A TolC-like protein is required for heterocyst development in

*Anabaena* sp. strain PCC 7120

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The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 forms heterocysts in a semi-regular pattern when grown on N\textsubscript{2} as sole nitrogen source. The transition from vegetative cells to heterocysts requires marked metabolic and morphologic changes. We show that a trimeric pore-forming outer membrane \(\beta\)-barrel protein of the TolC family, Alr2887, is upregulated in developing heterocysts and essential for diazotrophic growth. Mutants defective in *alr2887* did not form the specific glycolipid layer of the heterocyst cell wall, which is necessary to protect nitrogenase from external oxygen. Comparison of the glycolipid content of wild-type and mutant cells indicated that the protein is not involved in the synthesis of glycolipids but might instead serve as an exporter for the glycolipid moieties or enzymes involved in glycolipid attachment. We propose that Alr2887 together with an ABC transporter like DevBCA is part of a protein export system essential for the assembly of the heterocyst glycolipid layer. We designate the *alr2887* gene *hgdD* (from heterocyst glycolipid deposition protein).
Gram-negative bacteria use a type I export system to transfer proteins or other molecules like siderophores or fatty acids to the cell surface (37,50,55). The proteinaceous substrates contain a C-terminal secretion signal essential and sufficient for export. The information for targeting and translocation, however, seems to be presented in form of a secondary structure element rather than in the primary sequence as no clear amino acid motif has been identified. The machinery of type I export systems bridges the periplasm allowing the transfer of their substrates beyond the outer membrane. The export system is composed of a translocase spanning the plasma membrane with a α-helical, pore-forming domain and a cytosolic domain energizing the translocation process. Additionally, an adaptor protein associates with the translocase and, after substrate association, induces the interaction with an outer membrane TolC-like exit tunnel to conduct the secretion. Whereas TolC-like proteins from gram-negative proteobacteria have been amply investigated (37,50,54,55), nothing is known about these systems in cyanobacteria. Based on sequence similarity (40) and proteomic analysis of the cell-wall fraction (42), we have recently proposed the existence of a protein of this type, Alr2887, in *Anabaena* sp. strain PCC 7120 (hereinafter denoted *Anabaena* sp.), for which a function in lipid transfer has previously been proposed (40).

*Anabaena* sp. is a filamentous cyanobacterium, which starts a program of cell differentiation upon nitrogen starvation that results in the appearance of nitrogen fixing cells. These are called heterocysts and are arranged in a semi regular pattern along the filament (63). Three mechanisms protect the oxygen-labile nitrogenase from irreversible inactivation by O$_2$ produced by the oxygenic photosynthesis: (i) formation of extra envelop layers outside of the gram-negative cell wall, (ii) degradation of photosystem II (PSII) antennae and decrease of PSII activity, and (iii) increase of O$_2$-consuming respiration. The process of cell differentiation takes about one generation time of 20 h. Heterocysts provide the vegetative cells with their products of combined nitrogen and obtain from the vegetative cells...
metabolites that serve as reductant for \( N_2 \) fixation and C-skeletons for nitrogen assimilation (e.g. 1,20,63).

Formation of the extra envelope of the cell wall occurs in several steps. First, a homogenous layer of polysaccharides (heterocyst envelope polysaccharide: HEP) is formed by deposition of the material outside of the gram-negative cell wall (10,26). Subsequently, specific glycolipids (heterocyst envelope glycolipid: HGL) are made and deposited between this protecting layer and the outer membrane, forming the so-called laminated layer (26,31). This layer is the barrier for entry of gases (31,59,61). HGLs are polyhydroxy alcohols (26-28 C), glycosidically linked at C-1 to glucose (25 and references therein). In the synthesis of the glycolipids, fatty acid synthases, polyketide synthases, ketoreductases, dehydrases, acyl transferases or thioesterases are likely to be involved (19,32). How transport and deposition of the HGLs beyond the cell wall occurs is still unknown.

Several genes have been identified that are involved in the formation of heterocyst-specific cell-wall layers. \( \text{hepA} \), \( \text{hepB} \) and \( \text{hepC} \) encode proteins with similarity to ABC-type transporters, glycosyltransferases, and UDP-galactose-lipid carrier transferases, respectively (40,64). Furthermore, \( \text{alr2825} \), \( \text{alr2827} \), \( \text{alr2831} \), \( \text{alr2833} \), \( \text{alr2837} \), \( \text{alr2839} \) and \( \text{alr2841} \) are localized on a well-defined specific “HEP-island” in the chromosome encoding enzymes with putative function in the synthesis of the HEP layer (33). Additionally, \( \text{hglB} \), \( \text{hglC} \), \( \text{hglD} \) and \( \text{hglE} \) genes encode enzymes necessary for the synthesis of heterocyst glycolipids (4,9). In turn, the gene product of \( \text{hglK} \) is needed for the formation of the glycolipid layer (6). Finally, the \( \text{devBCA} \) operon codes for an exporter of the ABC-type transport family essential for the HGL layer formation (21). A TolC-like outer membrane protein like Alr2887 (40,42) would be the missing part of the export system that includes DevBCA, whose substrate is not yet known.

