyses of flash-induced absorbance changes in the visible region using a protocol developed for use with higher plant thylakoid membranes (21, 22). Cells were grown in BG-11-C supplemented with specified copper. Cell densities were determined by A$$_{490}$ and chlorophyll content was measured as the A$$_{660}$ of chlorophyll extracts. Similar chlorophyll contents and cell densities were determined for all genotypes and copper treatments. Cells were diluted in BG-11-C medium to a chlorophyll concentration of 25 $\mu$g ml$^{-1}$; required sample volume was 1.5 ml, and pathlength was 1 cm. Saturating actinic flashes were generated using a xenon flashlamp (15-microfarad capacitor at 1000 V; 6-µs half-width pulses with BG295 filters). Filters were inserted between the sample cuvette and the photomultiplier, and the photomultiplier was protected with BG39, OG530, and 580-nm cut-off filters. Transients were recorded sequentially at 542, 554, 563, and 575 nm after dark adaptation for 2 s before each train of flashes. The cycle of four flashes was repeated 20 times, and the transients at each wavelength were averaged. Changes (Δ$\Delta$) due to cytochrome b$_{563}$ at 563 nm, cytochromes f plus c$_6$ (which have sufficiently similar spectra that they are deconvoluted together) at 545 nm, cytochrome f at 554 nm, and plastocya$n$in at 575 nm were obtained by digitizing the transients and deconvoluting using the matrix of extinction coefficient values obtained for higher plants (21). This deconvolution can be applied to Synechocystis PCC 6803 without the need to dissipate any generated electric field, because there is no equivalent of the carotenoid bandshift, which would otherwise strongly overlap in this region. Each resultant trace represented the absorbance change at one wavelength of a single component. In all experiments, the measuring beam was switched on 50 ms before recording commenced, and during dark periods the photomultiplier was provided with light from a light-emitting diode of intensity equal to that of the measuring beam. In all samples, the size of the transients did not increase on successive flashes, indicating that there was sufficient P700 to cause a full photooxidation of $f_{645}$/plastocyanin with a single flash. Hence, only the 20-replicate average of the first flash transient is shown in the figures.

**RNA Isolation and Northern Analysis—**Total RNA was isolated from logarithmically growing cultures in BG-11-C medium containing 0.2 $\mu$M copper using an established method (23) and treated with DNase I. Equivalent amounts of total RNA (10 $\mu$g) were incubated (65 °C for 5 min) in 60% v/v formamide, 3.6 mM Tris-HCl (pH 7.8), 3 mM Na$_2$PO$_4$, and cell densities were determined for all genotypes and copper treatments.
RESULTS

Mutants of Synechocystis PCC 6803 with a Disrupted pacS Gene Have Reduced Tolerance to Copper but Enhanced Tolerance to Silver—Mutants, Synechocystis PCC 6803(pacS), were generated by integration of sequences derived from plasmid pIN-PACS, which contains ORF all1920 interrupted by a kanamycin resistance gene. Growth of Synechocystis PCC 6803(pacS) and wild type was tested in multiple liquid cultures supplemented with a range of levels of cadmium, zinc, cobalt, copper, and silver ions to determine maximum permissible concentrations (data not shown). Only resistance to copper appeared to be reduced in Synechocystis PCC 6803(pacS). Subsequently, growth was examined as a function of time in response to selected concentrations of copper (Fig. 1A). Unlike wild type, Synechocystis PCC 6803(pacS) is unable to grow in BG-11-C medium containing 1 μM copper and shows some inhibition of growth in medium containing 0.3 μM copper. In BG-11-C medium containing 0.2 μM copper, Synechocystis PCC 6803(pacS) has similar growth to wild type (data not shown). Restoration of tolerance to 3 μM copper was used as a selectable marker to identify mutants of Synechocystis PCC 6803(pacS) in which pacS had reintegrated into the chromosome by homologous recombination following incubation of cells with the corresponding DNA. The genotypes of Synechocystis PCC 6803(pacS), and cells with reintegrated pacS, were confirmed by Southern analysis: the band of 2.8 kb represents hybridization to pacS on a larger fragment because of the presence of the kanamycin resistance gene within pacS (Fig. 1B). Fig. 1C shows the phenotypes of Synechocystis PCC 6803(pacS), wild type, and cells with pacS reintroduced into the chromosome. There was no significant difference in the copper content of Synechocystis PCC 6803(pacS), compared with wild type, when cells were grown in media containing copper concentrations that allowed equivalent growth (data not shown).

