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# Functional Dependence between Septal Protein SepJ from *Anabaena* sp. Strain PCC 7120 and an Amino Acid ABC-Type Uptake Transporter

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**ABSTRACT**

**In the diazotrophic filaments of heterocyst-forming cyanobacteria, two different cell types, the $CO_2$-fixing vegetative cells and the $N_2$-fixing heterocysts, exchange nutrients, including some amino acids. In the model organism *Anabaena* sp. strain PCC 7120, the SepJ protein, composed of periplasmic and integral membrane (permease) sections, is located at the intercellular septa joining adjacent cells in the filament. The unicellular cyanobacterium *Synechococcus elongatus* strain PCC 7942 bears a gene, *Synpcc7942_1024* (here designated *dmeA*), encoding a permease homologous to the SepJ permease domain. *Synechococcus* strains lacking *dmeA* or lacking *dmeA* and expressing *Anabaena sepJ* were constructed. The *Synechococcus dmeA* mutant showed a significant 22 to 32% decrease in the uptake of aspartate, glutamate, and glutamine, a phenotype that could be partially complemented by *Anabaena sepJ*. *Synechococcus* mutants of an ATP-binding-cassette (ABC)-type transporter for polar amino acids showed >98% decreased uptake of glutamate irrespective of the presence of *dmeA* or *Anabaena sepJ* in the same strain. Thus, *Synechococcus* DmeA or *Anabaena* SepJ is needed to observe full (or close to full) activity of the ABC transporter. An *Anabaena sepJ* deletion mutant was significantly impaired in glutamate and aspartate uptake, which also in this cyanobacterium requires the activity of an ABC-type transporter for polar amino acids. SepJ appears therefore to generally stimulate the activity of cyanobacterial ABC-type transporters for polar amino acids. Conversely, an *Anabaena* mutant of three ABC-type transporters for amino acids was impaired in the intercellular transfer of 5-carboxyfluorescein, a SepJ-related property. Our results unravel possible functional interactions in transport elements important for diazotrophic growth.**

**IMPORTANCE**

**Membrane transporters are essential for many aspects of cellular life, from uptake and export of substances in unicellular organisms to intercellular molecular exchange in multicellular organisms. Heterocyst-forming cyanobacteria such as *Anabaena* represent a unique case of multicellularity, in which two cell types exchange nutrients and regulators. The SepJ protein located at the intercellular septa in the filaments of *Anabaena* contains a permease domain of the drug/metabolite transporter (DMT) superfamily that somehow contributes to intercellular molecular transfer. In this work, we have found that SepJ stimulates the activity of a polar amino acid uptake transporter of the ATP-binding-cassette (ABC) superfamily, which could itself affect an intercellular transfer activity related to SepJ, thus unraveling possible functional interactions between these different transporters.**

Some cyanobacteria grow as chains of cells known as filaments or trichomes that, when incubated in the absence of a source of combined nitrogen, present two cell types: vegetative cells that perform oxygenic photosynthesis and fix carbon dioxide and heterocysts that perform $N_2$ fixation (1–3). In species of the genera *Anabaena* and *Nostoc*, heterocysts are spaced along the filament, representing about 10% of total cells. In the $N_2$-fixing filament, the heterocysts provide the vegetative cells with fixed nitrogen and the vegetative cells provide the heterocysts with photosynthate. An exchange of glutamine (transferred from heterocysts to vegetative cells) for glutamate (transferred from vegetative cells to heterocysts) is thought to take place (4, 5). Additionally, sucrose (6–9) and alanine (10, 11) appear to be transferred from vegetative cells to heterocysts, and β-aspartyl-arginine appears to be transferred from heterocysts to vegetative cells (12).

The cyanobacteria are diderm bacteria bearing an outer membrane (OM) outside the cytoplasmic membrane (CM) and the peptidoglycan layer (13). In filamentous cyanobacteria, whereas the CM and peptidoglycan layer surround each cell, the OM is continuous along the filament, defining a continuous periplasm (13–17). In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (here designated *Anabaena*), genes encoding cell-cell joining proteins are known (18–21). These include genes in the *fraC-fraD-fraE* operon (18, 21) and *sepJ* (20), also known as *fraG* (19). By using fusions to the green fluorescent protein (GFP), FraC, FraD, and SepJ have been shown to be located in the intercellular septa of the *Anabaena* filaments (20, 21), and this has been corroborated for FraD by immunogold localization (22). Intercellular molecular exchange in *Anabaena* spp. can be probed with fluorescent tracers such as calcein (623 Da) or

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5-carboxyfluorescein (5-CF; 374 Da) (23, 24). Studies with these tracers have led to the proposal that SepJ and FraC/FraD are constituents of cell-cell joining complexes that facilitate intercellular molecular exchange in the cyanobacterial filament (21–24). Recent work has shown that these complexes are also involved in diffusion of esculin, a fluorescent sucrose analog (25). These complexes are reminiscent of the gap junction channels of metazoans (26, 27), but no sequence similarity exists between gap junction proteins and the cyanobacterial septal proteins.

SepJ consists of 751 amino acid residues and contains three well-differentiated domains (20, 24): a coiled-coil domain and a linker domain that appear to reside in the periplasm and an integral membrane domain that is homologous to proteins in the DMT (drug/metabolite transporter) superfamily (Transporter Classification Database number 2.A.7; http://www.tcdb.org). As deduced from the role of SepJ in the intercellular exchange of fluorescent tracers, this protein may provide a path for intercellular molecular transfer or may be part of a protein complex performing such a function. This activity should somehow involve transfer across the CM and channeling between the adjacent cells in the filament. For the transfer across the CM, the permease domain may have a role, as indicated by the specific requirement of the *Anabaena* permease to support diazotrophic function in strains expressing chimeras made of SepJ fragments from *Anabaena* and the filamentous, non-heterocyst-forming cyanobacterium *Trichodesmium erythraeum* (24). The identity of the SepJ physiological substrates is, however, unknown.

The DMT superfamily comprises a large number of families of proteins with diverse functions (28), including a large number of exporters grouped mainly in the drug and metabolite exporter (DME) family (Transporter Classification Database number 2.A.7.3) but also in some other families such as the putative tryptophan efflux (Trp-E) family (Transporter Classification Database number 2.A.7.23). Studies aimed at clarifying the evolution of these transporters have shown the role of gene duplication events starting from genes encoding a basic two-transmembrane segment element (29, 30). Interestingly, the SepJ permease shows several regions of homology to a two-transmembrane segment of EmrE from *Escherichia coli* K-12, a well-characterized protein of the small multidrug resistance (SMR) family (Transporter Classification Database number 2.A.7.1) (31). In particular, the fragment containing the last two predicted transmembrane segments of SepJ (amino acid residues 691 to 744) shows 20.4% identity (63% similarity) to the fragment containing the last two transmembrane segments of EmrE (amino acid residues 57 to 110). These similarities suggest that SepJ may participate in transport processes but do not permit us to anticipate its substrates or mechanism of action.

Different cyanobacteria have been shown to exhibit amino acid uptake activities (see, e.g., references 32 to 34), but these activities have been characterized mainly in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (35) and *Anabaena* (36). Both *Anabaena* and *Synechocystis* express an ABC-type transporter for basic amino acids, Bgt (36, 37), and an ABC-type transporter for neutral amino acids, N-I (37, 38). Additionally, *Synechocystis* expresses two $Na^+$-dependent glutamate transporters, which are the MFS (major facilitator superfamily) permease GltS and the TRAP-T (tripartite ATP-independent periplasmic transporter)-type transporter Gtr (37), and *Anabaena* expresses an ABC-type transporter for acidic and polar amino acids, N-II (39). The unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (here designated *Synechococcus*) expresses activities of uptake of acidic and neutral amino acids (34), but the molecular elements responsible have not yet been identified.