In this work, we analyzed the relation of Alr2887 to proteins of the TolC family and
investigated its possible function. Alr2887 is required for deposition of the glycolipid layer of the heterocyst envelope, and it could have a role in the secretion of proteinaceous substrates involved in this process. We therefore designate the alr2887 gene hgdD (from heterocyst glycolipid deposition protein).

MATERIALS AND METHODS

Bacterial strains and growth conditions. This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, also known as *Nostoc* sp. strain 7120, and several mutant derivatives described in Table 1. The wild-type *Anabaena* sp. was grown photoautotrophically at 30°C in liquid BG110 medium (51) or A&A medium in a 1:4 dilution (A&A/4; 2). All Fox- strains were grown in medium containing 5 mM NH₄NO₃ and 5 mM TES-NaOH (TES, N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid) (pH 8). Cultures of mutant strains NMP-alr2887-GFP, NME-alr2887-GFP and NMA-alr2887 contained 2 µg of streptomycin and 2 µg of spectinomycin ml⁻¹; cultures of DR181 contained 50 µg neomycin ml⁻¹ (21, 47). Undiluted medium was solidified with 1.5 % agar (Difco, Heidelberg, G). Heterocyst formation was induced in liquid cultures by washing the cells 3 times with combined nitrogen-free medium and incubating in BG110 or A&A/4 under growth conditions. The growth on agar-solified medium was determined, without antibiotics, in standard BG11 medium, BG110 or BG110 supplemented with 4 mM NH₄Cl / 8 mM TES-NaOH (pH 8). The amount of biomass in a culture was estimated as the concentration of chlorophyll *a*, determined in methanolic extracts.
Genetic procedures. Transformation of *E. coli* strains, and isolation and manipulation of plasmid DNA were standard (53). PCR was done with the Triple master PCR System (Eppendorf, Hamburg, G.). Total DNA from *Anabaena* sp. was isolated as described (8).

Mutant DR181 was generated by transfer of pIM181 (Table 2) from *E. coli* to *Anabaena* sp. by triparental mating (62). Plasmid pIM181 was constructed as follows: the entire open reading frame (orf) of *alr2887* was amplified from the chromosomal DNA of strain *Anabaena* sp. by PCR using primers 309 and 310 containing *Xho*I restriction sites (Table 3). The resulting fragment of ca. 2,300 bp was cloned into pGEMT and the C.K3 cassette, encoding neomycin phosphotransferase (13), was inserted with *Xba*I ends between the 2 *Nhe*I sites of pIM125 deleting a 513-bp long internal fragment of *alr2887*. The mutagenized gene was cut from that construct (pIM136) by endonuclease *Xho*I ligated into the unique *Xho*I-site of positive selection vector pRL271 (5) resulting in pIM181. After conjugal transfer, putative double recombinants were obtained by the positive selection method described by Cai and Wolk (8). Double recombination and complete segregation of the mutated gene was confirmed by PCR using primers 309 and 310.

An internal 600-bp fragment of the *alr2887* coding region was amplified by PCR on genomic DNA of strain PCC 7120 using oligonucleotides containing *Bam*HI restriction sites (Table 3, NMΔ-*alr2887*-F, -R). The restricted PCR product was cloned into pCSV3 (Table 1) containing a Sp<sup>R</sup>/Sm<sup>R</sup> gene cassette leading to the plasmid pNMΔ-*alr2887*. The plasmid was amplified through transformation into *E. coli* DH5α and the sequence confirmed by conventional sequencing. Transformation of *Anabaena* sp. by conjugal transfer of pNMΔ-*alr2887* was performed as described (14) generating plasmid-integration mutants by single recombination (NMΔ-*alr2887* strain). Segregation of the mutants was confirmed by Southern blotting of the genomic DNA according to the standard procedure (53) using DIG-
labeled DNA generated by PCR using the NMA primers (Table 3) as probe (Roche, Mannheim, G).

A 500-bp fragment of the alr2887 orf encoding the C-terminus of the protein was amplified by PCR on genomic DNA of strain PCC 7120 using primers with Clal/EcoRV restriction sites (Table 3; NMP-alr2887-GFP-F, -R). The restricted PCR product was cloned into pCSEL21 (48) to generate an in-frame product with the gene encoding for GFP. The plasmid was amplified through transformation into E. coli DH5α and the sequence confirmed by conventional sequencing. Subsequently, the fusion construct was excised by restriction with EcoRI. The resulting fragment was cloned into pCSV3 cargo plasmid generating pNMP-alr2887-GFP, in which the orf for gfp is added to the last coding triplet of gene alr2887. Conjugation with Anabaena sp. was performed as described above resulting in single recombinants (NMP-alr2887-GFP strain), whose genetic structure was confirmed by PCR using primer GFP1 or GFP2 (confirming insert) and P4 (testing for wild type) in combination with P1.