An estimation of the maximum permissible concentration of silver for growth of Synechocystis PCC 6803(pacS) suggested enhanced tolerance (data not shown). Growth of Synechocystis PCC 6803(pacS) was examined as a function of time in response to selected [silver] (Fig. 1A, right panel). Wild type cells were unable to grow in medium containing 0.5 μM silver, whereas Synechocystis PCC 6803(pacS) showed only slightly impaired growth. Growth of wild type cells was significantly impaired in 0.3 and 0.4 μM silver, concentrations that were not inhibitory to the pacS mutants.

Mutants of Synechocystis PCC 6803 with a DisruptedctaA Gene Have Unaltered Metal Tolerance but Reduced Accumulation of Copper—Mutants with disrupted ctaA were generated by integration of sequences derived from plasmid pIN-CTAA, which contains ORF slr1950 interrupted by a kanamycin resistance gene. Southern analysis confirmed integration of the antibiotic resistance gene into ctaA on all copies of the chromosome (Fig. 2A). Growth of Synechocystis PCC 6803(ctaA) and wild type was tested in multiple liquid cultures supplemented with a range of concentrations of cadmium, zinc, cobalt, copper, and silver ions to determine maximum permissible concentrations. Tolerance to all metals appeared unaltered (data not shown). Subsequently, growth was examined as a function of time in response to selected concentrations of copper (Fig. 2B). Neither cell line grew in BG-11 medium supplemented with 2 μM copper, whereas growth of both cell types was inhibited by 1 μM copper to a similar extent. In contrast, disruption of ctaA from Synechococcus PCC 7942 resulted in increased tolerance to copper ions compared with wild type (16).

Cultures of Synechocystis PCC 6803(ctaA) and wild type cells were exposed for 2 h to BG-11-C medium containing 0.2, 1, or 2 μM copper, and significantly less copper was detected in the mutant at the two higher metal concentrations (Fig. 3). The total copper content of the mutants after this “short exposure” to 0.2 μM copper was not significantly different from wild type. The cobalt and zinc contents of Synechocystis PCC 6803(ctaA) did not differ from wild type cells following 2 h of exposure to either 8 or 16 μM zinc or 5 or 10 μM cobalt (data not shown).

Photooxidation of Cytochrome c6 (PetJ) in Copper-containing Medium Is Greater in Cells Disrupted in ctaA or pacS—The observation that in some media Synechocystis PCC 6803(ctaA) contains less copper than wild type (Fig. 3) suggests a role for CtaA in copper import. Does CtaA supply copper for PetE, and...
what influence, if any, does PacS have on the substitution of
PfE for PfJ (Fig. 4A)?

