All0809/8/7 is a DevBCA-like ABC-type efflux pump required for diazotrophic growth in *Anabaena* sp. PCC 7120

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Efflux pumps export a wide variety of proteinaceous and non-proteinaceous substrates across the Gram-negative cell wall. For the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, the ATP-driven glycolipid efflux pump DevBCA–TolC has been shown to be crucial for the differentiation of N₂-fixing heterocysts from photosynthetically active vegetative cells. In this study, a homologous system was described. All0809/8/7–TolC form a typical ATP-driven efflux pump as shown by surface plasmon resonance. This putative exporter is also involved in diazotrophic growth of *Anabaena* sp. PCC 7120. A mutant in all0809 encoding the periplasmic membrane fusion protein of the pump was not able to grow without combined nitrogen. Although heterocysts of this mutant were not distinguishable from those of the wild-type in light and electron micrographs, they were impaired in providing the microoxic environment necessary for N₂ fixation. RT-PCR of all0809 transcripts and localization studies on All0807–GFP revealed that All0809/8/7 was initially downregulated during heterocyst maturation and upregulated at later stages of heterocyst formation in all cells of the filament. A substrate of the efflux pump could not be identified in ATP hydrolysis assays. We discuss a role for All0809/8/7–TolC in maintaining the continuous periplasm and how this would be of special importance for heterocyst differentiation.

The method by which HGLs traverse the Gram-negative cyanobacterial cell wall has been demonstrated recently (Staron et al., 2011). The ATP-binding cassette (ABC) exporter DevBCA and the outer-membrane protein TolC form an ATP-driven trans-envelope efflux pump to export HGLs across the Gram-negative cell wall. Like any typical Gram-negative trans-envelope pump, the *Anabaena* HGL exporter consists of an inner-membrane factor (IMF; comprising DevA as nucleotide binding domain and DevC as substrate-binding domain), a periplasmic membrane fusion protein (MFP; DevB) and an outer-membrane factor (OMF; TolC). Knockout mutations of *devBCA* or *tolC*, or mutations impairing the correct stoichiometric assembly of the efflux pump (2:6:3 of IMF:MFP:OMF), lead to immature heterocysts lacking the laminated layer (Fiedler et al., 1998; Moslavac et al., 2007a; Staron et al., 2011).

To further investigate the role of ATP-driven efflux pumps in *Anabaena* sp. PCC 7120, particularly with regard to heterocyst maturation, we started analysing gene clusters homologous to DevBCA. Strains with mutations in *all5346* (*hgdC*) or *all5347* (*hgdB*) have been shown to be unable to fix N₂ aerobically, and to aberrantly assemble HGL layers (Fan et al., 2005). In this work we have investigated the function of the gene cluster *all0809/8/7*. By in vitro

**INTRODUCTION**

*Anabaena* sp. strain PCC 7120 is a filamentous cyanobacterium that differentiates N₂-fixing cells upon nitrogen starvation. These heterocysts provide the photosynthetic filament with fixed nitrogen, and in return they obtain metabolites to fix N₂. Heterocysts protect the oxygen-labile N₂-fixing nitrogenase system by inactivating and degrading the oxygen-producing photosystem II, by increasing the respiratory O₂ consumption, and by forming two additional envelope layers outside the Gram-negative cell wall. The laminated layer consists of heterocyst-specific glycolipids (HGLs) and surrounds the outer membrane; it represents a barrier for O₂ diffusion. The outermost layer of heterocyst envelope polysaccharides (HEPs) protects the laminated layer from mechanical and chemical damage.