The 800-bp upstream region of alr2887 including the first 8 codons of the coding sequence were amplified by PCR on genomic DNA of strain PCC 7120 using primers with Clal/EcoRV restriction sites (Table 3; NME-alr2887-GFP-F, -R.) The restricted PCR product was cloned into pCSEL21 in front of the gfp open reading frame. The fusion fragment was excised by digestion with PstI/EcoRI and ligated into cargo vector pCSEL24 resulting in pNME-alr2887-GFP. Conjugation to Anabaena sp. was performed as described above resulting again in single recombinants (NME-alr2887-GFP strain), whose genomic structure was confirmed by PCR using primer GFP2 in combination with primer NME-alr2887-GFP-F.

RNA isolation and analysis. Total RNA was isolated from cells of 100-ml cultures starved for nitrogen for different time intervals as previously described (56). Electrophoretical
separation in denaturing agarose gels and blotting to Hybond+ membranes (GE-Healthcare, Freiburg, G) was performed as previously described (44). RNA size markers from “Roche” were visualized in the gel by staining with ethidium bromide. After blotting by vacuum, the membrane was hybridized with a $^{32}$P-labeled probe. The probe was a 2,300-bp PCR fragment containing the orf of \textit{alr2887}, amplified on DNA of plasmid pIM125 as a template and oligonucleotides 309 und 310 as primers. Hybridization and visualization was done as described (44).

**Microscopic visualization.** Filaments of \textit{Anabaena} sp. were visualized with a standard reverse light microscope (DM1000, Leica, G.). Heterocyst-containing cultures were stained with 0.5% Alcian blue in 50% ethanol solution prior to microscopy.

Fluorescence imaging of the strains expressing N-terminal and C-terminal protein-GFP fusions was performed on a TCS SP2 or TCS PS5 Leica confocal microscope (Wetzlar, G.; HCX PLAN-APO 63x 1.4 NA oil immersion objective). All images were taken using the same microscope setting in order to compare intensities. GFP was excited at 488 nm line supplied by an argon ion laser. GFP fluorescence was analyzed by collection through the window of 500-570 nm, and \textit{Anabaena} autofluorescence was monitored by collection through the window of 630-700 nm (45).

The promoter activity was further determined by GFP fluorescence measurements of NME-\textit{alr2887}-GFP strain in comparison to wild type by excitation at 480 nm and recording the emission in a window between 500 and 570 nm (Perkin Elmer LS55, Germany). The integral of each spectrum was determined and corrected for background fluorescence obtained by the wild-type strain. The results of three independent measurements and three independent clones carrying the NME-\textit{alr2887}-GFP construct are presented.
**Cell fractionation and treatment.** Cells were grown in 3 l of BG11 medium supplemented with 10 mM NaHCO₃ (and 2 µg of streptomycin ml⁻¹ and 2 µg of spectinomycin ml⁻¹ for the mutants) and bubbled with a mixture of air and CO₂ (1%, v/v) up to about 0.6 µg Chl ml⁻¹. The cells were washed twice in BG11₀ medium and re-inoculated in 1.5 l of BG11₀ medium, once again supplemented with 10 mM NaHCO₃ (and 2 µg of streptomycin ml⁻¹ and 2 µg of spectinomycin ml⁻¹ for the mutants), and incubated under growth conditions for 2 or 3 days. Heterocysts from the wild type and the mutants were isolated essentially as described (28). Fractionation was performed as described previously (42, 43).

Cell-wall fractions (60 µl) were washed by incubation with 8 M urea, 0.1 M sodium-carbonate or 1 M sodium chloride for 30 min on ice followed by sedimentation of the membrane fraction at 256,000 x g, 4°C, 10 min. Pelleted membranes and remaining supernatant were subjected to polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunodecorated by antibodies against the outer membrane protein Alr2269 (Omp85) or against GFP (rabbit anti-GFP, IgG, Invitrogen, Oregon, USA).

Secreted proteins were isolated from 1-l cultures that had been grown in BG11 to about 0.6 µg Chl ml⁻¹ and incubated in the absence of combined nitrogen for 9 h. Protease inhibitor PMSF and protease inhibitor cocktail (Complete, Roche, Manheim, G; 1 tablet / 500ml) was added to the suspension, and the cells were pelleted by centrifugation for 5 min at 4,000 x g at RT. The supernatant was cleared by two rounds of centrifugation at 9,000 x g for 10 min at 4°C. Ammonium sulfate was added to the supernatant to a final concentration of 60% (w/v), and the resulting solution was incubated for 30 min. The precipitate was collected by centrifugation at 30,000 x g for 10 min at 4°C. The clear pellet was dialyzed over night against 3 M urea, 20 mM Tris (pH 7.4), and subsequently half of the sample was subjected to SDS-PAGE analysis.
Electron microscopy. Preparation of the samples for transmission electron microscopy was performed as described (21,22) except that EPON was used for embedding. In short: fixation with glutaraldehyde and KMnO$_4$, dehydration with increasing concentrations of ethanol, post staining with uranyl acetate and lead citrate. The samples were examined with a Zeiss EM10C microscope at 80 kV.

