Septum-Localized Protein Required for Filament Integrity and Diazotrophy in the Heterocyst-Forming Cyanobacterium Anabaena sp. Strain PCC 7120

Enrique Flores,1* Rafael Pernil,1 Alicia M. Muro-Pastor,1 Vicente Mariscal,1 Iris Maldener,2‡ Sigal Lechno-Yossef,3 Qing Fan,3‡ C. Peter Wolk,3 and Antonia Herrero1

Instituto de Bioquímica Vegetal y Fotosíntesis, C.S.I.C., Universidad de Sevilla, E-41092 Seville, Spain1; Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, D-93040 Regensburg, Germany2; and MSU-DOE Plant Research Laboratory and Department of Plant Biology, Michigan State University, East Lansing, Michigan 488243

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Heterocysts, formed when filamentous cyanobacteria, such as Anabaena sp. strain PCC 7120, are grown in the absence of combined nitrogen, are cells that are specialized in fixing atmospheric nitrogen (N2) under oxic conditions and that transfer fixed nitrogen to the vegetative cells of the filament. Anabaena sp. mutants whose sepJ gene (open reading frame alr2338 of the Anabaena sp. genome) was affected showed filament fragmentation and arrested heterocyst differentiation at an early stage. In a sepJ insertion mutant, a layer similar to a heterocyst polysaccharide layer was formed, but the heterocyst-specific glycolipids were not synthesized. The sepJ mutant did not exhibit nitrogenase activity even when assayed under anoxic conditions. In contrast to proheterocysts produced in the wild type, those produced in the sepJ mutant still divided. SepJ is a multidomain protein whose N-terminal region is predicted to be periplasmic and whose C-terminal domain resembles an export permease. Using a green fluorescent protein translational fusion to the carboxyl terminus of SepJ, we showed that in mature heterocysts and vegetative cells, the protein is localized at the intercellular septa, and when cell division starts, it is localized in a ring whose position is similar to that of a Z ring. SepJ is a novel composite protein needed for filament integrity, proper heterocyst development, and diazotrophic growth.

All cyanobacteria perform oxygenic, plant-type photosynthesis. In some filamentous cyanobacteria, heterocysts develop from vegetative cells when the filaments sense nitrogen deprivation. Heterocysts are differentiated cells that are specialized for the fixation of N2 under oxic conditions and that transfer fixed nitrogen to the vegetative cells of the filament. Anabaena sp. mutants whose sepJ gene (open reading frame alr2338 of the Anabaena sp. genome) was affected showed filament fragmentation and arrested heterocyst differentiation at an early stage. In a sepJ insertion mutant, a layer similar to a heterocyst polysaccharide layer was formed, but the heterocyst-specific glycolipids were not synthesized. The sepJ mutant did not exhibit nitrogenase activity even when assayed under anoxic conditions. In contrast to proheterocysts produced in the wild type, those produced in the sepJ mutant still divided. SepJ is a multidomain protein whose N-terminal region is predicted to be periplasmic and whose C-terminal domain resembles an export permease. Using a green fluorescent protein translational fusion to the carboxyl terminus of SepJ, we showed that in mature heterocysts and vegetative cells, the protein is localized at the intercellular septa, and when cell division starts, it is localized in a ring whose position is similar to that of a Z ring. SepJ is a novel composite protein needed for filament integrity, proper heterocyst development, and diazotrophic growth.

Thus, the N2-fixing filament consists of two metabolically complementary cell types, photosynthetic vegetative cells and N2-fixing heterocysts, in a ratio of approximately 10 to 1 for Anabaena sp. strain PCC 7120 (hereinafter, Anabaena sp.) and some related cyanobacteria. Net fluxes of carbon (from vegetative cells to heterocysts) and nitrogen (from heterocysts to vegetative cells) take place in the filament. The mechanisms by which metabolites and regulatory molecules such as PatS are exchanged between the two cell types are unknown. One proposed hypothesis predicts transfer through an extracytoplasmic route, the periplasm, with the participation of uptake transporters and exporters in the two types of cells (8). We show here that the product of Anabaena sp. open reading frame alr2338 is located at the site of the division ring and the intercellular septa and that it is needed for filament integrity, proper heterocyst development, and diazotrophic growth.