In this work, we investigated a possible role in membrane transport of the *Anabaena* SepJ protein by expressing it in *Synechococcus*. Because this cyanobacterium is naturally transformable and oligoploid (40), it is a convenient host for expression of heterologous genes. *Synechococcus* open reading frame (ORF) *Synpcc7942_1024*, which we have termed gene *dmeA*, encodes a protein with some similarity to the permease domain of SepJ. To express the *Anabaena sepJ* gene in *Synechococcus*, we chose to replace *dmeA* with *sepJ*. Because we found effects of the deletion of *dmeA* and its complementation by *sepJ* on uptake of several amino acids, but mainly glutamate, by *Synechococcus*, we have also isolated *Synechococcus* mutants to identify the glutamate transporter of this cyanobacterium. Our results indicate that DmeA and SepJ influence the uptake of glutamate and some other amino acids mediated by the ABC-type transporter N-II. We corroborated this effect in *Anabaena* by studying the uptake of glutamate and a few other amino acids in the wild type, a *sepJ* mutant, and a mutant of three ABC-type transporters for amino acids. Conversely, we found an effect of an ABC-type transporter(s) on intercellular transfer of 5-CF, which is a SepJ-related property.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Synechococcus* strains were grown in BG11 medium (containing $NaNO_3$), and *Anabaena* strains were grown in BG11 medium or $BG11_0$ medium (lacking nitrate). All strains were grown at 30°C and illuminated with light from fluorescent lamps (25 μmol of photons [μE] $m^{-2}$ $s^{-1}$), in shaken (100-rpm) liquid cultures or in medium solidified with 1% Difco agar. For bubbled cultures, the growth medium was supplemented with 10 mM $NaHCO_3$, bubbled with a mixture of $CO_2$ and air (1%, vol/vol), and illuminated at 75 μE $m^{-2}$ $s^{-1}$. Antibiotics were used for *Synechococcus* strains at the following concentrations: 2 μg streptomycin sulfate (Sm) $ml^{-1}$, 2 μg spectinomycin dihydrochloride pentahydrate (Sp) $ml^{-1}$, and 25 μg kanamycin sulfate (Km) $ml^{-1}$. Antibiotics were used for *Anabaena* strains at the following concentrations: 2 μg Sm $ml^{-1}$ and 2 μg Sp $ml^{-1}$ for liquid cultures and 5 μg Sm $ml^{-1}$, 5 μg Sp $ml^{-1}$, and 150 μg neomycin sulfate (Nm) $ml^{-1}$ for solid cultures. *Escherichia coli* DH5α, used for plasmid constructions, was grown in LB medium, supplemented when appropriate with antibiotics at standard concentrations (41).

**Construction of mutants.** The *Anabaena* and *Synechococcus* genomic sequences can be seen at http://genome.microbedb.jp/cyanobase. To inactivate the *Synpcc7942_1024* gene, DNA fragments upstream and downstream from the gene were amplified by PCR using DNA from *Synechococcus* as the template and primer pairs syn1024-1/syn1024-6 (bearing a KpnI site) and syn1024-5 (bearing a KpnI site)/syn1024-4, respectively (all oligodeoxynucleotide primers are listed in Table 1). Both fragments were amplified by overlap extension PCR (OE-PCR) with the primers syn1024-1/syn1024-4 and cloned into pSpark to generate pCSLE1. A KpnI-ended fragment bearing Sm/Sp resistance gene cassette C.S3 was inserted into the KpnI restriction site of pCSLE1 to produce pCSLE2. To clone the *sepJ* gene (ORF *alr2338*) from *Anabaena*, *alr2338* was amplified by PCR using the primers alr2338-25/alr2338-34 (bearing a KpnI site) and an *alr2338*-containing plasmid template. The upstream and downstream sequences flanking the *Synpcc7942_1024* gene were amplified by PCR using DNA from *Synechococcus* as the template and the primer pairs syn1024-1/syn1024-2 and syn1024-3 (bearing a KpnI site)/syn1024-4, respectively. The three products were fused by OE-PCR using primers syn1024-1/syn1024-4 and cloned as one joint fragment into pSpark to generate pCSLE4. A KpnI-ended fragment bearing Sm/Sp resistance gene



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**TABLE 1** Oligodeoxynucleotide primers used in this work

| Primer | Sequence (5′→3′)[a] |
|---|---|
| alr2338-25 | ATCAACAACGAGATGGGGCGATTTGAG |
| alr2338-34 | CGTTTTTCCTGATCGGTACCTTAACCTTCTGCATT |
| syn0246-1 | CAAGACGTGATTGAATGAAGGCGATCCT |
| syn0246-4 | GCAGCAAACCGCTCTAGAGTTCCGATGT |
| syn0246-5 | ATGAAGGCGATCCTTTTATCCGG |
| syn0246-6 | AGACGAAAGGGCATCTAGAAAATCCTGCAGC |
| syn0246-7 | GCCACGATTCAGGCGGAGGAGT |
| syn0246-8 | CCCAGCTGTTGCCCGAGTTCAC |
| syn0247-1 | ATGCCCTTTCGTCTGAAATTACAAGGA |
| syn0247-2 | GCAGCAAACCGCTCTAGATGCCCTGTT |
| syn0247-3 | GCTGCAGGATTTTCTAGATGCCCTTTCGTCT |
| syn0247-4 | TGTTTGCTGAGATAGAGACTGCCCA |
| syn0248-1 | ACATCGGAACTCTAGAGCGGTTTGCTGC |
| syn0248-2 | AGAACAGATAGACCTCGGCATAGCGACC |
| syn0248-3 | CCTGCCGCTAGTCACCATCCTTTTCT |
| syn0248-4 | CCTCCCCGCACGGTTTCAGC |
| syn1024-1 | TTGATCAGAGCGTAGGGCTGGCTCTCA |
| syn1024-2 | AAATCGCCCCATCTCGTTGTTGATAAG |
| syn1024-3 | GCAGAAGGTTAAGGTACCGATCAGGAAAAACGC |
| syn1024-4 | TAGGCTAAGCGGTAGCTGCGGCCGACCTCA |
| syn1024-5 | ATCAACAACGAGGGTACCGATCAGGAAAAACGC |
| syn1024-6 | TTTTTCCTGATCGGTACCCTCGTTGTTGATAAG |
| syn1024-12 | GCCCATTCAGACCATAGGTTCCTT |
| syn1024-15 | GGTTGCCATTGGTCAAGACTCCT |
| syn1892-1 | TCAGACCGATCGCTTCC |
| syn1893-2 | CAGGCGGACCATCCACAAGGT |
| syn1893-3 | CAGTAGCGCGAGTCTAGATCGAGAAGCTG |
| syn1893-4 | GCGCGTTCTCCCTCCTCAGTG |
| syn1893-5 | GGCGGCCGGTTCATCCAA |
| syn1894-1 | TGGCTACATCATCCTGCTGGTCTG |
| syn1894-3 | CAGCTTCTCGATCTAGACTCGCGCTACTG |
| syn1894-4 | GCGCGAGTCGGGGGAAACAT |
| syn1894-5 | CAGAGCGCCCCCAGCAACA |
| syn1895-1 | CCGCCTAGACCAGAATACC |
| syn2209-1 | CGTGCCCCACTCAACTTTCAACTCC |
| syn2209-2 | TGGGTCCGGCAACTGTCACG |
| syn2209-3 | TGACAGTTGCCGGACCCACCAC |
| syn2209-4 | TCAACCACGAATTCGCAACCATCC |

[a] Introduced restriction enzyme cutting sites are underlined.

cassette C.S3 from pCSE33 was inserted into the KpnI restriction site of pCSLE4 to produce pCSLE5. Transformation of *Synechococcus* with pCSLE2 and pCSLE5 was carried out as previously described (42), the cells were incubated for 48 h at 30°C under illumination on nitrocellulose filters, and transformants were selected on Sm/Sp-containing BG11 plates. The genetic structure of the selected clones was corroborated by PCR with DNA from those clones and primers syn1024-12/syn1024-15. The clones were named strains CSLE2 and CSLE5, respectively.

