The proteome of the heterocyst cell wall in *Anabaena* sp. PCC 7120

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Abstract

Anabaena sp. PCC 7120 is a filamentous cyanobacterium that serves as a model to analyze prokaryotic cell differentiation, evolutionary development of plastids, and the regulation of nitrogen fixation. The cell wall is the cellular structure in contact with the surrounding medium. To understand the dynamics of the cell wall proteome during cell differentiation, the cell wall from *Anabaena* heterocysts was enriched and analyzed. In line with the recently proposed continuity of the outer membrane along the *Anabaena* filament, most of the proteins identified in the heterocyst cell-wall fraction are also present in the cell wall of vegetative cells, even though the lipid content of both membranes is different.

Keywords: *Anabaena*; β-barrel protein prediction; heterocyst; outer membrane; proteome analysis.

Introduction

The filamentous cyanobacterium *Anabaena* sp. PCC 7120 represents a model system for genetic and physiological studies of nitrogen fixation and prokaryotic cell differentiation (Wolk et al., 1984). When fixing N_2, the filament is composed of two different cell types, vegetative cells that host a thylakoid membrane system and perform oxygenic photosynthesis, and heterocysts that fix N_2. This cyanobacterium is also a model system for investigations of plastid evolution (Ertel et al., 2005; Bredemeier et al., 2007) because the gene content of plastomes is more closely related to bacteria of the genus *Nostoc*, e.g., *Anabaena* sp. PCC 7120, than to bacteria of the genus *Synechocystis*, e.g., *Synechocystis* sp. PCC 6803 (Martin et al., 2002). Generally, cyanobacterial cells are surrounded by two membranes, the plasma membrane and the outer membrane (Stanier and Cohen-Bazire, 1977). These are 30–40 nm apart and separated by the peptidoglycan layer. Recently it was suggested that in filamentous cyanobacteria the outer membrane is continuous along the filament (Flores et al., 2006), whereas the plasma membrane surrounds individual cells. The heterocyst cell wall carries an envelop that is external to the outer membrane and is composed of an inner glycolipid layer and an outer polysaccharide layer (Adams, 2000; Flores et al., 2006). The inner laminated layer, built of special glycolipids (hgl, heterocyst glycolipid), surrounds the outer membrane of the heterocyst and represents the actual barrier for gases. The layer consisting of heterocyst-specific polysaccharides (hep, heterocyst envelope polysaccharide) surrounds the laminated layer.

Based on the observed continuity of the outer membrane, metabolite exchange between vegetative cells and heterocysts through the periplasmic space was proposed (Flores et al., 2006). The consequence of this notion is a physiologically active periplasm, with major proteomic differences expected in the plasma membrane of the two cell-types. However, the formation of the heterocyst-specific glycolipid and polysaccharides layer (Adams, 2000) suggests a cell-type specific change in the outer membrane as well. Therefore, an outer membrane continuum raises several important questions: How are the specific glycolipids restricted to heterocysts? Are lipids or proteins of the outer membrane exchangeable between the cells of the filament? Is the outer membrane proteome of the two different cell-types comparable? To explore the latter question, the cell wall properties of vegetative cells and heterocysts have to be compared. As a first step, the proteome of vegetative cells of *Anabaena* sp. PCC 7120 was analyzed (Moslavac et al., 2005a). We then fractionated isolated heterocysts and analyzed the cell wall to develop a biochemical tool to investigate the adaptation after cell differentiation. We subsequently compared the lipid distribution and protein content of the two membranes and discuss the consequences.

Results

Isolation of cell wall fractions from heterocysts

First, we aimed to established nOmp85 (Alr2269; Ertel et al., 2005; Moslavac et al., 2005b; Bredemeier et al., 2007) as a cell wall-marker for both heterocysts and vegetative cells, because no heterocyst-specific outer membrane protein is known to date. nOmp85 was found in vegetative cells (Ertel et al., 2005) and the gene is expressed independently of the nitrogen source, as determined by microarray analysis (Ehira and Ohmori, 2006). The protein

contains a classical targeting signal for translocation via the Sec translocon (Moslavac et al., 2005a). The NMP-alr2269-GFP strain encodes nOmp85 with a C-terminal green fluorescent protein (GFP) attached by a GATatcCAT linker. Analysis of the GFP fluorescence (Figure 1, GFP and overlay) indicates that nOmp85 is present in both vegetative cells (Figure 1; identified by autofluorescence) and heterocysts (Figure 1; identified in bright field image). The ring-like fluorescence (Figure 1, enlargement) confirms the presence of GFP on the periphery of the cell, which is consistent with an outer membrane localization. However, the cell wall localization results in a weak fluorescence signal. For this reason, the GFP signal is only obtained in the focal plane and not in cells out of focus, as found for the cyanobacterial autofluorescence (Figure 1). The outer membrane localization of the protein was confirmed by Western blot analysis (data not shown). This observation confirmed the suitability of the use of nOmp85 antibodies for the quality control of heterocyst membranes.