Previous workers detected no cytochrome c₆ in Synechocystis
PCC 6803 containing ~4 × 10⁶ copper atoms cell⁻¹ grown in
0.3 μM copper (4). Under these conditions, the decrease in ΔA
(absorbance deconvoluted at a defined wavelength for a defined
component) at 554 nm upon exposure to a pulse of actinic light
is attributable to cytochrome f alone, whereas in the absence of
added copper this change becomes greater because of an addi-
tional contribution at 554 nm because of transient oxidation of
cytochrome c₆ (4) (at rest the carrier is reduced). Growth of
Synechocystis PCC 6803(pacS) is inhibited by 0.3 μM copper
(Fig. 1A) precluding the use of this [copper] in subsequent
experiments. Growth is not inhibited at 0.2 μM copper. Pho-
oxidation of cytochrome c₆ (plus cytochrome f) has been
compared in BG-11-C containing 0, 0.2, 0.3, and 1 μM copper (Fig.
4B). The transient decrease in ΔA at 554 nm is less in 0.2 μM
copper compared with no added copper and similar to that observed
in 0.3 μM copper. This implies that plastocyanin replaces cyto-
oxidase (Cyt c Ox) (4). Panel B, the kinetics of light-induced absorbance
change, deconvoluted at 554 nm for cytochrome c₆, plus f (ΔA) in
response to a 6-μs pulse of actinic light (coincident with the drop in ΔA)
in cells grown in BG-11-C with different added [copper] (0, 0.2, 0.3, and
1 μM). The subsequent rise in ΔA is because of re-reduction of cyto-
orhodin by the cytochrome b₆f complex. Panel C, the magnitude of the
decrease in ΔA at each [copper] and hence the relative amount of
photooxidation of cytochrome c₆. The residual change in ΔA in 1 μM
copper is the contribution of cytochrome f.

Fig. 3. Synechocystis PCC 6803(ctaA) accumulates less copper.
Cell-associated copper in Synechocystis PCC 6803(ctaA) (filled columns)
and wild type cells (open columns). Cells were grown in BG-11-C me-
dium and then exposed to the indicated copper concentrations for 2 h
prior to harvesting, acid digestion, and determination of copper content
by atom absorption spectroscopy.

Fig. 4. Kinetics and magnitude of photooxidation of cyto-
orhodin c₆ in Synechocystis PCC 6803 grown in different [copper].
Panel A, a diagrammatic representation of electron flow within the
thylakoid lumen in Synechocystis PCC 6803. Upon light (hv) activ-
ation of photosystems (PS) I and II, electrons are donated to a mem-
brane complex that includes PS I from a reduced electron carrier
located within the thylakoid lumen. The carrier is subsequently re-
duced by the cytochrome b₆f complex (Cyt b₆f), associated with PS II,
and must shuttle between the sites of electron acquisition and donation.
In copper sufficiency the carrier is plastocyanin-encoded by petE,
whereas under copper limitation this is substituted with cytochrome c₆
encoded by petJ. Both carriers can also donate electrons to cytochrome
c oxidase (Cyt c Ox.) Panel B, the kinetics of light-induced absorbance
change, deconvoluted at 554 nm for cytochrome c₆, plus f (ΔA) in
response to a 6-μs pulse of actinic light (coincident with the drop in ΔA)
in cells grown in BG-11-C with different added [copper] (0, 0.2, 0.3, and
1 μM). The subsequent rise in ΔA is because of re-reduction of cyto-
orhodin by the cytochrome b₆f complex. Panel C, the magnitude of the
decrease in ΔA at each [copper] and hence the relative amount of
photooxidation of cytochrome c₆. The residual change in ΔA in 1 μM
copper is the contribution of cytochrome f.

Fig. 5. Disruption of pacS or ctaA increases the magnitude of
photooxidation of cytochrome c₆ and reduces petE transcript
abundance. Panel A, the kinetics of light-induced absorbance change
deconvoluted at 554 nm in response to a 6-μs pulse of actinic light
in wild type cells and each of the individual transporter (pacS, ctaA,
coaT, and ctaA) mutants grown in BG-11-C supplemented with 0.2 μM
copper. Panel B, the magnitude of the decrease in ΔA deconvoluted at
554 nm for each of the mutants and hence the relative amount of
photooxidation of cytochrome c₆. Equivalent trends have been observed
on two further occasions. Panel C, Northern analysis showing plasto-
ocyanin (petE) (upper panel) and cytochrome c₆ (petF) (lower panel),
transcript abundance in Synechocystis PCC 6803(ctaA) (lane 1), Syn-
echocystis PCC 6803(pacS) (lane 2), and wild type (lane 3) cells grown
in BG-11-C containing 0.2 μM copper. DNase-treated RNA was resolved on
a 1.2% w/v formaldehyde-agarose gel and probed with petE.
partly restoring the use of plastocyanin.