Lipid analysis. Lipid analysis was performed as previously described (21). In brief: lipids were extracted from filaments, isolated heterocysts and cell-wall fractions by addition of methanol–chloroform (1:2). The organic solvent was evaporated in a stream of nitrogen. Lipids were dissolved in 200 µl of chloroform and chromatographed on thin-layer plates of silica gel (Kieselgel 60 F$_{254}$, Merck, Darmstadt, G.) using 170 ml chloroform, 30 ml methanol, 20 ml acetic acid and 7.4 ml distilled water as running medium. Lipids were visualized by sprinkling with 25% sulfuric acid, and exposing the plate to 220°C, 30 s.

Nitrogenase activity. *Anabaena* sp. strains (Table 1) were grown photoautotrophically in a shaker in BG11 medium to a density of 3-5 µg Chl ml$^{-1}$ and induced by incubation in BG11$_0$ medium for 48 h. Nitrogenase activity was determined by the acetylene reduction assay under light (150 µE m$^{-2}$ s$^{-1}$) in an atmosphere of 14% acetylene in air or under anoxic conditions (24,57). For anoxic conditions, 10 µM DCMU was added to the cell suspension, and the flask containing the cells was sealed with a rubber stopper, bubbled with argon for 3 min and further incubated, under culture conditions, for 1 h before the assay was started by addition of acetylene. Ethylene production was found to be linear under these conditions for at least 1 h.

Homology modeling. According to the PHYRE server (http://www.sbg.bio.ic.ac.uk/~phyre/) TolC of *E. coli* (PDB:1EK9 (36)) is the best template for homology modeling of Alr2887.
The suggested alignment was manually adjusted and a homology model was built with Modeller v8.2 in a multiple templates approach (23,52). In Alr2887 the situation with the length of the two extracellular loops is the reverse of that in the crystal structure of TolC (1EK9). In order to avoid de novo modeling of long loops, we have taken the long loop of 1EK9 (aa257-278) as a template for the long loop in Alr2887 (supp. Fig. 1). Coiled coil prediction was performed by the REPPER server (30).

**Phylogenetic analysis.** Sequences of TolC-like proteins were collected (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein) and filtered for a maximal sequence identity of 95% with CD-HIT (38) revealing 178 sequences. Sequences with uncertain annotation were controlled by InterProScan (65). A multiple alignment was produced with MAFFT v5.861 (35) and IQPNNI v3.0.1 (41,58) was used to reconstruct a maximum likelihood phylogeny, assuming the WAG model (60) and constant rates across sites.

**RESULTS**

**Expression of hgdD.** The hgdD (alr2887) gene is up-regulated during nitrogen step-down (15,40). Transcripts of about 2.8 to 3 kb were detected with a labeled hgdD probe in RNA isolated after different time points of nitrogen starvation. A clear increase of the message after 6 and 9 hours and a relative decline after 12 hours were observed (Fig. 1A). Consistently, the activity of the hgdD promoter, tested with an N-terminal HgdD-GFP fusion (present in strain NME-alr2887-GFP), was highest in developing heterocysts, which were frequently found as doubles (Fig. 1B).
**HgdD is a TolC-like outer membrane protein.** A relation of HgdD (the Alr2887 protein) to proteins of the TolC family can be proposed based on amino acid similarity (40,42) or homology modeling (Fig. 2A). We identified HgdD as a TolC-like protein according to the analysis performed by the PHYRE server. In accordance with TolC structures, a homotrimer is proposed where each monomer contributes four transmembrane β-strands to the channel. The trimeric structure of the channel assembled by HgdD corroborates the results obtained by BN-PAGE analysis (42). Following this prediction, a model of HgdD containing large α-helical regions, which extend this channel deep into the periplasm towards the cytoplasmic membrane, was created (Fig. 2A). Constructing a phylogenetic tree of TolC-like proteins (Fig. 2B) indicated a clustering of HgdD into the group of the TolC-like proteins involved in secretion (3), especially with members of the CyaE group secreting cyclolysin (27).

To test the proposed outer membrane localization (42,43), we analyzed a C-terminal HgdD-GFP translational fusion that is expressed from the native hgdD promoter of the *Anabaena* chromosome (present in strain NMP-arl2887-GFP). By microscopic analysis, we did not observe a localization at the periphery of the cells as expected for an outer membrane protein, perhaps because of a low fluorescence signal of the exported protein (not shown). However, using isolated cell-wall fractions from NMP-arl2887-GFP, the outer-membrane localization of HgdD-GFP was confirmed by immunodecoration with αGFP antibodies (Fig. 2C, lane 1). The antibodies did not recognize any protein in *Anabaena* not containing a GFP tagged protein (not shown). To confirm the membrane insertion of the fusion protein, the cell-wall fraction was washed with 8 M urea (lane 2), 0.1 M sodium carbonate (lane 3) or 1 M sodium chloride (lane 4). The outer membrane protein Alr2269 (Omp85-like protein; 7,18) was detected in the membrane pellet independent of the treatment applied (Fig. 2C, lane 1-4, P). The same holds true for HgdD-GFP, which was only detected in minor amounts in the supernatant after incubation with 8 M urea. The migration of the HgdD-GFP fusion protein is
consistent with its calculated size of about 110 kDa. Therefore, the HgdD-GFP fusion is present in the cell wall and HgdD appears to be membrane inserted.