MATERIALS AND METHODS

Bacterial strains. Anabaena (Nostoc) sp. strain PCC 7120 was used in this work. Mutant strains 216 (2) and CSE2 (9) have previously been described. An internal fragment of open reading frame alr2338 (sepJ) was amplified by PCR using the primers 5′-ATT CCT TCC CAG CAG TGT TCT C-3′ and 5′-GAA CAG AAG CCA GGA TAA CAC C-3′, with DNA from wild-type Anabaena sp. as the template, and cloned into plasmid pGEM-T Easy (Promega Corp.). Insertion of a PstI-cat-emr-oriT(RK2)-PstI cassette from pRL2665b (12) into the unique PstI site of the resulting plasmid yielded plasmid pRL2787a. pRL2787a was transferred to Anabaena sp. by triparental mating with selection for erythromycin (Em) resistance, yielding mutant strain SR2787a. Single-crossover homologous recombination with sepJ was confirmed by Southern blotting and by PCR with primers 5′-TAA CCT GAC GAT CGC TTA T-3′ and 5′-GAT GGA CTT TAG CCC CAC AT-3′.
The sepJ-green fluorescent protein gene (gfp) strain CSAM137 was constructed as described above. Plasmid pCSAM134 contains a PCR-amplified, 515-bp DNA fragment from the C-terminal portion of sepJ cloned into the pGEM-T Easy plasmid. The amplified fragment, generated with primers 5'-TTT TCT GTG TTG AGG TGC C-3' (alr2338-2) and 5'-GGC AGG TTT GTT GAA ATC TT G-3' (alr2338-EcoV-2), corresponds to nucleotides 527 to 13 with respect to the translational stop site of sepJ and contains an EcoRV site (underlined in primer alr2338-EcoV-2) introduced into the sequence encoding amino acids 9 and 10 from the stop codon. A promoterless gfp gene was excised from pCEL19 (18) as an EcoRV-Apal fragment and introduced into EcoRV- and Apal-digested pCSAM134, producing pCSAM135. A PstI fragment from pCSAM135, containing the translational fusion between the C-terminal region of sepJ and the gfp gene, was cloned into the polylinker of pCSV3 (23), yielding pCSAM137. Plasmid pCSAM137 was transferred to Anabaena sp. by conjugation as previously described, and colonies resistant to streptomycin and spectinomycin were selected. The entire plasmid was integrated by single-crossover homologous recombination, resulting in an altered sepJ gene, with the gfp gene substituting for the last 27 bp of the gene. The genomic structure was confirmed by PCR performed with primers 5'-CGA ATG TAT AAC CAA CAG CAG C-3' (alr2338-3; corresponding to nucleotides 505 to 526 with respect to the translational start site of the sepJ gene) and 5'-CAA GAA TTT GGA CAA CTC C-5' (gfp-4). Using DNA from the exconjugants as the template, these oligonucleotide primers amplify a DNA fragment of 1,774 bp only if wild-type chromosomes are present in the template DNA.

**Northern analysis.** Total RNA from Anabaena sp. and its derivatives was isolated as previously described from cultures grown or incubated with the indicated nitrogen sources and bubbled with air supplemented with 1% CO$_2$ (20). Northern analysis was carried out as described previously (19).

**Microscopy.** For standard light microscopy, Anabaena sp. and SR2787a were grown in BG11 medium (in the presence of 5 μg Em/ml for the mutant) and incubated in BG11$_0$ medium (which lacks a source of combined nitrogen) for 48 h at the light (75 μE m$^{-2}$ s$^{-1}$) on a rotary shaker (90 rpm). Filaments were harvested and fixed with glutaraldehyde and KMnO$_4$, dehydrated with increasing concentrations of ethanol, and embedded in EPON, and ultra-thin sections were stained with uranyl acetate and lead citrate (7). The samples were examined with a Zeiss EM101C transmission electron microscope at 80 kV.