Next, heterocysts were isolated by destruction of vegetative cells (Golden et al., 1991) of filaments (Figure 2A, heterocysts visualized by Alcian blue staining) grown for 48 h in $BG11_0$. The isolation of heterocysts was initially confirmed by microscopy (Figure 2B). The proteome of isolated heterocysts and vegetative cells differed significantly, as documented by SDS-PAGE analysis (Figure 2C, lanes 1 and 2), supporting the purity of the heterocyst fractions. Furthermore, heterocysts contain a modified inner membrane system that lacks components of the oxygen-evolving photosystem II, such as D1 (Baier et al., 2004). Immunodecoration of isolated vegetative cells and heterocysts with D1 antibodies (Figure 2C; lane 1, 2 bottom) confirmed the absence of this protein in the heterocyst fraction and thus the purity of these cells. Analysis of the lipid content of cells grown on BG11 or $BG11_0$ (Figure 2D, lane 1, 2) and isolated heterocysts (Figure 2D,
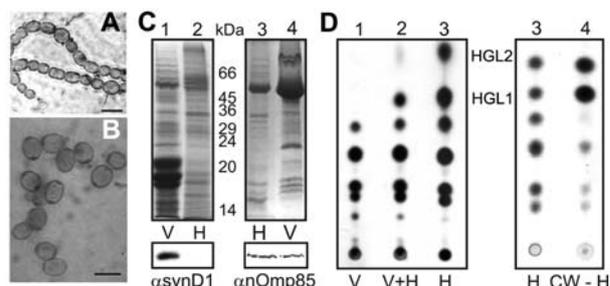
Figure 2   Heterocyst and heterocyst cell wall isolation.
(A) Filaments of *Anabaena* sp. PCC 7120 grown in $BG11_0$ were stained by Alcian blue and visualized by light microscopy. The scale indicated represents 5 μm. (B) Isolated heterocysts were analyzed by light microscopy after Alcian blue staining. The scale indicated represents 5 μm. (C) Samples (10 μg of protein) of vegetative cells (lane 1), heterocysts (lane 2), cell wall of heterocysts (lane 3) or cell wall of vegetative cells (lane 4) were subjected to SDS-PAGE, followed by Coomassie Blue staining or immunodecoration with D1 (lanes 1 and 2; bottom) or nOmp85 antibodies (lanes 3 and 4; bottom). (D) The lipid content in vegetative cell (lane 1), entire filament (lane 2), heterocyst (lane 3) and heterocyst cell wall fraction (lane 4) was analyzed. HGLs are indicated.

lane 3) documented the enrichment of the heterocyst-specific glycolipids (HGL1 and HGL2).

For fractionation of the nitrogen-fixing cells, isolated heterocysts were broken by extensive sonication because they remain intact during mild treatment (Figure 2B). After fractionation, the cell wall fraction was isolated, as monitored by immunoassay with nOmp85-specific antibodies (Figure 2C, lanes 3 and 4; bottom). The protein content of the cell wall is largely comparable to that of the cell wall of vegetative cells (Figure 2C, lanes 3 and 4) although a few different bands are observed. Comparison of the lipids present in heterocysts (Figure 2D; lane 3) and in the cell wall fraction of heterocysts (Figure 2D; lane 4) shows enrichment of the heterocyst-specific glycolipids in the cell wall fraction. We therefore conclude that we have isolated a cell wall fraction of heterocysts.