Mutants of hgdD do not form the glycolipid layer of heterocysts. Having assigned HgdD to the TolC protein family, we aimed to explore the function of the protein. Two independent approaches for site-directed mutagenesis of hgdD were performed. Strain DR181 resulted from partial deletion of the gene and insertion of cassette C.K3 into the gene by double recombination with the chromosome. In strain NMAΔ-alr2887, the pCSV3 plasmid was inserted into the chromosome at the hgdD locus by single homologous recombination. None of these insertions compromised the expression of the downstream orf alr2888 as determined by northern blot analysis (not shown). These insertions resulted in a clear Fox- phenotype (not shown), which confirms the finding for a transposon mutant of alr2887 (17,40). However, one of the mutants, NMAΔ-alr2887, was tested and found to develop nitrogenase activity when incubated under anoxic atmosphere. Under oxic conditions, the NMAΔ-alr2887 mutant showed a nitrogenase activity of about 0.07 nmol of ethylene/µg Chl·h compared to 5.32 nmol of ethylene/µg Chl·h in the wild-type Anabaena sp. However, when incubated in an anoxic atmosphere in the presence of DCMU to inhibit oxygen-evolving photosynthesis, the activity in the mutant increased 5 times to about 0.34 nmoles of ethylene/µg Chl·h while the wild type developed 6.42 nmol of ethylene/µg Chl·h. In prolonged anaerobic incubations in the presence of acetylene (1 to 3 days), the mutant released substantial amounts of ethylene, close to the levels produced by the wild type. This suggests that new nitrogenase was synthesized and accumulated during the time of anaerobic incubation.

The morphology of heterocysts in the mutant strains grown without combined nitrogen for two days was analyzed with respect to the presence of heterocyst-specific envelope layers (Fig. 3). In ultra-thin sections, the homogenous layer was clearly visible
encompassing the enlarged immature heterocyst. The laminated layer, consisting of glycolipids, was deposited on top of the outer membrane in the wild type, but could not be detected in any of the mutants (Fig. 3C and D, not shown for strain NMΔ-alr2887). The rearrangement of the inner thylakoid membranes and formation of the so-called honeycomb membranes did not occur in the mutants. Instead, the membranes dissolved to an irregular short confluent appearance. The number of glycogen granules increased strongly in both vegetative cells and heterocysts, which is an indication for depletion of combined nitrogen (16). Polar structures as in the heterocysts of the wild type were not made. The lack of the HGL layer leads to an early arrest in heterocyst differentiation of the hgdD mutants similar to that observed in HGL− mutant M7, which is defective in devA (21, 22).

**HgdD functions in the formation of the laminated layer.** HgdD could act on the formation of the laminated layer by different mechanisms. It could be involved in export of the HGLs, lipid moieties or enzymes needed for assembly of the constituents of the laminated layer. It could also influence the HGL synthesis, or the obtained phenotype could be pleiotropic because HgdD might be important for the biogenesis of the outer membrane per se. For the latter we did not find any evidence. No significant changes of the outer membrane proteome occurred as analyzed by SDS-PAGE (not shown), and whole-cell functions analyzed were not altered. For instance, we could not obtain a significant change of the uptake of amino acids like arginine, phenylalanine, glutamine, aspartic acid or glutamic acid (49) in the deletion mutant NMΔ-alr2887 (not shown).

In wild-type filaments, heterocyst-specific glycolipids accumulated to a detectable level 6 hours after induction of heterocyst formation and their amount increased with time (Fig. 4A). The synthesis of HGLs preceded the formation of the laminated layer, because 14 hours after nitrogen step-down this layer was not yet observed (Fig. 4B). The first evidence
for the HGL layer in electron micrographs was found 17 hours after nitrogen step-down (not shown) and the layer was fully developed at 20 hours (Fig. 4C). Interestingly, rather than following the presence of HGLs in lipid extracts, the appearance of the HGL layer took place after the production of HgdD as determined by the GFP intensity in N-terminal HgdD-GFP fusion strain NME-\textit{alr2887}-GFP (Fig. 4D). This observation would be consistent with HgdD being involved in HGL layer formation rather than in HGL production.