For the construction of clones with deletions comprising *Synpcc7942_0246*, *Synpcc7942_0247*, and *Synpcc7942_0248*; *Synpcc7942_0247* and *Synpcc7942_0248*; and *Synpcc7942_0246*, DNA fragments upstream and downstream from each of those sequences were amplified by PCR using DNA from *Synechococcus* as the template. Primer pairs syn0246-1/syn0246-4 (bearing an XbaI site), syn0247-1/syn0247-2 (bearing an XbaI site), and syn0246-5/syn0246-6 (bearing an XbaI site) were used, respectively, to amplify the upstream fragments from each sequence. In the same way, syn0248-1 (bearing an XbaI site)/syn0248-2 and syn0247-3 (bearing an XbaI site)/syn0247-4 were used to amplify the downstream fragments from *Synpcc7942_0248* and *Synpcc7942_0246*, respectively. Both upstream and downstream fragments from *Synpcc7942_0246-Synpcc7942_0247-Synpcc7942_0248* and from *Synpcc7942_0247* and *Synpcc7942_0248* were amplified by OE-PCR with primer pairs syn0246-1/syn0248-2 and syn0247-1/syn0248-2, respectively. The PCR products were cloned into pSpark to produce pCSLE9 and pCSLE15. The XbaI-ended fragment bearing the C.K3 gene cassette (Km$^r$) from pCSAL2a (A. López-Lozano and A. Herrero, unpublished data) was inserted in both orientations into XbaI-digested pCSLE9 and pCSLE15. The produced plasmids were: pCSLE10a and pCSLE16a (reverse orientation) and pCSLE10b and pCELE16b (direct orientation), respectively. The upstream and downstream fragments from the *Synpcc7942_0246* gene were digested with XbaI and cloned together with the C.K3 XbaI-ended fragment into pSpark. The selected plasmid, pCSLE19, carried the C.K3 cassette in direct orientation.

For inactivation of both *Synpcc7942_1893* and *Synpcc7942_1894*, an internal fragment of each gene was amplified by PCR using DNA from *Synechococcus* as the template and primer pairs syn1893-2/syn1893-3 (bearing an XbaI site) and syn1894-3 (bearing an XbaI site)/syn1894-1, respectively. Both fragments were amplified by OE-PCR with the primers syn1894-1 and syn1893-2 and cloned into pSpark to produce pCSLE24. The XbaI-ended fragment bearing the C.K3 cassette was inserted in both orientations into the XbaI site, producing pCSLE25a (reverse orientation) and pCSLE25b (direct orientation).

Plasmids pCSLE15a/b, pCSLE19, and pCSLE25a/b were transferred to *Synechococcus* wild type, and plasmids pCSLE10a/b were transferred to the wild-type, CSLE2, and CSLE5 strains by transformation as described above. Transformants were selected on required antibiotic-containing BG11 plates. The genetic structure of the selected clones was checked by PCR analysis using the following primers: syn0246-1/syn0248-2, syn0247-1/syn0248-2, syn0246-5/syn0247-4, and syn1892-1/syn1895-1. The mutants obtained were named, respectively, CSLE15a/b, CSLE19, CSLE26a/b, CSLE10a/b, CSLE11a/b, and CSLE12a/b.

**Immunofluorescence analysis.** For detection of SepJ by immunofluorescence, a sample of 150 μl of *Synechococcus* liquid cultures was placed atop poly-L-lysine-precoated microscope slides and covered with a 45-μm-pore-size Millipore filter. After that, the filter was removed and the slide was allowed to dry at room temperature and then immersed in 70% ethanol for 30 min at −20°C and dried for 10 min in an oven at 80°C. The cells were then washed twice by covering the slide with PBS-T buffer (140 mM NaCl, 1.5 mM $KH_2PO_4$, 2.7 mM KCl [pH 7.4], Tween 20 [0.05%, vol/vol]) (2 min each time, room temperature) and treated with blocking buffer (5% milk powder in PBS-T) for 15 min. The cells on the slides were then incubated with a primary antibody raised against the coiled-coil domain of SepJ (24) for 90 min, washed three times with PBS-T, incubated for 45 min in the dark with secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (Sigma; 1:500 dilution in PBS-T), and washed three times with PBS-T. After drying, several drops of FluorSave (Calbiochem) were added on top, covered with a coverslip, sealed with nail polish, and allowed to dry overnight at 4°C. Fluorescence was imaged using a Leica DM6000B fluorescence microscope and an Orca-ER camera (Hamamatsu). Fluorescence was monitored using an FITC L5 filter (excitation, band-pass [BP] 480/40 filter; emission, BP 527/30 filter), and images were managed with the ImageJ software (http://imagej.nih.gov/ij).

**Substrate uptake assays.** Cell suspensions from the different *Synechococcus* strains were inoculated at 1 μg chlorophyll *a* (Chl) $ml^{-1}$ and grown in BG11 medium (supplemented with appropriate antibiotics in the case of the mutants) for 24 h. Cells were harvested by low-speed centrifugation at room temperature, washed with 10 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7), and resuspended to a final concentration of 5 to 8 μg Chl $ml^{-1}$ in the same buffer. Uptake assays were carried out at 30°C in the light (175 μE $m^{-2}$ $s^{-1}$). Amino acid uptake was determined essentially as previously described (34). An amount of 25 μl of L-U-$^{14}$C-amino acid or [U-$^{14}$C]glycine solution was added to 600 μl of cell suspension to give a final concentration of 10 μM. After an incubation of 10 min, a sample of 550 μl was filtered and the radioactivity in the filter was measured in a scintillation counter. The uptake of [U-$^{14}$C]sucrose, [U-$^{14}$C]fructose, and [U-$^{14}$C]glucose was determined at a 50 μM sub-

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strate concentration. Uptake of 3 μM L-[U-$^{14}$C]aspartate, L-[U-$^{14}$C]glutamate, L-[U-$^{14}$C]glutamine, L-[U-$^{14}$C]proline, and α-[1-$^{14}$C]aminoisobutyric acid (AIB) in *Anabaena* wild-type and mutant strains was determined in mechanically fragmented filaments (43). Filament suspensions were passed through a needle the number of times necessary to give a filament length of about 8 cells per filament for the different tested strains. In every case, the radioactivity retained by boiled cells was used as a blank. All radiolabeled chemicals were purchased from PerkinElmer or American Radiolabeled Chemicals, Inc. Significance in the differences of uptake between strains was assessed by unpaired Student's *t* tests, assuming a normal distribution of the data. Data sets with *P* values of ≤0.05 are considered significantly different.