For confocal microscopy, strain SR2787a was grown in BG11 medium in the presence of 5 μg Em/ml and incubated in BG11$_0$ medium for 48 h in the light (75 μE m$^{-2}$ s$^{-1}$) on a rotary shaker (90 rpm). Filaments were harvested and fixed with glutaraldehyde and KMnO$_4$, dehydrated with increasing concentrations of ethanol, and embedded in EPON, and ultra-thin sections were stained with uranyl acetate and lead citrate (7). The samples were examined with a Zeiss EM101C transmission electron microscope at 80 kV.

**Lipid and nitrogenase analyses.** Filaments of Anabaena sp. and SR2787a grown in BG11 medium (in the presence of 5 μg Em/ml for the mutant) were harvested, washed with nitrogen-free medium, and incubated in BG11$_0$ medium for 48 h. Lipids were extracted from whole filaments with chloroform-methanol (2:1, vol/vol), concentrated under N$_2$, and chromatographed on thin layers of silica gel (22). Heterocyst glycolipids were identified as described previously (20). Nitrogenase activity was determined by the acetylene reduction technique as described previously (17). For anaerobic assays, 10 μM M-(3,4-dichlorophenyl)-1,1-dimethylethylamine was added to the cell suspension, the flask containing the cells was sealed with a rubber stopper, bubbled with argon for 15 min, and further incubated, under culture conditions, for 1 h before the assay was started by the addition of acetylene.

**RESULTS**

**Identification of the sepJ gene.** Fox$^{−}$ mutants are defined as those that are unable to fix N$_2$ under oxic conditions. In a set of 1,076 random Fox$^{−}$ transposon mutants (6), 21 transposi-

![FIG. 1. Scheme showing the different domains of SepJ and the approximate location of the fusion to GFP.](image)

The 751-amino-acid, predicted protein SepJ comprises (i) a 200-residue N-terminal coiled-coil region such as is normally engaged in protein-protein interactions; (ii) an internal, 211-amino-acid sequence that is rich in Pro and Ser and shows similarity to plant extensins and mammalian mucins; and (iii) an ~340-amino-acid C-terminal region that is homologous to proteins in the drug/metabolite exporter (DME) family of *Bacteria* and *Archaea* (15), including the RhtA amino acid exporter of *Escherichia coli* (15) (Fig. 1). Protein topology and subcellular localization predictions, made using programs that allow predictions for gram-negative bacteria (http://www.expasy.org/tools/#topology), suggest that the 411-residue N-terminal region of SepJ is extracytoplasmic and that the C-terminal portion is a cytoplasmic membrane protein. Most predicted topologies place the carboxyl terminus of the protein in the cytoplasm, but whether the protein bears 9 or 11 α-helical transmembrane segments is uncertain.

**Phenotype of a sepJ mutant.** In nitrate-containing liquid medium, the wild-type strain forms mostly long filaments whereas the sepJ mutant SR2787a forms filaments with a somewhat homogeneous distribution of lengths from a few cells to more than 80 cells (Fig. 2). When transferred to medium without combined nitrogen, filaments of the wild type remain long
whereas filaments of the mutant strain fragment, within a few days, into filaments that are mostly ≤10 cells in length (Fig. 2). When prepared for transmission electron microscopy, filaments of the mutant strain fragment even more extensively (Fig. 3a). Many of its cells appear to initiate differentiation, with deposition of what may be a heterocyst polysaccharide layer (Fig. 3b). Some cells in cultures of the mutant incubated for 48 h without combined nitrogen were stained with Alcian