Plastocyanin (petE) Transcripts Are Less Abundant, and Cytochrome c₆ (petJ) Transcripts Are More Abundant, in Cells Deficient in Functional ctaA or pacS—It has previously been shown (4) that a decline in photooxidation of cytochrome c₆ in copper-containing medium corresponds with a decline in abundance of the PetJ polypeptide and petJ transcript. The abundance of plastocyanin transcripts and polypeptides shows a reciprocal response, increasing in copper (2, 4). The data in Fig. 5B imply more electron flow through cytochrome c₆ in Synechocystis PCC 6803(pacS) and Synechocystis PCC 6803(ctaA) and hence predict less plastocyanin and less petE transcripts. In cells grown in BG-11-C plus 0.2 μM copper, petE transcripts were less abundant in Synechocystis PCC 6803(pacS) and Synechocystis PCC 6803(ctaA) than wild type (Fig. 5C). Cytochrome c₆ and petJ transcripts were more abundant in Synechocystis PCC 6803(pacS) and Synechocystis PCC 6803(ctaA) than wild type at this copper (Fig. 5C).

Disruption of ctaA or pacS Increases Sensitivity to Low Iron—It was speculated that a greater dependence upon PetJ rather than PetE for photosynthetic electron transport in Synechocystis PCC 6803(pacS) and Synechocystis PCC 6803(ctaA) may confer a greater dependence on iron. Iron deficiency, generated by subculture either in BG-11-FC media with no added iron or by addition of the iron chelator deferoxamine mesylate, slowed the growth of all cell lines (compare y axis on Fig. 7A with Fig. 1A and 2B). Fig. 7A shows that Synechocystis PCC 6803(pacS) and Synechocystis PCC 6803(ctaA) are more sensitive to low iron than wild type cells; this was observed using both types of low iron media and in two further replicates (not shown) of each of these experiments.

Disruption of ctaA Increases Dependence upon petJ and Reduces Cytochrome Oxidase Activity in Low Copper—Despite the observed enhanced dependence upon iron, it was possible to obtain, on copper-replete medium, double mutants (petJ,ctaA and petJ,pacS) in which petJ was insertionally inactivated on all copies of the Synechocystis PCC 6803 chromosome (Fig. 7B). However, Synechocystis PCC 6803(petJ,ctaA) was sensitive to copper depletion, with (slightly) reduced growth on BG-11-C (data not shown) and severely impaired growth in the presence of the copper chelator bathocuproinedisulfonic acid (Fig. 7C).

Copper is implicated in respiratory electron transport, as well as photosynthetic electron transport. In Synechocystis PCC 6803, cytochrome c oxidase resides at both the plasma and thylakoid membranes, can accept electrons from plastocyanin, as well as from cytochrome c₆, and requires copper (1). However, cytochrome oxidase activities were not significantly different from wild type in membranes isolated from either Synechocystis PCC 6803(pacS) or Synechocystis PCC 6803(ctaA) grown in 0.2 μM copper (data not shown). When cells were grown in BG-11-C medium, cytochrome oxidase activities were lower (mean 58%) in membranes from Synechocystis PCC 6803(ctaA) compared with wild type in each of eight independent experiments. It is noted that a related copper transporter has recently been implicated in the biogenesis of cytochrome c oxidase in Rhodobacter capsulatus (26).
**DISCUSSION**

Several lines of evidence support roles for PacS and CtaA in the delivery of copper for photosynthetic electron transport in *Synechocystis* PCC 6803. Plastocyanin is the electron carrier in the thylakoid lumen of higher plant chloroplasts (2), organelles that share close common ancestors with cyanobacteria (27). It is speculated that equivalent copper transporters act in chloroplasts.