**Intercellular transfer of tracers.** Staining of the cells in the filaments with calcein and 5-CF was performed as previously reported (22, 23). Filament suspensions were spotted onto agar and then placed in a custom-built temperature-controlled sample holder with a glass coverslip on top. Measurements were carried out at 30°C. Cells were imaged with a Leica HCX Plan-Apo 63× 1.4-numerical-aperture (NA) oil immersion objective attached to a Leica TCS SP2 confocal laser-scanning microscope using a 488-nm-line argon laser as the excitation source. Fluorescent emission was monitored by collection across windows of 500 to 541 nm with a 150-μm pinhole. After an initial image was recorded, the bleach was carried out by switching the microscope to X-scanning mode, increasing the laser intensity by a factor of 10, and scanning a line across one cell for 0.137 s. The laser intensity was then reduced, the microscope was switched back to XY-imaging mode, and a sequence of images was recorded at intervals of about 1 s. Data from fluorescence recovery after photobleaching (FRAP) were analyzed using ImageJ 1.46q software (http://imagej.nih.gov/ij/) and quantified using the recovery rate constant, *R* (22, 25).

**RT-qPCR.** RNA was isolated as described previously (41) from 800 ml of bubbled *Synechococcus* cultures. RNA was treated with Ambion Turbo DNA-free DNase according to the manufacturer's protocol. Three independent RNA samples were analyzed from each strain (the wild type and the *dmeA* mutant CSLE2), and three technical replicates were carried out for each sample. RNA (200 ng) was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen) with random primers as indicated by standard protocols of the manufacturer. Quantitative real-time PCR (qPCR) was performed on an iCycler iQ real-time PCR detection system equipped with the software iCycler iQ v 3.0 from Bio-Rad. PCR amplification was performed in a 20-μl reaction mix according to standard protocols of the SensiFAST SYBR and fluorescein kit (Bioline). The qPCR conditions were as follows: 1 cycle at 95°C for 2 min and 30 cycles of 95°C for 15 s, 71°C for 20 s, and 72°C for 30 s. PCR products were checked by a single-peak melting curve. The threshold cycle ($C_T$) of each gene was determined and normalized to that of *rpoA* (ORF *Synpcc7942_2209*), tested with two independent primer pairs, to obtain a $\Delta C_T$ value from each sample. Relative gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method (44). In order to determine statistically significant differences between gene expression levels in the mutant and the wild type, reverse transcription (RT)-qPCR data were processed by REST-2009 software (http://rest.gene-quantification.info/). The following primer pairs were used: syn0246-7/syn0246-8 (for *Synpcc7942_0246*), syn0248-3/syn0248-4 (for *Synpcc7942_0248*), syn1893-4/syn1893-5 (for *Synpcc7942_1893*), syn1894-4/syn1894-5 (for *Synpcc7942_1894*), and syn2209-1/syn2209-2 and syn2209-3/syn2209-4 (for *Synpcc7942_2209*).

## RESULTS

**Expression of *Anabaena sepJ* in *Synechococcus*.** The permease domain of *Anabaena* SepJ is homologous to proteins in the DMT superfamily, and in the current version of the Transport Classification Database (http://www.tcdb.org), it is placed in the putative tryptophan efflux (Trp-E) family. *Synechococcus* ORF *Synpcc7942_1024*, which we designate gene *dmeA*, encodes a 330-residue protein that also belongs to the DMT superfamily, specifically to the DME family. To investigate possible natural substrates of *Anabaena* SepJ, we prepared a *Synechococcus* strain in which the *Anabaena sepJ* gene substituted for the *dmeA* gene (Fig. 1A). To select for gene replacement in *Synechococcus*, *sepJ* was cloned adjacent to the Ω cassette that provides Sm/Sp resistance, producing strain CSLE5. As a control, a *dmeA* deletion mutant, strain CSLE2, in which the *dmeA* gene was replaced only by the Ω cassette, was prepared. The integration of *Anabaena sepJ* at the *dmeA* locus was corroborated by PCR analysis (data not shown). Expression of *sepJ* in strain CSLE5 should take place from the *dmeA* gene promoter. Production of SepJ in CSLE5 was tested with antibodies raised against the coiled-coil domain of SepJ (24). Immunofluorescence analysis showed that SepJ was found in the periphery of the cells of strain CSLE5 but not of CSLE2 or wild-type *Synechococcus* (Fig. 1B). This is consistent with correct expression of the *sepJ* gene and localization of the SepJ protein in the cytoplasmic membrane of strain CSLE5.

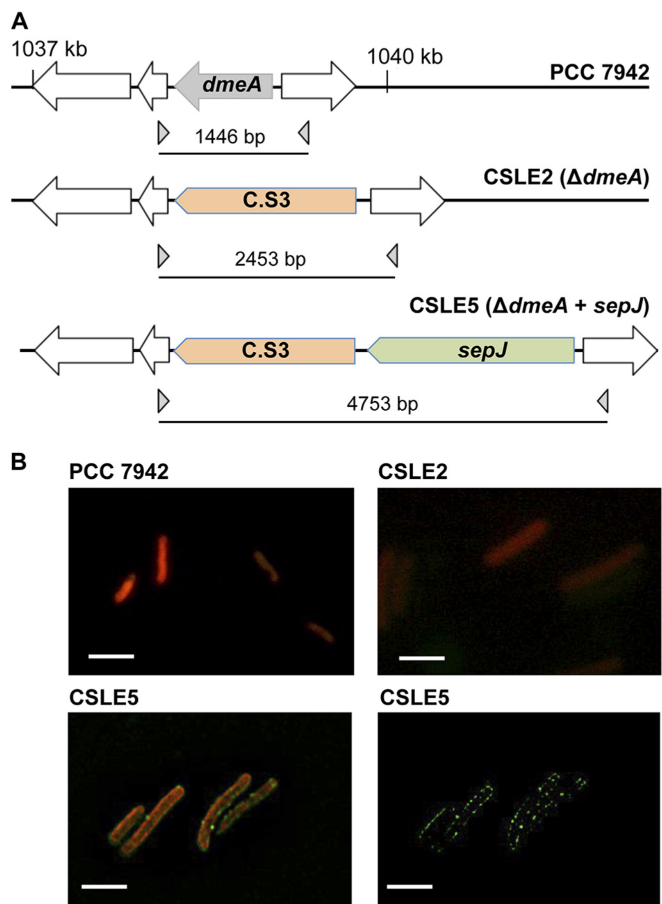


**FIG 1** Removal of *dmeA* and expression of *Anabaena sepJ* in *Synechococcus*. (A) Scheme of the *Synechococcus dmeA* genomic region (*Synpcc7942_1024*) with indication of the insertion and orientation of the C.S3 (Ω) gene cassette, which bears transcriptional terminators close to both of its edges, and the *sepJ* gene. Sizes of the wild-type and inserted fragments, analyzed by PCR (data not shown), are indicated. (B) Immunofluorescence detection of SepJ using antibodies to the coiled-coil domain of SepJ. Overlays of cyanobacterial autofluorescence (red) and immunofluorescence (green) are shown for the three strains, and the immunofluorescence alone is also shown for strain CSLE5. Bars, 3 μm.