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To further investigate the role of ATP-driven efflux pumps in *Anabaena* sp. PCC 7120, particularly with regard to heterocyst maturation, we started analysing gene clusters homologous to DevBCA. Strains with mutations in *all5346* (*hgdC*) or *all5347* (*hgdB*) have been shown to be unable to fix N₂ aerobically, and to aberrantly assemble HGL layers (Fan et al., 2005). In this work we have investigated the function of the gene cluster *all0809/8/7*. By in vitro

**Abbreviations:** HEP, heterocyst envelope polysaccharide; HGL, heterocyst-specific glycolipid; IMF, inner-membrane factor; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; MFP, membrane fusion protein; OMF, outer-membrane factor; SPR, surface plasmon resonance.

Two supplementary figures are available with the online version of this paper.
protein–protein interaction studies, we could show that All0809/8/7–TolC form a typical ATP-driven efflux pump. A knockout strain lacking a functional copy of all0809 encoding the central periplasmic MFP was not able to grow diazotrophically under aerobic conditions. All0809/8/7–TolC were neither restricted to heterocysts in localization studies nor directly involved in the formation of the additional heterocyst cell wall layers. A distinct substrate of the IMF All0807/8 could not be identified in ATP hydrolysis assays. Therefore, this efflux pump could play a general but important maintaining role in the periplasm of mature heterocysts.

METHODS

Anabaena strains and growth conditions. All Anabaena strains used in this study are listed in Table 1; the respective plasmids are listed in Table 2. Wild-type Anabaena sp. PCC 7120 grew phototrophically at 28 °C in liquid BG11 medium (Rippka et al., 1979). Mutants unable to fix N₂ grew in BG11 medium supplemented with 5 mM NH₄Cl and 5 mM MES-NaOH buffer, pH 7.8. Respective mutant strains were cultivated in the presence of the appropriate antibiotics listed in Table 1 (for concentrations employed see Fiedler et al., 1998; Maldener et al., 2003; Moslavac et al., 2007a). Media were solidified with 1.5% (w/v) agar (Difco). Induction of heterocyst formation and isolation were performed as described earlier (Moslavac et al., 2007b).

Generation of mutant Anabaena strains. Anabaena mutants were generated by triparental mating and double recombination by using pRL271 (Black et al., 1993), as described earlier (Elhai & Wolk, 1988a; Wolk et al., 1984). Double recombinants were selected by the use of sacB, as described by Cai & Wolk (1990). The C.K3-cassette was derived as a blunt-end fragment from pRL442 (Elhai & Wolk, 1988b), and it was inserted into a central EcoRI site of all0809 (mutant M0809F in Table 1; plasmid pIM391 in Table 2) and against the gene orientation (mutant M0809R; plasmid pIM392). The respective oligonucleotides (KO) used for gene amplification and plasmid construction are listed in Table 3.

Mutant M0809R was complemented via single recombination of pRL271 containing all0809 and its promoter bearing upstream region Pall0809 (plasmid pIM450) into the downstream part of the disrupted chromosomal all0809::C.K3. In this way, the disrupted version of all0809 localized upstream of the integrated vector, followed by a fully restored operon comprising the promoter part followed by the entire all0809 gene, all0808 and all0807 in the single recombinant strain C0809 (Table 1). The oligonucleotides used for cloning and analysis (CP) are listed in Table 3.

Mutants encoding GFP fusions were generated by single recombination of pRL271 containing translational fusions of either all0807 (plasmid pIM521) or devA (plasmid pIM522) to GFP amplified from pCSEL19 (Muro-Pastor et al., 2006) into the respective wild-type gene. Fusions of GFP to the C terminus of the respective genes were generated by a PCR overlap procedure (Davidson et al., 2002). The respective oligonucleotides (GFP) are listed in Table 3. By single recombination into the last gene of the putative operon, expression of the other two genes was not affected and the expression of the original all0807 gene was not likely, since the gene was separated from the promoter and the upstream genes of the putative operon by insertion of the vector after recombination.