Analysis of the proteome

The proteomic composition of different heterocyst cell-wall fractions was analyzed using two strategies: peptide mass fingerprint analysis and peptide sequencing (Tables 1 and 2). For comparison, cell wall fractions prepared from vegetative cells were analyzed as well (Table 1, column 6, a). A total of 18 proteins could be identified in the fraction from heterocysts, 13 of which were also identified in the cell wall fraction of vegetative cells (Table 1). The function and localization of these proteins were previously discussed (Moslavac et al., 2005a). However, the remaining five proteins might represent heterocyst-specific proteins. Indeed, NifH2 (Table 1, protein 17) is likely, as NifH (Elhai and Wolk, 1990), a heterocyst-specific protein, and the level of NtcA (Table 1, protein 4) is enhanced in heterocysts compared to vegetative cells (Olmedo-Verd et al., 2005). However, both proteins likely represent cytosolic contamination. All1427 (Table 1, protein 15) contains two cystathionine-β synthase domains (CBS), which are usually in tandem repeats and are found in
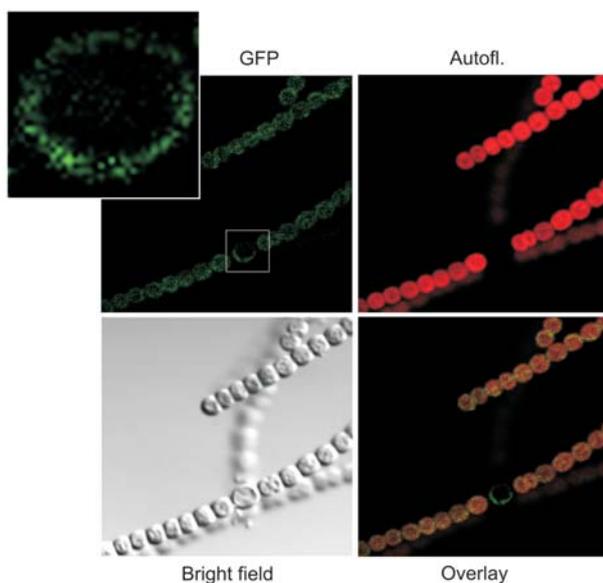

Figure 1   Expression of an Alr2269-GFP fusion.
GFP signal, autofluorescence, bright field image and overlay analysis of strain NMP-alr2269-GFP. One heterocyst signal is enlarged (left-upper corner).

Table 1   Proteins identified in the outer membrane fraction of heterocysts.

No.	Accession number	Predicted function	Exp.	Peptides	Found in VC	Mol. mass (kDa) pre/mat	Up-regulation
1	alr4550	Probable porin	MF/PS	21 (173)/12	a/b	60.5/57.8	-
2	all4499	Probable porin	MF/PS	22 (173)/9	a/b	58.5/55.4	-
3	alr0834	Porin	MF/PS	13 (88)/5	-/b	54.3	+
4	alr4392	Nitrogen-responsive regulatory protein NtcA	PS	6	-/-	24.9	+
5	alr2269	nOmp85	PS	8	-/b	89.5/87.8	-
6	alr0397	Similar to ferric aerobactin receptor	PS	7	-/b	94.2/90.2	-
7	all3984	Predicted protein	MF/PS	12 (108)/4	-/b	24.7/21.0	+
8	alr5055	Hypothetical protein	PS	3	-/-	21.6/19.02	+
9	alr2887	Outer membrane efflux protein	MF/PS	11 (85)/3	a/b	80.7/78.0	+
10	all1861	Peptidoglycan-binding protein	MF/PS	12 (90)/4	a/b	28.4/27.1	+
11	all3585	Hypothetical protein	MF/PS	7 (69)/3	a/b	29.4/25.7	-
12	all0089	Hypothetical protein	MF/PS	6 (65)/3	-/b	55.8	+
13	all0268	Predicted Zn-dependent hydrolases	MF/PS	5 (57)/2	-/b	27.3/24.2	-
14	all4388	Involved in polysaccharide export	MF/PS	9 (67)/4	-/b	51.5/48.0	+
15	all1427	Hypothetical protein	PS	3	-/-	21.6	+
16	alr0668	Hypothetical protein	PS	4	-/-	23.0	+
17	alr0874	Nitrogenase reductase NifH2	PS	3	-/-	32.6	+
18	all3826	*N*-Acetylmuramoyl-L-alanine amidase cwlL precursor-like	PS	3	-/b	25.4/22.3	-

Data presented are ORF numbers (column 2; Kaneko et al., 2001), predicted function (column 3), experiment in which the protein was detected (column 4; MF, mass fingerprint; PS, peptide sequencing), number of peptides detected (column 5) and detection score for MF, detection in cell wall of vegetative cells here (a) or previously (b) (Moslavac et al., 2005a; column 6), molecular mass (column 7), and significant up-regulation of gene expression (column 8; according to Ehira and Ohmori, 2006).

cytosolic and membrane proteins with diverse functions (Ignoul and Eggermont, 2005). Alr5055 (Table 1, protein 8) contains a domain found in proteins involved in spore germination. It was speculated that the homolog of Alr5055 in *Bacillus subtilis*, GerM, has a role in peptidoglycan synthesis during sporulation (Slynn et al., 1994). For Alr0668 (Table 1, protein 16), no significant homology to a protein or domain could be identified.