\[ \text{FIG. 2. Filament lengths of } \textit{Anabaena} \text{ sp. and SR2787a.Filaments grown in BG11 medium (in the presence of 5 μg Em/ml for the mutant) were harvested, washed with nitrogen-free medium, and re-suspended in BG11 or BG11o medium with antibiotic for the mutant. After incubation for the indicated times at } 30^\circ \text{C in the light on a shaker, samples, which were taken with great care to prevent disruption, were visualized by standard light microscopy. Ninety to 120 filaments were counted in each case. WT, wild type.} \]

\[ \text{FIG. 3. Ultrastructures of strain SR2787a incubated in nitrogen-free medium for 48 h (a, b) and of a heterocyst of wild-type } \textit{Anabaena} \text{ sp. induced for 48 h in nitrogen-free medium (c). Size markers, 1 μm. C, carboxysomes; Hep, heterocyst polysaccharide layer; Hgl, heterocyst glycolipid layer; Sep, septum; S. c., small cell.} \]
blue, suggesting that they had produced extracellular polysaccharides, although staining was less intense than that shown by wild-type heterocysts of a 48-h induction (results not shown). However, a polysaccharide-like shell was the only morphological sign of differentiation that was evident in nitrogen-deprived SR2787a. Unlike in wild-type heterocysts from a 48-h induction (Fig. 3c), intracellular membranes were not reorganized, carboxysomes were not degraded, and no heterocyst glycolipids were deposited in the mutant cells (Fig. 3). Consistently, no heterocyst-specific glycolipids were detected in extracts of SR2787a cells incubated for 48 h in the absence of combined N (Fig. 4). Cells of SR2787a continued to divide inside the newly synthesized envelope, a phenomenon never seen in the wild type, giving rise to groups of two or three encapsulated cells (Fig. 3a and b). The shape of these cells differed from that of normal vegetative cells, with occasional production of very small cells (Fig. 3a), and the septum between the cells was broader than between vegetative cells or between heterocysts and neighboring vegetative cells in the wild type (Fig. 3 and not shown). In conclusion, SepJ enhances the integrity of the filament, especially when it is nitrogen deprived, and is necessary for heterocyst maturation.

Some Anabaena mutants that produce defective heterocysts show nitrogenase activity when assayed under micro-oxic conditions, a Fix+ phenotype (28). To check whether the pro-heterocysts observed in cultures of SR2787a could develop a heterocyst-like metabolism independently of their abortive morphological differentiation, we tested for nitrogenase activity under both oxic and anoxic conditions in cells incubated for 48 h without combined nitrogen. Under assay conditions in which the wild type produced about 3.9 μmol ethylene · mg chlorophyll⁻¹ · h⁻¹ (aerobic activity) and 6.6 μmol ethylene · mg chlorophyll⁻¹ · h⁻¹ (anaerobic activity), no production of ethylene was detected with mutant strain SR2787a, which thus exhibits a Fix⁻ phenotype. Therefore, not only morphological heterocyst maturation but also metabolic differentiation was arrested as a result of the inactivation of sepJ.

Expression of sepJ and localization of the SepJ protein.
Because the SR2787a mutant shows some fragmentation even during growth on nitrate, we investigated the growth conditions under which sepJ is expressed. We performed Northern blot analysis with two different probes of sepJ and with RNA isolated from wild-type cells grown with N₂, nitrate, or ammonium as the nitrogen source. Hybridization was strongest with RNA from the N₂-grown cells, but it was also appreciable with RNA from ammonium- and nitrate-grown cells (data generated with the longer probe are shown in Fig. 5a). Although some signals that corresponded to degraded RNA were observed, signals corresponding to the largest mRNA molecules indicated that the gene was expressed as a monocistronic transcript of about 2.3 kb. When wild-type cells were grown with ammonium and incubated for up to 24 h in the absence of...
combined nitrogen, expression increased during the period of 3 to 12 h of nitrogen deprivation (data generated with the shorter probe are shown in Fig. 5b). To test whether this increase in expression requires the heterocyst development regulators NtcA and HetR, RNA isolated from mutants of the corresponding genes was used. Expression in the \textit{ntcA} mutant CSE2 (9) was greater than in the wild type at all time points tested. In contrast, expression in the \textit{hetR} mutant 216 (2) was similar to that in the wild type. Thus, the expression of \textit{sepJ} does not require these heterocyst development regulators, although it is affected by NtcA.