**Metabolite uptake in *Synechococcus dmeA* and complemented mutants.** *Synechococcus* shows relatively low activities of

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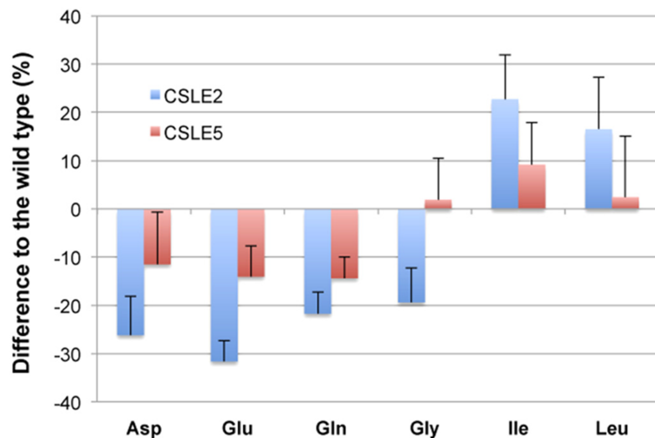


**FIG 2** Uptake of some representative amino acids in *Synechococcus* mutants CSLE2 and CSLE5. The difference between uptake in each mutant and the wild type is indicated, including error bars. The number of uptake assays with independent cultures for each amino acid was 9 to 16 for strain CSLE2 and 8 to 20 for CSLE5. Wild-type values in nanomoles milligram of $Chl^{-1}$ $minute^{-1}$ (mean ± standard deviation of the mean; *n*, number of assays performed with independent cultures) were as follows: Asp, 6.09 ± 0.41 ($n = 12$); Glu, 4.88 ± 0.30 ($n = 17$); Gln, 4.12 ± 0.19 ($n = 18$); Gly, 1.87 ± 0.17 ($n = 8$); Ile, 6.72 ± 0.44 ($n = 13$); Leu, 5.81 ± 0.56 ($n = 8$). Student's *t* tests for the comparison of uptake between pairs of strains indicated that the difference between wild type and CSLE2 was significant for Asp ($P = 0.027$), Glu ($P = 0.0004$), and Gln ($P = 0.003$); the difference between wild type and CSLE5 was significant for Gln ($P = 0.036$); and the difference between CSLE2 and CSLE5 was significant for Glu ($P = 0.037$).

uptake of some sugars (45) and amino acids (34), making it suitable to study the effect of heterologous SepJ on metabolite uptake. Activities of uptake of 50 μM [$^{14}$C]fructose or [$^{14}$C]glucose were very low in wild-type *Synechococcus* (about 0.3 and 0.6 nmol mg $Chl^{-1}$ $min^{-1}$, respectively) and were affected in neither the *dmeA* deletion mutant (CSLE2) nor the strain complemented with *sepJ* (CSLE5). Uptake of sucrose in wild-type *Synechococcus* was about 3.2 nmol mg $Chl^{-1}$ $min^{-1}$, and it was not significantly affected in strain CSLE2 or CSLE5.

The uptake of a number of tested amino acids was, in contrast, affected when *dmeA* was deleted. The activities of uptake of aspartate, glutamate, glutamine, and glycine were lower, by 19 to 32%, in CSLE2 than in the wild type, whereas the activities of uptake of isoleucine and leucine were 17 to 23% higher in CSLE2 than in the wild type (Fig. 2). The differences were statistically significant for aspartate ($P = 0.027$), glutamate ($P = 0.0004$), and glutamine ($P = 0.003$). In strain CSLE5, the uptake of the six tested amino acids was partially or completely recovered, and the corresponding uptake activities were closer to the wild-type activities than those observed in strain CSLE2 (Fig. 2). In particular, the uptake of glutamate was significantly different between strains CSLE5 and CSLE2 ($P = 0.037$), corroborating that *Anabaena* SepJ complemented the *dmeA* mutation for this activity. Because strain CSLE5 bears C.S3 in the same position as strain CSLE2 (Fig. 1A), complementation by SepJ indicates that the effect of the *dmeA* mutation resulted from lack of DmeA rather than from a polar effect of the C.S3 insertion. These results showed that the *Synechococcus* DmeA permease is needed for full uptake of some amino acids, including acidic amino acids, and that *Anabaena* SepJ can fulfill, at least partly, the role of DmeA. Additionally, these results showed that DmeA negatively affects the uptake of amino acids with nonpolar side chains such as isoleucine and leucine.

***Synechococcus* amino acid uptake mutants.** A substantial activity of acidic amino acid uptake remained in the *Synechococcus dmeA* mutant. To study the amino acid transporters that could be responsible for this activity, BLAST searches of the *Synechococcus* genome were performed using as queries the protein sequences of the amino acid transporters N-I, N-II, and Btg of *Anabaena* and Nat, Btg, GltS, and Gtr of *Synechocystis* (Table 2). The components of the main *Anabaena* transporter for acidic amino acids, which is the ABC-type transporter N-II encoded by *natFGH-bgtA* (39), retrieved a cluster of genes, *Synpcc7942_0246* to *Synpcc7942_0249*, from the genome of *Synechococcus* (Table 2). *Synpcc7942_0246* is predicted to encode a periplasmic substrate-binding protein similar to *Anabaena* NatF (56.8% identity), *Synpcc7942_0247* and *Synpcc7942_0248* are predicted to encode permeases similar to NatG (41.6% identity) and NatH (49.7% identity), respectively, and *Synpcc7942_0249* is predicted to encode an ATPase protein with high identity to BgtA (74.6%).

To study the role of these gene products in amino acid uptake and their possible functional relation to DmeA and SepJ, a deletion comprising *Synpcc7942_0246*, *Synpcc7942_0247*, and *Synpcc7942_0248* was generated in *Synechococcus* wild type and strains CSLE2 and CSLE5. Deletion was accompanied by insertion of the C.K3 gene cassette that does not bear transcriptional terminators and therefore can promote the expression of downstream genes (46). The C.K3 was inserted in both reverse (a) and direct (b) orientations. The generated strains, CSLE10a/b, CSLE11a/b, and CSLE12a/b, respectively, showed an uptake activity for gluta-

**TABLE 2** Genes of *Synechococcus* sp. strain PCC 7942 encoding the proteins with highest similarity to amino acid transport proteins from *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803

| Organism and transporter | Gene | ORF | Synechococcus homolog: ORF (*Synpcc7942_*) | Synechococcus homolog: *P* value |
|---|---|---|---|---|
| *Anabaena* | | | | |
| N-I | *natA* | *all1046* | *1893* | e−96 |
| | *natB* | *alr1834* | *1861* | e−109 |
| | *natC* | *all1047* | *1894* | e−99 |
| | *natD* | *all1248* | *2177* | e−93 |
| | *natE* | *all2912* | *0815* | e−78 |
| N-II | *natF* | *alr4164* | *0246* | e−106 |
| | *natG* | *alr4165* | *0247* | e−58 |
| | *natH* | *alr4166* | *0248* | e−102 |
| Bgt | *bgtA* | *alr4167* | *0249* | e−98 |
| | *bgtB* | *alr3187* | *0248* | e−28 |
| *Synechocystis* | | | | |
| Nat | *natA* | *slr0467* | *1893* | e−90 |
| | *natB* | *slr0559* | *1861* | e−99 |
| | *natC* | *sll0146* | *1894* | e−95 |
| | *natD* | *slr0949* | *2177* | e−93 |
| | *natE* | *slr1881* | *0815* | e−74 |
| Bgt | *bgtA* | *slr1735* | *0249* | e−63 |
| | *bgtB* | *slr1270* | *1761* | e−76 |
| GltS | *gltS* | *sll1145* | *2238* | 0.13 |
| Gtr | *gtrA* | *sll1102* | *0187* | 0.076 |
| | *gtrB* | *sll1103* | *1257* | 0.0008 |
| | *gtrC* | *sll1104* | *1276* | e−42 |