We next analyzed the five heterocyst-specific proteins for putative membrane localization using bioinformatics programs. Helical membrane segments could not be identified by TMHMM (data not shown). Next, we compared predictions by Signal P (Bendtsen et al., 2004), P-Sort (Gardy et al., 2005), our own program based on the β-barrel score (BBS275; Mirus and Schleiff, 2005), MCMBB (Bagos et al., 2004a) and Pred-TMBB (Bagos et al., 2004b; data summarized in Table 2). For comparison, we show the results for nOmp85, a protein clearly localized in the outer membrane (Figure 1). The results confirm the cytosolic localization of NifH2 and NtcA (Table 2)

and suggest a cytosolic localization of the CBS-containing protein All1427. The localization of Alr5055 (protein 8) and Alr0668 (protein 16) is not reliably predictable. The presence of a secretion signal (Table 2) and the selection by two programs favors Alr5055 as being localized or attached to the outer membrane. For Alr0668, only our program suggests a β-barrel fold, except for the 0% false-prediction method (Table 2), which also suggests that Alr0668 is soluble.

When expression of the genes encoding the identified proteins was inspected according to Ehira and Ohmori (2006), we found that nine genes belong to the class induced as a starvation response or up-regulated during proheterocyst formation and four to the class specific for heterocysts (Figure 3; Ehira et al., 2003), considering the significance of the changes obtained. For *alr*2887 this up-regulation was independently observed by analyzing expression in the T22 mutant (Maldener et al., 2003). Most of the cytosolic proteins identified (3 out of 4) belong to the latter group. Remarkably, the genes encod-

Table 2   Predicted localization of proteins not identified in the cell wall of vegetative cells and of Alr2269.

No.	Acc. no.	Signal P 3.0	Loc	BBS275	dep/ind.	0%	MCMBB	Pred-TMBB	Final
4	alr4392	–	Cyt	0.00	-	-	-0.044	–	–
5	alr2269	ANA-QT (18)	OM	2.79	+	+	0.033	19	OM
8	alr5055	TVA-GG (26)	OM/PP	0.03	-	-	0.002	2	?
15	all1427	–	Cyt	0.03	-	-	-0.038	–	–
16	alr0668	–	Cyt	1.37	+	-	-0.031	–	?
17	alr0874	–	Cyt	0.05	-	-	-0.065	–	–

Data presented are ORF numbers (column 2), putative cleavage site (column 3; Bendtsen et al., 2004), Psortb classification (column 4; Gardy et al., 2005), β-barrel score 275 (column 5; Mirus and Schleiff, 2005), selection by the dependent/independent (column 6; Mirus and Schleiff, 2005) or 0% false-prediction method (column 7; Mirus and Schleiff, 2005), MCMBB score for mature proteins (selection >0; column 8; Bagos et al., 2004a) and number of transmembrane β-strands predicted by Pred-TMBB (column 9; Bagos et al., 2004b). Column 10 shows the final conclusion (cyt, cytosol; PP, periplasm; -, no selection/prediction; ?, no reliable prediction possible).
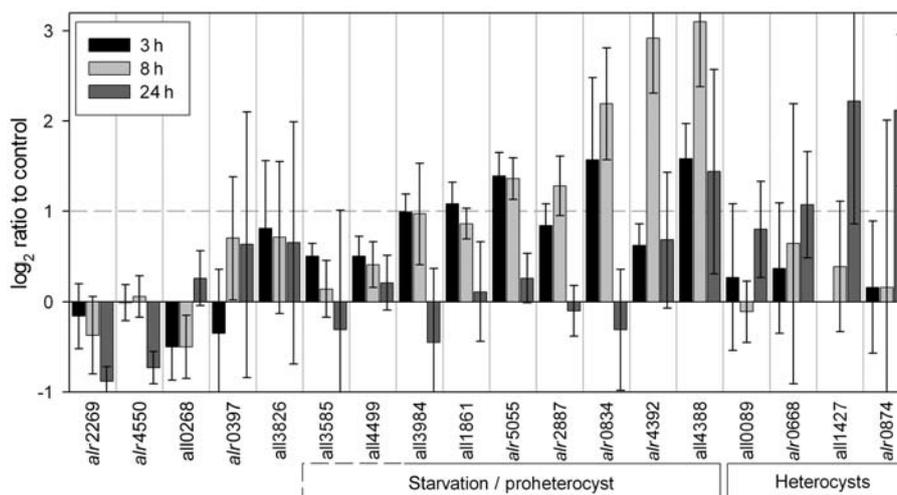
Figure 3 Expression of genes encoding proteins identified in the heterocyst outer membrane fraction.
Log$_2$ ratio of expression at indicated times after nitrogen step-down in comparison to untreated cells. Values are taken from Ehira and Ohmori (2006). Classification into the three groups is according to Ehira et al. (2003) and the gray dashed line shows the two-fold change range.