The in vivo localization of SepJ was studied using a SepJ-GFP translational fusion protein in which GFP was added at amino acid 742 of the protein, 9 amino acids from the C terminus (Fig. 1). According to the predicted topology of SepJ, this construct should place GFP in the cytoplasm adjacent to the cytoplasmic face of the membrane. In strain CSAM137 carrying the \textit{sepJ-gfp} fusion, the fusion gene substituted for the \textit{sepJ} gene so that no intact \textit{sepJ} gene was present and \textit{sepJ-gfp} would be transcribed from the \textit{sepJ} promoter (see Materials and Methods for details). Strain CSAM137 developed heterocysts and grew diazotrophically, indicating that the SepJ-GFP polypeptide retains SepJ function. Fluorescence from SepJ-GFP was observed in the septa joining vegetative cells in ammonium-, nitrate-, and \textit{N}_2-grown filaments (Fig. 6a) and also at the apposed ends of vegetative cells and heterocysts in \textit{N}_2-grown filaments (Fig. 6b). Only one spot of increased fluorescence was observed between each pair of vegetative cells, suggesting that the proteins from separate cells are closely clustered in those septa. However, two separate spots of increased fluorescence could be distinguished in the junctions between vegetative cells and heterocysts (Fig. 6b), suggesting...
that there are separate foci of the protein in the vegetative-cell cytoplasmic membrane and within the neck of the heterocyst. Perhaps the poles of adjacent cells contribute to the cluster of that protein, with the clusters closely appressed in the case of the vegetative cells but more disjointed when the two types of cells are juxtaposed.

In cells in the process of division, fluorescence was also observed in what appeared to be a ring at the site of division. Figure 6c shows examples of dividing cells in two stages: in the left panels, two dividing cells show the GFP fluorescence at the periphery, midway between the ends of the cells; in the right panels, the GFP fluorescence appears to originate at a division plane as the dividing cell constricts.

**DISCUSSION**

Inactivation of *Anabaena* sp. open reading frame *alr2338* (gene *sepJ*) results in a complex phenotype that includes filament fragmentation, arrested heterocyst development (with lack of heterocyst glycopolysaccharide production), and the lack of nitrogenase activity even under anoxic conditions (Fix<sup>−</sup> phenotype). This gene (and an identical mutant phenotype) has also recently been identified in an independent work, in which the authors denoted *alr2338 fraG* (21). We prefer the name *sepJ* because it better reflects our findings on the subcellular localization of the product of *alr2338*.

We have shown that a SepJ-GFP fusion protein is localized at the poles of mature *Anabaena* sp. cells in the intercellular septa. Because the SepJ-GFP protein retains SepJ function, we think that the observed localization of the fusion protein corresponds to the physiological position of SepJ. The fragmentation phenotype of the *sepJ* inactivation mutants and the localization of SepJ in the intercellular septa suggest that this protein plays a role in linking cells of the *Anabaena* sp. filament. In bacteria, high fluorescence of GFP fused to the C-terminal region of cytoplasmic membrane proteins is observed only if the GFP is located in the cytoplasm as a result of the particular topology of the investigated protein (5). Therefore, the fact that GFP fluorescence was observed with the construct used here is consistent with predictions of a SepJ topology in which the carboxyl terminus of the protein is cytoplasmic and the GFP is in the cytoplasm, anchored to the cytoplasmic face of the cytoplasmic membrane by the C-terminal region of SepJ. The N-terminal extracytoplasmic domains of SepJ may join cells by means of protein-protein interactions, since coiled-coil domains can bind proteins. Whether SepJ monomers produced by neighboring cells interact and whether other proteins participate with SepJ in a cell-to-cell joining complex are questions that deserve future research. Direct SepJ interactions are more likely between vegetative cells than between vegetative cells and heterocysts, since separate loci of GFP fluorescence from the SepJ-GFP fusion protein are observed in the latter case.