To further test this observation, we compared the lipid distribution of the wild-type strain PCC 7120 and mutant strains DR181 and NM\textDelta-\textit{alr2887}. All strains produced the major heterocyst-specific glycolipid (HGL1) of \textit{Anabaena} sp. (25,61) when incubated in combined nitrogen-free medium for 3 days (Fig. 5A), even though the amount of glycolipids was reduced in the mutant strains and only detectable in isolated heterocysts (Fig. 5A, lane 4 vs. lanes 6 & 7). The heterocyst-specific glycolipid 2 (HGL2) could also be detected in isolated heterocysts of the \textit{hgdD} mutants (lanes 6, 7). Because cell-wall lipids are synthesized at the plasma membrane (12), the reduction of the glycolipids could be interpreted as a defect in synthesis or in correct deposition into the cell wall. Discriminating between both possibilities can be achieved by the analysis of the lipid content of isolated cell walls. If the mutation causes a defect in synthesis, the amount of glycolipids detectable in heterocysts and cell walls should be comparable. In case of a defect in the deposition of glycolipids as a special envelope layer of the heterocyst, the lipids might accumulate in the plasma membrane after synthesis, but not any longer in the cell wall. Isolated cell walls from heterocysts of the wild-type \textit{Anabaena} sp. had a high content of HGL1 and also showed the spot for HGL2, indicating that the laminated layer of the heterocyst envelope co-purified with our cell-wall preparations. Analysis of the lipid content of the cell walls isolated from heterocysts of the mutants showed that the glycolipids were drastically reduced in them (Fig. 5A, lanes 9 and
Therefore, the heterocyst-specific glycolipids were produced but did not appear in the cell wall and did not assemble as a laminated layer.

**Altered protein secretion.** The phylogenetic analysis suggested that HgdD is a protein-secreting TolC-like protein (Fig. 2). To test this possibility, we compared the distribution of the proteins secreted from wild-type *Anabaena* sp. and the mutant strain NMA- *alr2887* after 9 h of nitrogen deprivation (Fig 5B). At this stage of development, HGLs are synthesized but the laminated layer is not yet deposited (Fig. 4). At least 3 proteins of high molecular weight, which were secreted from wild type, were not observed in supernatants of the mutant (Fig. 5B, white arrows). This observation, which was corroborated with samples from later time points (not shown), would be consistent with HgdD being involved in the secretion of specific proteins.

**DISCUSSION**

HgdD (Alr2887) was previously found in a proteomic analysis of cell walls from vegetative cells (42) and heterocysts (43) of *Anabaena* sp. suggesting a role of the protein in both types of cells. However, the expression of *hgdD* (*alr2887*) is up-regulated during nitrogen step-down (Fig. 1A, 4D; 15,40), especially in proheterocysts (Fig. 1B), suggesting a further or specific function in heterocyst differentiation. It has been reported that transposition of Tn5-1063 into this gene abolishes diazotrophic growth (17,40). Confirming the phenotype of the previously isolated transposon mutant, site directed insertional mutants of *hgdD* (strains DR181 and NMA-*alr2887*) showed a Fox- phenotype with abolished growth on N₂ and subsequent filament fragmentation (not shown).

In the *Anabaena* chromosome, the *hgdD* gene is part of a cluster of genes encoding proteins of unknown function (*alr2887, alr2888, alr2889, alr2890*) that are located immediately downstream of the genes *kaiA, kaiB* and *kaiC* (34). With an *hgdD* probe, a
transcript of about 2.8 to 3 kb is detected that can cover hgdD, its 5’ promoter region and alr2888 (Fig. 1A). A transcript of a similar size was detected with an alr2888 probe (results not shown). The latter probe also detected a transcript of about 1 kb that can cover alr2888 and alr2889. Whereas the 2.8 to 3-kb transcript was not observed with RNA isolated from the DR181 and NMAΔ-alr2887 mutants, the 1-kb transcript was present in these RNA preparations. Therefore, although alr2888 is co-transcribed with hgdD in wild-type Anabaena sp., an hgdD-independent transcription of alr2888 that is not impaired in the mutants also takes place. This makes it unlikely that the Fox− phenotype of the hgdD mutants results from a polar effect of the insertions in hgdD, although the possibility remains that Alr2888 and Alr2889 contribute to the same function as HgdD.

Growth on nitrate or ammonia is not affected in the hgdD mutants, suggesting that HgdD has not an essential role of in vegetative cells under standard laboratory conditions (not shown). An hgdD mutant exhibits a Fox− Fix+ phenotype suggesting that these type of mutants are unable to provide the micro-oxic environment necessary for nitrogenase activity. This phenotype has also been described for mutants with aberrant heterocyst envelope (17,46) or mutants affected in respiration like the cox2/cox3 double mutants defective in heterocyst-specific terminal respiratory oxidases (57). Even though the hgdD mutants show normal synthesis of the HGLs, they cannot form the laminated HGL layer (Fig. 3, 5). This phenotype could be explained by a reduction of the incorporation of the HGLs into the heterocyst-specific envelope that is external to the gram-negative cell wall. The maximal expression of HgdD fits between the developmental stages of accomplished synthesis of the glycolipids and appearance of the laminated layer (Fig. 4). As predicted by protein modeling and phylogenetic clustering, HgdD belongs to the TolC family of outer membrane proteins (Fig. 2). TolC forms a trimeric 12-stranded β-barrel in the outer membrane and spans the periplasmic space by forming a α-barrel, and our results are consistent with a cell-wall localization of HgdD (Fig.
2C). The TolC-like proteins are commonly involved in the export from the cells of gram-negative bacteria of various molecules like drugs and proteins (37,50,54,55), and HgdD specifically associates in phylogenetic analysis with the protein-secretion TolC-like proteins (Fig. 2B). We have observed changes in the pool of secreted proteins of an hgdD mutant (Fig. 5), which would be consistent with HgdD belonging to the protein-secreting TolC-like proteins. The proteins that are not secreted in the hgdD mutant remain to be identified, and the possibility that they are not secreted because of an indirect effect of the mutation on development should also be explored.