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**TABLE 3** Glutamate transport in *Synechococcus* sp. strain PCC 7942 and some mutant strains

| Strain | Genotype | Glutamate uptake (nmol mg $Chl^{-1}$ $min^{-1}$) ($n$)[a] |
|---|---|---|
| PCC 7942 | Wild type | 4.88 ± 0.30 (17) |
| CSLE2 | Δ*dme*::Ω cassette | 3.34 ± 0.21 (15) |
| CSLE5 | Δ*dme*::Ω cassette::*sepJ* | 4.20 ± 0.23 (16) |
| CSLE10a | Δ*Syn_0246-Syn_0248*::C.K3 (reverse) | 0.08 ± 0.01 (4) |
| CSLE10b | Δ*Syn_0246-Syn_0248*::C.K3 (direct) | 0.07 ± 0.01 (4) |
| CSLE11a | Δ*dme*::Ω cassette Δ*Syn_0246-Syn_0248*::C.K3 (reverse) | 0.08 ± 0.00 (2) |
| CSLE11b | Δ*dme*::Ω cassette Δ*Syn_0246-Syn_0248*::C.K3 (direct) | 0.06 ± 0.01 (2) |
| CSLE12a | Δ*dme*::Ω cassette::*sepJ* Δ*Syn_0246-Syn_0248*::C.K3 (reverse) | 0.05 ± 0.00 (2) |
| CSLE12b | Δ*dme*::Ω cassette::*sepJ* Δ*Syn_0246-Syn_0248*::C.K3 (direct) | 0.05 ± 0.01 (2) |
| CSLE15a | Δ*Syn_0247-Syn_0248*::C.K3 (reverse) | 0.10 ± 0.02 (2) |
| CSLE15b | Δ*Syn_0247-Syn_0248*::C.K3 (direct) | 0.06 ± 0.02 (2) |
| CSLE19b | Δ*Syn_0246-*::C.K3 (direct) | 0.22 ± 0.03 (4) |
| CSLE26a | Δ*Syn_1893-Syn_1894*::C.K3 (reverse) | 3.11 ± 0.98 (3) |
| CSLE26b | Δ*Syn_1893-Syn_1894*::C.K3 (direct) | 4.94 ± 0.76 (3) |

[a] Data are means and standard deviations of the means of the results from assays performed with the number of independent cultures indicated in parentheses. Student's $t$ tests indicated that glutamate uptake is significantly different in each mutant and the wild type ($P \leq 0.01$), except in the case of CSLE5 ($P = 0.136$), CSLE26a ($P = 0.0577$), and CSLE26b ($P = 0.95$). *Syn*, *Synpcc7942*.

mate that was 1 to 2% of the wild-type activity (Table 3). These results indicate that the *Synpcc7942_0246* to *Synpcc7942_0249* gene cluster encodes the major high-affinity glutamate transporter in *Synechococcus*, which we designate system N-II.

Because the uptake of glutamate is decreased in the *Synechococcus dmeA* mutant by about 32% but in the *Synpcc7942_0246-Synpcc7942_0247-Synpcc7942_0248* deletion mutant by 98 to 99%, we asked whether DmeA could be involved in transport assisted by the periplasmic binding protein Synpcc7942_0246. A *Synpcc7942_0247-Synpcc7942_0248* deletion strain might therefore show uptake mediated by DmeA-Synpcc7942_0246. We prepared *Synechococcus* strains in which the C.K3 gene-cassette replaced *Synpcc7942_0247* and *Synpcc7942_0248* (strain CSLE15b, gene cassette in direct orientation; strain CSLE15a, gene cassette in reverse orientation) (Fig. 3A). Strains CSLE15a and CSLE15b showed glutamate uptake activities very similar to those of strains CSLE10a and CSLE10b (1 to 2% of the wild-type activity [Table 3]), indicating that the permease components of the N-II system are essential for the uptake activity.

We then asked whether DmeA, complementing the activity of the periplasmic binding protein Synpcc7942_0246, could provide substrate for the permease components of the N-II system. If this were the case, a *Synpcc7942_0246* deletion strain could show uptake mediated by DmeA-Synpcc_0247/Synpcc7942_0248. We therefore prepared a *Synechococcus* strain in which the C.K3 gene cassette replaced *Synpcc7942_0246* (strain CSLE19b, gene cassette in direct orientation) (Fig. 3A). Strain CSLE19b showed a glutamate uptake activity that was 4.5% of the wild-type activity (Table 3), which is substantially lower than the activity associated with DmeA (32% of the wild-type activity). This indicates that DmeA (present in strain CSLE19b) cannot substitute for the periplasmic binding protein of the N-II system (missing in strain CSLE19b). Nonetheless, the low but significant level of glutamate uptake observed in strain CSLE19b (4.5% of the wild-type activity) suggests that the membrane complex made of permeases and ATPases has some activity in the absence of a periplasmic binding protein or that another periplasmic binding protein can partially substitute for Synpcc7942_0246. A possible increased expression of the *Synpcc7942_0247-Synpcc7942_0248-Synpcc7942_0249* genes in strain

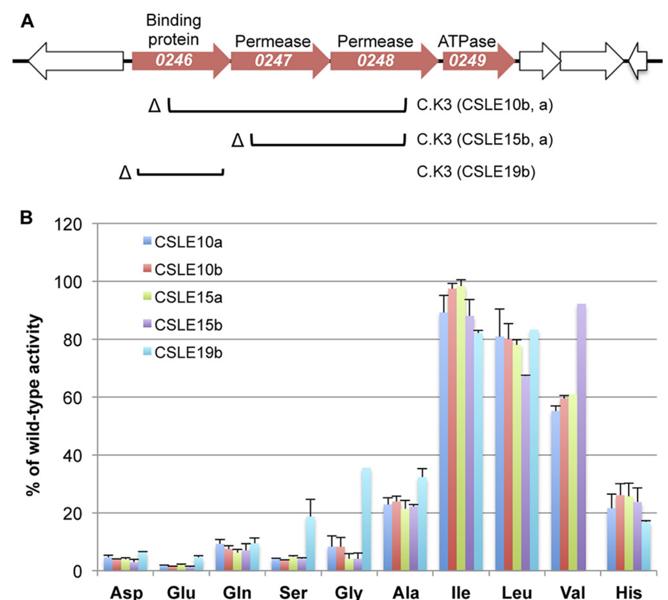


**FIG 3** Uptake of some representative amino acids in mutants of a *Synechococcus* ABC-type transporter. (A) Scheme of the *Synpcc7942_0246* to *Synpcc7942_0249* genomic region with indication of the predicted function of gene products, deleted regions (Δ), and strain names (a, C.K3 gene cassette in reverse orientation; b, C.K3 in direct orientation). (B) The uptake of the indicated amino acid in each mutant is represented, including error bars, as a percentage of the wild-type activity. The number of uptake assays with independent cultures for each amino acid was 2 to 5 for strains CSLE10a and CSLE10b, 2 for CSLE15a and CSLE15b (except for Val, $n = 1$), and 3 to 4 for CSLE19b (except for Gly and Leu, $n = 1$, and Val, not tested). Wild-type values in nanomoles milligram $Chl^{-1}$ minute$^{-1}$ (mean ± standard deviation of the mean; $n$, number of assays performed with independent cultures) were as follows: Asp, 6.09 ± 0.41 ($n = 12$); Glu, 4.88 ± 0.30 ($n = 17$); Gln, 4.12 ± 0.19 ($n = 18$); Ser, 2.24 ± 0.12 ($n = 4$); Gly, 1.87 ± 0.17 ($n = 8$); Ala, 6.94 ± 0.37 ($n = 6$); Ile, 6.72 ± 0.44 ($n = 13$); Leu, 5.81 ± 0.56 ($n = 8$); Val, 9.81 ± 0442 ($n = 2$); His, 3.78 ± 0.26 ($n = 6$). Student's $t$ tests indicated that uptake of the following amino acids was significantly different between each mutant and the wild type: Asp, Glu, and Gln ($P < 10^{-4}$); Ser ($P < 10^{-3}$); Gly ($P < 10^{-3}$; statistical analysis not done for strain CSLE19b); Ala ($P < 10^{-4}$); and His ($P < 10^{-3}$).