ing Alr2269 and Alr4550 are down-regulated upon nitrogen step-down (Figure 3). However, these two proteins were among the most abundant in vegetative cells (Moslavac et al., 2005a) and down-regulation of their gene expression does not preclude the appearance of these proteins. Analysis of the expression of the genes coding for the β-barrel outer membrane proteins identified in proteomic analysis of vegetative cells but not of heterocysts shows that four genes are up-regulated. However, only two genes, all4026 and alr2588, showed significant up-regulation after 24 h. Nevertheless, the analysis of gene regulation was performed using entire filaments (Ehira and Ohmori, 2006) containing approximately ten-fold more vegetative cells than heterocysts (Adams, 2000). Hence, this analysis supports the idea of functional modification of the outer membrane proteome, but does not exclude the possibility that some of the changes in gene expression observed occur in vegetative cells rather than in heterocysts.

Discussion

A GFP fluorescence signal was obtained on the periphery of the cells with the Alr2269-GFP fusion analyzed in this work. Although the fluorescence signal was relatively weak, this observation is fully consistent with the location of Alr2269 in the outer membrane (Ertel et al., 2005; Moslavac et al., 2005b; Bredemeier et al., 2007). Because Alr2269 bears a signal peptide for export through the Sec translocation system, which is known to hardly render active GFP in the periplasm (Feilmeier et al., 2000; Thomas et al., 2001), weak fluorescence might result from poor maturation of GFP in the periplasm of the cyanobacterium. It is thought that disulfide bond formation during protein folding in the periplasmic space of non-filamentous bacteria causes the inactivation of GFP fluorescence. Hence, it might be suggested that the reducing capacity in the periplasmic space of Anabaena sp. PCC 7120 is different from that of non-filamentous bacteria. This notion is in line with a more active meta-

bolic role in Anabaena than in other bacteria (Flores et al., 2006) requiring a different environment or a higher concentration of chaperones to allow significant folding of GFP. Alternatively, Alr2269 might be massively exported so that some GFP molecules fold properly, as GFP folds within seconds (Jackson et al., 2006). Nonetheless, it appears that folding of a Sec-translocated GFP takes place in the periplasm of Anabaena.

Using D1 as a marker for vegetative cells (Baier et al., 2004) and Alr2269 as a marker protein for outer membrane fractions (Figure 1; Ertel et al., 2005), we developed a protocol to isolate cell wall fractions from the nitrogen-fixing heterocysts. Approximately 50% of the proteins identified can clearly be assigned as outer membrane proteins, which is similar to the value previously reported for preparation of the cell wall fraction of vegetative cells (Moslavac et al., 2005a). All outer membrane proteins, with the possible exception of Alr5055, were identified in the cell wall of vegetative cells, suggesting similar proteome functionality of the two regions of the continuous outer membrane. In contrast, proteins with a highly probable cytosolic localization are not found in vegetative cell preparations and are products of genes up-regulated during nitrogen starvation (Figures 3 and 4; Ehira and Ohmori, 2006).