Consistent with the requirement of its protein product for diazotrophic growth, the *sepJ* gene is expressed at higher levels in *N₂*-grown filaments than in filaments grown in the presence of combined nitrogen (Fig. 5). However, expression under the latter conditions is substantial and apparently permits sufficient accumulation of SepJ-GFP that the resulting fluorescence can be observed in ammonium- and nitrate-grown cells as well as in *N₂*-grown cells (Fig. 6). Although Nayar et al. have shown a possible moderate increase in expression in mature heterocysts (21), increased expression in the absence of combined nitrogen is dependent on neither NtcA nor HetR. Why NtcA appears, rather, to have a negative effect on expression is unknown but may be related to the presence of a canonical NtcA binding site (10) within the coding sequence of *sepJ*. The expression data and the evident fragmentation of the *sepJ* mutant SR2787a in cultures with combined nitrogen are consistent with SepJ having a role also in nitrogen-replete vegetative cells. The question then arises whether the fragmentation and developmental phenotypes are related or are independent effects of the inactivation of *sepJ*.

Mutations in other genes are known to lead to the fragmentation of filaments of *Anabaena* sp. (1), and therefore other proteins may have a related role in filament integrity. The phenotype of a *fraC* mutant is similar to that of the *sepJ* mutant in that the mutant fragments and exhibits cell division after the synthesis of a putative heterocyst polysaccharide layer, but whereas a *sepJ* mutant is Fox<sup>−</sup> and Fix<sup>−</sup>, a *fraC* mutant is Fox<sup>−</sup> but Fix<sup>+</sup>, i.e., it exhibits anaerobic nitrogenase activity (1). Filaments of a *fraH* mutant also fragment, but mature heterocysts are observed in combined nitrogen-free cultures of this mutant (1). Therefore, fragmentation does not necessarily lead to the severe heterocyst development arrest observed in the Fix<sup>−</sup>*sepJ* mutant.

The similarity of the C-terminal domain of SepJ to transporters, and the localization of this protein at the junction between cells, raises the possibility that it is involved in the movement of metabolites or regulatory compounds between neighboring cells. This movement could take place directly from cell to cell, mediated by a junction complex that includes SepJ, or could involve a periplasmic route (8). Using a *hetR promoter-gfp* fusion construct as a reporter for the start of heterocyst differentiation, Nayar et al. (21) showed that SepJ is not needed for pattern formation. However, their and our results show that development is arrested between the developmental steps of the production of heterocyst wall polysaccharides and of heterocyst-specific glycolipids. Perhaps the multidomain SepJ has two differentiated functions: (i) a function in joining cells in both combined nitrogen-replete and -free media, possibly related to the extracytoplasmic region of the protein, and (ii) a function in heterocyst differentiation, possibly related to the permease portion of the protein.

Heterocyst-forming cyanobacteria are truly multicellular organisms in which different cells, namely, heterocysts and vegetative cells, contribute complementary functions to the performance of the organism as a whole. The results presented in this work illustrate the novel finding of the subcellular localization of the SepJ protein that has a role in filament integrity and heterocyst development. Our results suggest that SepJ has a specific localization in the cytoplasmic membrane, where it forms a ring during cell division; when cell division has been completed, it is localized at the center of the septum, occupying a position corresponding to a cell pole (Fig. 6). The apparently ring-like distribution of SepJ during division suggests that SepJ may interact with the divisional Z ring observed in cyanobacteria (16, 25). The actin-related bacterial cytoskeletal protein MreB is involved in subcellular protein localization (4), and the MinCDE system regulates septation in diverse bacteria.