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CSLE19b could also contribute to the observed glutamate uptake activity in this strain.

To gain a wider view of the amino acid uptake activities mediated by the transporter encoded in the *Synpcc7942_0246* to *Synpcc7942_0249* gene cluster, nine additional amino acids were tested in relevant *Synechococcus* mutants. Aspartate was tested (in addition to glutamate) as an acidic amino acid; glutamine and serine were tested as amino acids with neutral polar side chains; and glycine, alanine, isoleucine, leucine, and valine were tested as amino acids with nonpolar side chains. Histidine, which is only partly basic at neutral pH, was also tested. Arginine and lysine were not tested because strain PCC 7942 shows negligible transport activities of these basic amino acids (34). Results summarized in Fig. 3B show that the tested mutants were defective in the uptake not only of glutamate but also of aspartate, glutamine, serine, and glycine. Interestingly, in every case the effect of inactivating the permeases of the system was higher than the effect of inactivating the periplasmic binding protein, as described above for glutamate. Although to a lower degree than the uptake of the amino acids just mentioned, uptake of alanine and histidine was also affected. In contrast, uptake of leucine, isoleucine, and valine was not significantly different in the mutants and the wild type. These results show that the *Synpcc7942_0246* to *Synpcc7942_0249* gene cluster encodes a transporter for acidic amino acids and amino acids with neutral polar or small side chains and that *Synechococcus* additionally expresses at least one more amino acid transporter with specificity for amino acids with nonpolar, aliphatic side chains.

Using the *Anabaena* NatA protein (38) as a query for searching the *Synechococcus* genomic sequence (Table 2), ORF *Synpcc7942_1893* was identified as encoding a protein highly similar to NatA (68.7% identity). *Synpcc7942_1893* is clustered together with *Synpcc7942_1894*, which is homologous to NatC (49.7% identity). *Synpcc7942_1893* and *Synpcc7942_1894* were jointly removed, with simultaneous insertion of the C.K3 gene cassette in both orientations producing *Synechococcus* strains CSLE26b (gene cassette in direct orientation) and CSLE26a (gene cassette in reverse orientation). These strains were not significantly impaired in glutamate uptake (Table 3). Whereas strain CSLE26b showed activities of uptake of isoleucine and leucine about 35% lower than the wild-type activity, with low statistical significance (Student's *t* tests, $P = 0.053$ for Ile and 0.083 for Leu; data not shown), strain CSLE26a showed activities of uptake of isoleucine and leucine about 70% lower than the wild-type activity, likely of statistical significance ($P = 0.0006$ for Ile and 0.0043 for Leu; data not shown). These results suggest that *Synpcc7942_1893* and *Synpcc7942_1894* encode elements of an ABC-type transporter able to mediate isoleucine and leucine uptake. Still another transporter for Ile, Leu, and Val could, however, be encoded in the *Synechococcus* genome. The *Synpcc7942_2492* to *Synpcc7942_2496* gene cluster may encode such a transporter, but its inactivation was not pursued in this work.

To investigate whether the effect of the *dmeA* mutation on the uptake of amino acids in *Synechococcus* could result from an effect on gene expression, we performed RT-qPCR analysis of the expression of two N-II-encoding genes (*Synpcc7942_0246* and *Synpcc7942_0248*) and of the two isoleucine/leucine transporter-encoding genes *Synpcc7942_1893* and *Synpcc7942_1894*, using *rpoA* as a housekeeping gene for normalization. Relative expression of those genes in the *Synechococcus dmeA* mutant compared to wild-type *Synechococcus* was studied with RNA samples isolated from three independent cultures of each strain (three technical replicates carried out for each RNA sample). According to REST analysis, *P* values were 0.382 for *Synpcc7942_0246*, 0.524 for *Synpcc7942_0248*, 0.523 for *Synpcc7942_1893*, and 0.634 for *Syn pcc7942_1894*, indicating that the differences in expression between the *dmeA* mutant and the wild type were not significant.

**Amino acid uptake in *Anabaena*.** We then investigated whether SepJ could influence the uptake of aspartate, glutamate, or glutamine in *Anabaena*. We previously found that the outer membrane of *Anabaena* is relatively impermeable to acidic amino acids and glutamine and that it can be bypassed by subjecting filaments to mechanical fragmentation (43). We therefore investigated the uptake of the three amino acids in fragmented filaments of wild-type *Anabaena* and *sepJ* deletion strain CSVM34 (24). Results in Table 4 show that inactivation of *sepJ* significantly decreased aspartate and glutamate uptake. Glutamine uptake was also affected, but the effect was of uncertain statistical significance. Assays performed with strain CSX60-R10, an *Anabaena natA bgtA* double mutant that lacks activity of the N-I, N-II, and Bgt transporters (39), showed that uptake of aspartate, glutamate, or glutamine required these transporters, which is consistent with previously known data (39, 43). Thus, deletion of *sepJ* affects uptake of aspartate and glutamate in *Anabaena*, but uptake requires the amino acid ABC-type transporters of this organism to take place.

In *Anabaena*, whereas glutamine is taken up by the N-I, N-II, and Bgt transporters, glutamate is taken up by N-I and N-II, and aspartate is taken up mostly (about 95% of the total uptake activity) by the N-II transporter (39). The results shown above with aspartate suggest therefore an influence of SepJ on the activity of N-II. To test whether an effect of SepJ on N-I also occurs, two substrates for which this transporter is highly specific, proline (38) and the nonmetabolizable α-aminoisobutyric acid (AIB [47]), were tested. As shown in Table 4, filaments of strain CSVM34 subjected to fragmentation showed proline and AIB uptake activities that were similar to those of the wild type, whereas fragmented filaments of strain CSX60-R10 showed negligible uptake of these amino acids as expected. These results show that inactivation of *sepJ* specifically affects transport mediated by the N-II ABC-type transporter.

**Intercellular transfer of calcein and 5-CF.** SepJ-related channels appear to contribute to intercellular transfer of the fluorescent tracers calcein and 5-CF (22, 25). Because SepJ influences an ABC-type amino acid transporter, we asked whether, conversely, an *Anabaena* mutant of the amino acid ABC-type transporters, strain CSX60-R10, was affected in transfer of those tracers. In FRAP analysis carried out as described in Materials and Methods, the recovery rates of calcein fluorescence in bleached cells were similar in strain CSX60-R10 and the wild type (Table 5). Transfer of 5-CF was, however, about 54% in strain CSX60-R10 compared to the wild type, a significant difference (Table 5). Of note, the recovery rate constant for 5-CF in strain CSX60-R10 ($R$, 0.058 $s^{-1}$), determined here, is close to that previously determined in the *sepJ* deletion mutant ($R$, 0.054 $s^{-1}$) (25).