Although we cannot exclude the possibility that some proteomic alterations might have escaped our analysis, no significant amount of heterocyst-specific outer membrane proteins seems to exist, which is in line with continuity of the outer membrane along the filament (Flores et al., 2006). The difference in the number of proteins observed in cell wall fractions from vegetative cells (Moslavac et al., 2005a) and heterocysts (Table 1) could support the notion that not all outer membrane proteins of the heterocyst cell wall were identified, as large-scale mass spectrometry approaches favor the detection of abundant and non-hydrophobic proteins (Eichacker et al., 2004) and is dependent on the methodology used (Moslavac et al., 2005a). Alternatively, this observation might point to a higher diversity of the outer membrane proteome of vegetative cells. However, it cannot be
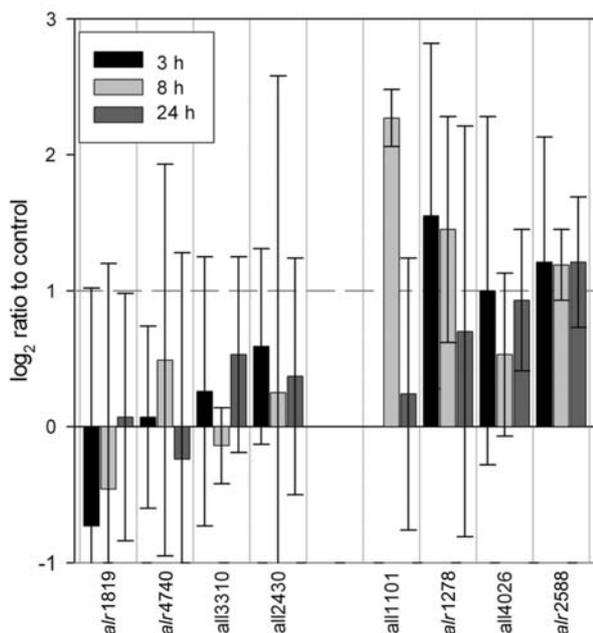
Figure 4 Expression of genes encoding outer membrane proteins identified in the outer membrane fraction of vegetative cells.

Log$_2$ ratio of expression at indicated times after nitrogen step-down in comparison to untreated cells for genes encoding for outer membrane-inserted β-barrel proteins identified in Moslavac et al. (2005a). Values are taken from Ehira and Ohmori (2006). The gray dashed line shows the two-fold change range.

excluded that the cell wall fraction of vegetative cells was contaminated by heterocysts, as heterocyst induction could have occurred as a result of cyanobacterial growth in a dense culture. However, heterocysts show low abundance when filaments are grown in the presence of NO$_3^-$ and are robust against the solubilization strategy used for vegetative cells (Figure 2). In addition, up-regulation of the expression of genes coding for outer membrane proteins, resulting in increased protein levels during heterocyst formation, might be an alternative to the insertion of novel proteins to regulate the functionality of the outer membrane (Table 1, Figures 3 and 4). For instance, the presence of the Omp85 homolog Alr2269 in the outer membrane of both cell types has been experimentally confirmed (Ertel et al., 2005; Bredemeier et al., 2007; Figure 1). Furthermore, the involvement of gene products in proheterocyst or heterocyst formation (e.g., *alr*4388; Maldener et al., 2003) does not necessarily imply that the protein has no function in vegetative cells, even though this function might not be essential. HetR, for example, seems to be essential for heterocyst differentiation (Herrero et al., 2004), but is also expressed in nitrate-grown cells (Ehira and Ohmori, 2006). However, the presence and function of gene products essential for heterocyst development in vegetative cells has to be confirmed in the future.

Materials and methods

Bacterial strains and culture conditions

Segments of 500 bp of the *alr*2269 open reading frame encoding the C-terminus of the Alr2269 protein were amplified from

genomic DNA of *Anabaena* sp. PCC 7120 (also termed *Nostoc* sp. PCC 7120) by PCR using primers with ClaI/EcoRV restriction sites (alr2269-Cterm-GFP-F, 5′-ATC GAT CGA TAC GGG AGA AGA TGA CTT ATT ACT AG-3′; alr2269-Cterm-GFP-R, 5′-ATC GGA TAT CAA ACC TTT CTC CAA TAC CGA AAT TGA T-3′). The restricted PCR product was cloned into pCSEL21 (Olmedo-Verd et al., 2006). The C-terminal GFP fusion construct generated was excised by restriction with EcoRI and cloned into pCSV3 (Olmedo-Verd et al., 2006) to yield pNMP-alr2269-GFP. The plasmid was transferred to *Anabaena* sp. PCC 7120 by conjugation (Elhai and Wolk, 1988) to generate the NMP-alr2269-GFP strain. Single recombinants were selected on the basis of Sp/Sm resistance conferred by the C.S3 cassette.