Microscopic visualization. Light micrographs were captured by a DM 5500 B microscope (Leica) using a DFC420 C camera (Leica). Reduction of INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride] to formazan crystals [1-(4-iodophenyl)-3- (phenyl)-5-(4-nitrophenyl)-formazan] was performed by incubating culture aliquots with 2 mM INT for 10 min prior to microscopy (Fay & Kulosaariyi, 1972). GFP was excited at 480 nm, and fluorescence was captured at 525 nm.

Samples for transmission electron microscopy were prepared as described previously (Fiedler et al., 1998). Fixation and post-fixation were performed using glutaraldehyde and potassium permanganate. Ultrathin sections were stained with uranyl acetate and lead citrate. Micrographs of the samples were taken with a Tecnai electron microscope (Philips) at 80 kV.

Expression analysis. Total RNA was extracted from 50 ml samples of Anabaena cultures before and at 3, 6, 9, 12 and 24 h after combined nitrogen step-down, and RT-PCR was performed as described previously (Staron et al., 2011). The respective oligonucleotides (RT) are listed in Table 3.

Construction, overexpression and purification of recombinant proteins. Recombinant proteins were overexpressed and purified as described previously (Staron et al., 2011). In brief, proteins were overexpressed as glutathione S-transferase (GST) tag fusions in Escherichia coli strain Rosetta-Gami DE3 using the vector pET42a (Merck). GST fusions were purified, and the N-terminal GST was cleaved off the respective protein. The respective oligonucleotides (OEX) are listed in Table 3. Internal modifications of protein variants or of protein–protein fusions were generated by PCR overlap extension. The fusion of All0807 and All0808 (All0807/8) was performed according to the fusion of DevA and DevC (DevAC; Staron et al., 2011). all0807 and all0808 were amplified separately, and subsequently fused by self-aligning sequences created by the reverse

Table 1. Anabaena strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Resistance(s)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>PCC 7120</td>
<td>Wild-type</td>
<td>–</td>
<td>C. P. Wolk</td>
</tr>
<tr>
<td>DR74</td>
<td>all0809::C.K3</td>
<td>Nm’</td>
<td>Fiedler et al. (1998)</td>
</tr>
<tr>
<td>M0809F</td>
<td>all0809::C.K3f</td>
<td>Nm’</td>
<td>This study</td>
</tr>
<tr>
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<td>all0809::C.K3r</td>
<td>Nm’</td>
<td>This study</td>
</tr>
<tr>
<td>C0809</td>
<td>all0809::pIM450</td>
<td>Nm’, Cm’, Em’</td>
<td>This study</td>
</tr>
<tr>
<td>0807-GFP</td>
<td>all0807::pRL271::all0807::gfp</td>
<td>Cm’, Em’</td>
<td>This study</td>
</tr>
<tr>
<td>3112-GFP</td>
<td>all3112::pRL271::all3112::gfp</td>
<td>Cm’, Em’</td>
<td>This study</td>
</tr>
</tbody>
</table>
Flow cells (FC1 and FC2) of the sensor chip at a flow rate of 0.05% (v/v) Triton X-100 pHs from 6.0 to 6.6, or HEPES-NaOH at pH 7.0; 150 mM NaCl, and