Outer membrane efflux proteins like TolC function together with a traffic ATPase, also known as ABC transporter (37,50,54,55). The phenotype of the hgdD mutants is remarkably similar to that of the mutants defective in the DevBCA traffic ATPase (21,22). Mutants in devA (alr3712), devB (alr3710) or devC (alr3711) fail to deposite the laminated layer, even though they are not impaired in HGL synthesis. DevA represents an ATP binding cassette and DevC a membrane channel of an ABC transporter. Thereby they are similar to the plasma membrane localized protein translocase HlyB (21,22,37,39), which combines these two domains (supp. Fig. 1). Therefore, a 1:1 stoichiometry between DevC and DevA is expected (Fig. 6). DevC contains an additional domain (amino acid 40 to amino acid 254), which is similar to the periplasmic exposed region of AcrB from E. coli (amino acid 590 to amino acid 869; ref. 11), which is not found in HlyB. By similarity, this domain could present a contact site for the periplasmic membrane fusion protein DevB. This protein shows similarity to HlyD (37) in the proposed N-terminal transmembrane domain, which is not present in AcrA, and its HlyB docking region (Fig. 6, supp. Fig. 1). One difference to HlyD is remarkable: DevB contains an extension of the coiled coil domain with a periodicity of 18 as predicted by the REPPER server. According to the nomenclature of coiled coil domains, it might be a hemaglutinin-like domain (29). The estimated length of this additional coiled coil
system is in the range of 12-15 nm assuming a dimeric antiparallel coiled coil domain (Fig. 6). Hence, DevB and HgdD together would be able to bridge a distance of about 30 nm, which corresponds to the estimated distance between outer and plasma membrane of Anabaena sp. Therefore, HgdD is a good candidate for a component of a complex together with DevBCA, which bridges the two membranes in order to export molecules essential for formation of the laminated layer of the heterocyst envelope.

Homologues of devB (hgdB, all5347) and devC (hgdC, all5346) that are involved in temporal and spatial aspects of HGL deposition have been described (19). However, their cell walls are different from the one described here, since the laminated layer is found even though aberrantly distributed (19). HglK, a protein with unknown function and putative membrane localization is directly or indirectly involved in deposition of the HGLs (6). Mutants in hglK develop thylakoid distensions that could have accumulated non-exported glycolipids. Therefore, the phenotype of the hgdD mutants described here resembles the phenotype of mutants in the DevBCA exporter rather than of other hgl or hgd genes.

To summarize, the outer membrane-localized TolC-like protein HgdD may have a non-essential constitutive function, but is essential during nitrogen starvation. The mutant phenotype and sequence homology supports a TolC-like function of HgdD in export of enzymes involved in the assembly of the laminated layer outside of the gram-negative outer membrane in the heterocyst envelope. In addition to the three proteins observed to be absent in the secretome of the mutant (Fig. 5), All2736, a protein of unknown function, might also be a substrate for HgdD. The all2736 gene is induced similarly as hgdD (15), and All2736 has previously been found to co-migrate in a blue native PAGE with HgdD (42).
ACKNOWLEDGMENTS

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REFERENCES


**FIGURE LEGENDS**

Fig. 1. HgdD is up-regulated in proheterocysts upon nitrogen step-down. (A) RNA was isolated from NH$_4^+$ grown cells (lane 1) and from cells that had been starved for combined nitrogen for 6, 9 or 12 hours (lanes 2-4). Samples had 30 µg RNA. The blots were successively hybridized with $^{32}$P labeled probes of *hgdD* (upper panel) and *rnpB* (lower panel), which was used as a loading and transfer control, respectively. Sizes of RNA standards are indicated on the left. (B) The N-terminal HgdD-GFP translational fusion in strain NME-*alr2887*-GFP was visualized by confocal microscopy. The cyanobacterial auto-fluorescence (AUF.), GFP fluorescence (GFP), the overlay of both signals (overl.) and the bright field image (BFI) of the NME-*alr2887*-GFP strain 16 hours after nitrogen step-down is shown.