Cyanobacteria were grown photoautotrophically at 30°C in BG11 medium under illumination at 70 μmol photons m^{-2} s^{-1} and bubbling with air containing 1% CO$_2$. Vegetative cells were fractionated as previously described (Moslavac et al., 2005a). To induce heterocyst formation, cells were collected at approximately 25 μg chlorophyll *a* ml^{-1}, centrifuged at 3000 *g* for 10 min at room temperature, washed twice with BG11$_0$ medium and further grown in nitrate-free BG11$_0$ medium.

Heterocyst isolation and fractionation

Heterocysts were isolated according to Golden et al. (1991). In brief, cells were collected by centrifugation (3000 *g*) after 48-h induction in BG11$_0$ medium. The presence of heterocysts was assessed by microscopy. The pellet was resuspended in ice-cold 8% sucrose, 5% Triton X-100, 50 mM EDTA, pH 8.0, 50 mM Tris/HCl, pH 8.0, 1 mg/ml lysozyme, and vortexed vigorously for 2–3 min at room temperature. The suspension was mildly sonicated (Sonopuls sonifier; Bandelin Electronic, Berlin, Germany; MS73 tip; 3 min on ice) to break the vegetative cells. Heterocysts were collected by centrifugation at 3000 *g* for 5 min at 4°C and washed four times in 8% sucrose, 50 mM EDTA, pH 8.0, 50 mM Tris/HCl, pH 8.0. The fraction was assessed by 0.5% Alcian blue (50% ethanol solution) staining.

Isolated heterocysts were resuspended in 5 mM HEPES/NaOH, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), sonicated five times on ice (2 min with a 10-min break) using a Branson 450 sonifier (Branson Ultrasonic Corp., Danburg, CT, USA; macro tip, output 5) followed by four French press (SLM Instruments Inc., Rochester, NY, USA) cycles at 1100 psi. The suspension was mixed with Complete Protease Inhibitor (Roche, Mannheim, Germany) and centrifuged at 15 000 *g* at 4°C for 1 h. The pellet was resuspended in 15 ml of 55% sucrose solution, overlaid by 8 ml of 40% sucrose, 3 ml of 30% sucrose and 7 ml of 10% sucrose solution in 20 mM HEPES, pH 8.0, 0.2 mM PMSF and centrifuged at 130 000 *g* at 4°C for 16 h. The orange cell-wall pellet was collected from the bottom (Omata and Murata, 1984), washed twice in 20 mM HEPES/NaOH, pH 8.0, 1 mM PMSF, resuspended in the same buffer, frozen in liquid nitrogen and stored at -20°C. Lipid analysis is described in Fiedler et al. (1998).

Microscopic visualization of protein localization

Fluorescent imaging of the NMP-alr2269-GFP strain was performed on a TCS SP2 Leica confocal microscope (Wetzlar, Germany; HCX PLAN-APO 63× 1.4 NA oil immersion objective). GFP was excited using the 488-nm line of an argon ion laser and GFP fluorescence was analyzed in the window 500–570 nm, while autofluorescence was monitored at 630–700 nm (Muro-Pastor et al., 2006).

Digestion of cell wall proteins and mass spectrometric analysis

Cell wall fractions were centrifuged at 80 000 *g* for 10 min at 4°C. The pellet was resuspended in 80% acetone, 5% formic

acid and 0.1% deoxycholic acid and incubated on ice for 10 min. After centrifugation at 80 000 g for 15 min at 4°C, the pellet was washed with 20 µl of 80% acetone and pelleted again by centrifugation for 10 min. The pellet was resuspended in 100 mM Tris/HCl, pH 8.5, 1 mM $CaCl_2$, 10 µg/µl trypsin with an enzyme/substrate ratio of approximately 1:100. Probes were transferred into LoBind tubes (Eppendorf, Hamburg, Germany) and incubated for 16 h at 37°C with gentle shaking. Digestion was stopped by addition of formic acid (5% final concentration). Preparations were submitted to peptide sequencing by mass spectrometric analysis and proteins were identified by mass fingerprinting (Schmitt et al., 2006) and peptide sequencing (Moslavac et al., 2005a).

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