## DISCUSSION

To investigate a possible role of the *Anabaena* SepJ protein in transport of metabolites across the cytoplasmic membrane, we introduced the *sepJ* gene at the *Synechococcus dmeA* locus. DmeA is a permease that, as is the case for the SepJ permease domain,

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**TABLE 4** Amino acid transport in filaments of *Anabaena* sp. strain PCC 7120 and some mutant strains subjected to mechanical fragmentation

| Strain | Uptake rate[a] for: Asp, nmol mg Chl$^{-1}$ min$^{-1}$ ($n$) | % | $P$ | Glu, nmol mg Chl$^{-1}$ min$^{-1}$ ($n$) | % | $P$ | Gln, nmol mg Chl$^{-1}$ min$^{-1}$ ($n$) | % | $P$ | Pro, nmol mg Chl$^{-1}$ min$^{-1}$ ($n$) | % | $P$ | AIB, nmol mg Chl$^{-1}$ min$^{-1}$ ($n$) | % | $P$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PCC 7120 | 1.69 ± 0.18 (13) | 100 | | 1.09 ± 0.12 (15) | 100 | | 4.24 ± 0.55 (13) | 100 | | 3.46 ± 0.42 (3) | 100 | | 8.81 ± 1.51 (4) | 100 | |
| CSVM34 | 1.00 ± 0.09 (6) | 59 | 0.022 | 0.55 ± 0.04 (6) | 50 | 0.006 | 2.94 ± 0.18 (6) | 69 | 0.091 | 4.08 ± 0.23 (3) | 118 | 0.259 | 10.46 ± 0.25 (3) | 119 | 0.341 |
| CSX60-R10 | 0.03 ± 0.00 (6) | 1.8 | $<10^{-5}$ | 0.00 ± 0.00 (6) | 0 | $<10^{-5}$ | 0.02 ± 0.00 (6) | 0.5 | $<10^{-4}$ | 0.03 ± 0.00 (3) | 0.9 | 0.001 | 0.02 ± 0.00 (3) | 0.2 | 0.002 |

[a] Data are means and standard deviations of the means of the results from assays performed with the number of independent cultures indicated in parentheses. Student's $t$ test $P$ values are indicated for the comparison of each mutant and the wild type for the uptake of each amino acid.

**TABLE 5** Intercellular exchange of calcein and 5-carboxyfluorescein in wild-type *Anabaena* and strain CSX60-R10

| Strain | Genotype | Transfer ($R$, s$^{-1}$)[a]: Calcein | 5-CF |
|---|---|---|---|
| PCC 7120 | Wild type | 0.070 ± 0.010 (29) | 0.108 ± 0.009 (33) |
| CSX60-R10 | *natA bgtA* | 0.073 ± 0.010 (25) | 0.058 ± 0.006 (41) |
| $P$, PCC 7120 vs CSX60-R10[b] | | $P$ = 0.820 | $P = 2 \times 10^{-6}$ |

[a] Intercellular exchange of tracers was investigated by FRAP analysis as described in Materials and Methods. Data (recovery rate, $R$) are means and standard deviations of the means from the number of filaments subjected to FRAP analysis indicated in parentheses. Filaments were from three independent cultures for each strain.
[b] Differences between the two strains were assessed by the Student $t$ test; $P$ is indicated for each tracer.

belongs to the DMT superfamily. We found that the *Synechococcus dmeA* mutant is significantly impaired in the uptake of glutamate, aspartate, and glutamine but also that its activity of transport of glycine was lower, and that of isoleucine and leucine was higher, than the wild-type activity. In the *Synechococcus* transformant strain CSLE5, the SepJ protein is observed by immunofluorescence analysis at the periphery of the cells, consistent with localization in the cytoplasmic membrane. This strain exhibits recovered uptake of aspartate, glutamate, glutamine, and glycine, indicating that SepJ stimulates the transport of these amino acids in *Synechococcus*, used as a heterologous host. However, the transport of those amino acids in *Synechococcus* is mostly dependent on the presence of a specific ABC-type transporter. We will first discuss this transporter and then the relation of the transport of glutamate and the other amino acids with DmeA/SepJ and ABC-type transporters.

Different unicellular cyanobacteria, including *Synechococcus* strains, are known to exhibit some capability of amino acid uptake, which may be relevant for supplementary amino acid nutrition of these photoautotrophs (see, e.g., references 34 and 48 to 50). The *Synpcc7942_0246* to *Synpcc7942_0249* gene cluster of *Synechococcus* encodes a transporter composed of a periplasmic binding protein (Synpcc7942_0246), two integral membrane proteins or permeases (Synpcc7942_0247 and Synpcc7942_0248), and an ATPase (Synpcc7942_0249), components of a typical ABC-type uptake transporter. Prokaryotic amino acid uptake ABC-type transporters fall into two families of the ABC superfamily: the polar amino acid uptake transporter (PAAT) family (Transporter Classification Database number 3.A.1.3) and the hydrophobic amino acid uptake transporter (HAAT) family (Transporter Classification Database number 3.A.1.4). The Synpcc7942_0246 to Synpcc7942_0249 proteins clearly constitute a transporter that belongs to family 3.A.1.3. Because this transporter is most similar to the *Anabaena* N-II transporter and at least one transporter for amino acids with nonpolar side chains is present in *Synechococcus*, we term the transporter identified in this work N-II. This transporter is the main system responsible for uptake of acidic amino acids, neutral amino acids with polar side chains, and glycine in this cyanobacterium. This identification adds an example to the relatively high abundance of ABC-type transporters in cyanobacteria compared to other bacteria (51).

As described above, the uptake of glutamate and a few other amino acids in *Synechococcus* is influenced by the DmeA and SepJ proteins, but the activity of transport of those amino acids is

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mostly dependent on the ABC-type transporter N-II. Inactivation of *dmeA* does not significantly affect the expression of two N-II-encoding genes that were tested, implying an effect of DmeA on the activity rather than on the expression of N-II. This could be a direct or indirect effect, but the possibility of a general requirement of DmeA for ABC transporters to develop their activity in *Synechococcus* is unlikely, because the *dmeA* mutant shows increased incorporation into the cells of isoleucine and leucine, which are taken up also by ABC-type transporters. These observations lead us to suggest that DmeA specifically influences the N-II system, stimulating its activity, and, on the other hand, the system(s) responsible for the transport of leucine and isoleucine, restricting its activity. Uptake of glutamate, aspartate, and glutamine is also decreased in *Anabaena sepJ* mutants, but the uptake of these amino acids in *Anabaena* is also fully dependent on ABC-type transporters. Specifically, SepJ appears to facilitate the function of ABC-type transporter N-II but not of ABC-type transporter N-I in *Anabaena*. Proteins that influence the activity of ABC-type transporters, involving quite diverse mechanisms of regulation, are known in eukaryotic systems (see, e.g., references 52 and 53). A possible molecular mechanism for the effect of *Synechococcus* DmeA and *Anabaena* SepJ on specific ABC-type importers present in these organisms is, however, currently unknown.

In the filaments of *Anabaena*, whereas the amino acid transporter N-I is present only in vegetative cells (38), the N-II transporter is present in both vegetative cells and heterocysts (39). Whereas no data on subcellular localization are available for N-I, N-II has been shown localized throughout the cytoplasmic membrane, including the septal regions between cells in the filament (39). Because SepJ is localized to the intercellular septa (20), interactions between SepJ and the N-II transporter would be possible in the septal regions. As mentioned in the introduction, SepJ is a proposed constituent of cell-cell joining complexes that mediate intercellular molecular exchange and can be probed with fluorescent tracers. Although intercellular transfer of calcein is not affected in the *natA bgtA* mutant strain CSX60-R10, intercellular transfer of 5-CF is impaired in this mutant (Table 5) to a similar extent as in a *sepJ* mutant (25). Thus, one or several of the amino acid ABC-type transporters of *Anabaena* appears to influence at least one property related to SepJ, the intercellular transfer of 5-CF. This represents an effect complementary to that described above of SepJ on the N-II transporter. The septal junction complexes, to which SepJ contributes, could be envisaged as channels connecting the adjacent cells, and substrates such as glutamate and glutamine could be assumed to diffuse through such channels (23). The results presented here unravel possible functional interactions of SepJ with at least one amino acid transporter. Although the mechanism(s) of such interactions remains to be investigated, they might improve intercellular molecular exchange in the filaments of *Anabaena*, which would be consistent with the observation that *Anabaena* mutants with inactivated amino acid ABC-type transporters, such as strain CSX60-R10, are strongly impaired in diazotrophic growth (39).

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