Fig. 2. The sequence of HgdD is related to TolC proteins. (A) A model of HgdD was generated as described in the text. It shows structural homology to the outer membrane protein TolC. Side, top, and bottom views are given. Further details are posted as supplemental material. The coordinates are available upon request. (B) The tree of 178 sequences of the TolC family (for details see supp. Figure 2) represents a maximum likelihood phylogeny, assuming the WAG model and constant rates across sites. It is based on a multiple alignment. Scale '0.1' indicates 0.1 amino acid substitutions. The functional categories were assigned according to (3). (C) Isolated cell walls from lysed heterocysts (lane 1) were treated with 8 M urea (lane 2), 0.1 M sodium carbonate (lane 3) or 1 M NaCl (lane 4). The pelleted membrane fraction (P) or the supernatant (S) were probed with antibodies against the outer membrane protein Alr2269, which was used as a control, or GFP. The migration of some molecular weight markers (size in kDa) is indicated on the left side for the pellet fraction probed with αGFP.

Fig. 3. The ultra structure of heterocysts of *hgdD* mutants. Transmission electron micrograph of ultra thin sections of a connection of a heterocyst and a vegetative cell of the wild type *Anabaena* sp. (A) and the mutant DR181 (C) are shown. Magnifications of the cell wall and heterocyst envelope, indicated by squares in (A) and (C), are shown for the wild type (B) and DR181 (D). In (A), the empty white space surrounding the cell wall results from dehydration of the protoplast during sample preparation. Scale bar shows 1 µm for A and C. GL, laminated layer; P, homogenous layer, CM, cytoplasmic membrane, OM, outer membrane.

Fig. 4. The progress of HGL synthesis and HGL layer formation. (A) TLC of lipids of the wild type *Anabaena* sp. before (lane 1) or at indicated times after transfer to BG11$_0$ is shown (lanes 2-7). Representative transmission electron micrographs of ultra thin sections of filaments of wild type *Anabaena* sp. 14 hours (B) or 20 hours (C) after transfer to BG11$_0$ are depicted. The arrow in C points...
to the HGL layer seen after 20 hours. The bar indicates 1 μm. (D) The fluorescence of the NME-alr2887-GFP strain was determined and the difference to the background of the wild-type strain of three independent measurements and three independent NME-alr2887-GFP clones is shown for the indicated times after nitrogen step-down.

Fig. 5. The hgdD mutants synthesize the heterocyst-specific glycolipids. (A) TLC of lipids of the wild type Anabaena sp. (lanes 1, 3, 5, 8), the mutant NMA–alr2887 (lane 2, 4, 6, 9) and the mutant DR181 (lane 7, 10) is shown. Lipids were extracted from filaments grown in BG11 (lane 1, 2), filaments 3 days after transfer to BG11₀ (lanes 3, 4), isolated heterocysts (lanes 5, 6, 7) or isolated heterocyst cell walls (lanes 8, 9, 10). The lipids of the cell wall fractions show a slightly altered migration behavior due to edge effects of the TLC plate. The HGL lipids are indicated by arrows: open arrows for HGL₁ and grey for HGL₂. In lanes 9 and 10 the area where HGL₂ would be expected is also indicated. (B) Proteins secreted from the wild type (wt) or NMA–alr2887 mutant (Δ) 9 hours after nitrogen step-down were concentrated and subjected to SDS-PAGE followed by silver staining. The molecular weight standards are indicated on the left, and proteins not secreted from the mutant are indicated by white arrows.

Fig. 6. A proposed functional arrangement of HgdD. The structural composition of the HgdD / DevABC complex is shown as discussed. HgdD was modeled as in Fig 2A, DevA using MJ0796, a bacterial ATP binding cassette (PDB:1L2T), as template and sections of DevB using MexA (PDB:1VF7) as template. The pink boxes represent an extension of the two-helix coiled coil of DevB to bridge the periplasm and attached to TolC. The circle with question mark documents that further elements stabilizing the complex might exist. OM, outer membrane; PM, cytoplasmic membrane.
# Tables

## TABLE 1. *Anabaena* strains used in this study

<table>
<thead>
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<th>Resistance</th>
<th>Genotype</th>
<th>Relevant Properties</th>
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<td><em>alr</em>2887::C.K3</td>
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<td>Fox&lt;sup&gt;+&lt;/sup&gt;, Het&lt;sup&gt;+&lt;/sup&gt;, Hgl&lt;sup&gt;+&lt;/sup&gt;, Hen&lt;sup&gt;-&lt;/sup&gt;</td>
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<tr>
<td>NMP-<em>alr</em>2887-GFP</td>
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<td><em>alr</em>2887::gfp</td>
<td>C-terminal GFP-protein fusion</td>
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<tr>
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<td>Sp&lt;sup&gt;+&lt;/sup&gt;/Sm&lt;sup&gt;+&lt;/sup&gt;</td>
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## TABLE 2. Plasmids used in this study

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<td>pCSV3</td>
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**TABLE 3. Primers used for cloning**

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Figure 1: Moslavac et al.
Figure 2: Moslavac et al.
Figure 3: Moslavac et al.
Figure 4: Moslavac et al.
Figure 5: Moslavac et al.
Figure 6: Moslavac et al.