Evidence that CtaA is involved in (i) copper import and (ii) copper supply for plastocyanin includes a reduction in the copper content of *Synechocystis* PCC 6803(*ptaA*) compared with wild type (Fig. 3), an increase relative to wild type in photooxidation of cytochrome *c*_p* in* Synechocystis* PCC 6803(*ptaA*) at 0.2 μM copper (Fig. 5, A and B), a decrease relative to wild type in the abundance of *petE* transcripts, and an increase in *petJ* transcripts in *Synechocystis* PCC 6803(*ptaA*) at 0.2 μM copper (Fig. 5C). Impaired copper acquisition in *Synechocystis* PCC 6803(*ptaA*), and impaired ability to switch (at 0.2 μM copper) from use of iron in cytochrome *c*_p* to* copper in plastocyanin, predicts increased iron dependence of *Synechocystis* PCC 6803(*ptaA*). This is consistent with an observed reduction in growth of this genotype in low iron (at 0.2 μM copper) (Fig. 7A). More specifically, this predicts enhanced dependence upon copper and plastocyanin in the presence of copper. However, in common with *Synechocystis* PCC 6803(*ptaA*), *Synechocystis* PCC 6803(*pacS*) also shows an increase in photooxidation of cytochrome *c*_p* to* copper at 0.2 μM copper (Fig. 5C), and a reduction in growth in low iron relative to wild type at 0.2 μM copper (Fig. 7A). The requirement for two transporters for efficient switching to plastocyanin is consistent with (i) copper traversing two membranes before holoplastocyanin is made in the thylakoid lumen, (ii) inward transport by both PacS and CtaA, and (iii) location of PacS within thylakoid membranes (Fig. 8). Why does PacS confer resistance to copper? Copper may promote fewer adverse interactions within the thylakoid than elsewhere in the cell because of sequestration by plastocyanin and/or an abundance of anti-oxidant systems in this photosynthetic compartment. Silver resistance of *Synechocystis* PCC 6803(*pacS*) may be caused by less silver binding to, and inhibiting of, thylakoid proteins. Deletion of *pacS* in *Synechococcus* PCC 7942 conferred the opposite phenotype, silver sensitivity (15). This could relate to differences in the expression of plastocyanin, or differences in the direction of transport by PacS, in the two cyanobacteria. The decline in *petE* transcript levels in *Synechocystis* PCC 6803(*pacS*) suggests that transcriptional switching also requires copper to reach the thylakoid.

The presence of multiple CPx-type ATPases in a single organism but differing in metal specificity provides an opportunity to identify structural elements that discriminate between metals. The *E. coli* zinc exporter (*ZntA*) is known to also transport lead (11), and deletion of ZiaA confers lead (in addition to zinc) sensitivity, a phenotype not detected in any of the other CPx-type ATPase mutants of *Synechocystis* PCC 6803 (data not shown). As illustrated in Fig. 8, the known metal preferences are CoaT, cobalt; ZiaA, zinc and lead; PacS, copper and silver; and CtaA, copper. Both PacS and CtaA contain an extended motif CPICALGLATP surrounding the sequence (CPC) thought to associate with metals during transport. This sequence is conserved in the majority of the CPx-type ATPases that have been assigned a role in the transport of copper. In ZiaA, it is replaced with the motif CPCALVISTP, and a similar extended motif is present in other zinc and cadmium transporters.

The amino-terminal cytoplasmic domains of most CPx-type ATPases contain the sequence GMXGX(G where X is any residue), sometimes repeated (11). Both PacS and CtaA contain a single copy of this motif, but this is absent from CoaT, whereas in ZiaA it is associated with a second putative metal binding region containing repeated HXH motifs (Fig. 8). To what extent (and how) do differences in these regions influence metal discrimination? By analogy to the interaction between CCC2 and ATX1 in yeast (5), it is speculated that metal donors, metallochaperones, deliver (or acquire) metals (to/from) these amino-terminal domains, at least of PacS and CtaA. There is now a quest for ORFs encoding metallochaperones.

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