**Table 3.** Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO all0809</td>
<td>5’: CTCGAGATATAGGTTAATATTTCGGG</td>
</tr>
<tr>
<td></td>
<td>3’: CTCGAGTCAATATTGCTTGAGGCTCG</td>
</tr>
<tr>
<td>CP all0809</td>
<td>5’: GATTTCAGTAGTAATATCTGTTGCTTTG</td>
</tr>
<tr>
<td></td>
<td>3’: GTGCAGGTTTGTAGGCACACATCCATCCGACG</td>
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<tr>
<td></td>
<td>5’: GTCGAGAACCCATTAAAGCTGCGGCTCAGAGT</td>
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<tr>
<td></td>
<td>3’: CTCGAGTCAAGTCTTACATCCATCCGACG</td>
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<tr>
<td>RT all0809</td>
<td>5’: TACCGCGGAACCCACACAGC</td>
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<td></td>
<td>3’: ACCCTCTACTTCCTTCTACAC</td>
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<tr>
<td>RT rnpB</td>
<td>5’: GGTAGCTTTAAGTGTATAGG</td>
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<td>3’: CCCCTAGTCCCCAGTCCCCAACATCTG</td>
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<tr>
<td>GFP all0807</td>
<td>5’: CTCGAGATGTCAGAAGATCTTTTCGG</td>
</tr>
<tr>
<td></td>
<td>3’: CGAAGCGGATCAGAAGATCTTTTCGG</td>
</tr>
<tr>
<td>GFP alr3712</td>
<td>5’: GGGTTCTGGATCCGCTGATGAGTAAGGAGAGAACCATTTTC</td>
</tr>
<tr>
<td></td>
<td>3’: CTCGAGTTATTTGATAGTTGATTCGAGT</td>
</tr>
<tr>
<td>OEX All0809</td>
<td>5’: GGATCGCCGAGTTCCAGGAGGTCG</td>
</tr>
<tr>
<td></td>
<td>3’: GAACCCATTCGCGAAGCTGACAGTCCGAGT</td>
</tr>
<tr>
<td>OEX All0807/8</td>
<td>5’: GGGTTCTGGATCCGCTGATGAGTAAGGAGAGAACCATTTTC</td>
</tr>
<tr>
<td></td>
<td>3’: CTCGAGTTAAATCCGCTGAGGTCG</td>
</tr>
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**Cell fractionation and glycolipid purification.** Cell fractions (Moslavac et al., 2007b) and heterocyst glycolipids (Winkenbach et al., 1972) were prepared as described elsewhere. In brief, enriched heterocysts were broken by at least five passes through a French pressure cell (24 000 p.s.i.; 166 MPa) and separated into a soluble cytoplasmic and an insoluble membrane fraction by centrifugation (45 000 g, 30 min, 4°C). To obtain cytoplasmic membrane, thylakoid membrane and outer membrane, the pellet was separated by a discontinuous sucrose gradient (Moslavac et al., 2007b). To obtain heterocyst glycolipids, the pellet was separated by a continuous sucrose gradient (Winkenbach et al., 1972).

**ATP hydrolysis assay.** The assays were performed as described previously (Staron et al., 2011). In brief, 0.1 μg ml⁻¹ DevAC or All0807/8, and 0.2 μg ml⁻¹ DevB or All0809, and 2 μg of the respective cell fractions or 8 μg of heterocyst glycolipids at indicated concentrations were mixed in ATPase reaction buffer supplemented with an ATP regeneration system and a reaction detection system. The rate of hydrolysis in units (U) was calculated as moles ATP hydrolysed per minute and per milligram of the ATPase DevAC or the ATPase All0807/8.

**RESULTS**

**Clustered genes all0809–07 are essential for diazotrophic growth of Anabaena sp. PCC 7120**

At least six gene clusters closely homologous to devBCA (also referred to as alr3710–3712) can be predicted from the genome sequence of Anabaena sp. PCC 7120: (i) all0809–0807, (ii) all2652–2651, (iii) alr3647–3649, (iv) alr4280–4282,
(v) *alr4973–alr4975* and (vi) *all5347–5346*. Clusters (ii) and (vi) do not encode a DevA-like protein. All clusters are predicted to encode MFPs, so their gene products presumably form an ATP-driven efflux pump together with the only OMF encoded in the genome of *Anabaena* sp. PCC 7120: TolC (also referred to as Alr2887 or HgdD; Moslavac et al., 2007a). Like *devBCA*, *all0809/8/7* are predicted to encode subunits of an ABC exporter. In the same order as the operon *devBCA*, *all0809* encodes a Dev-like MFP, *all0808* the substrate-binding domain of a DevAC-like IMF, and *all0807* the nucleotide-binding domain of the same IMF (Fig. 1a).

To elucidate the function of *all0809/8/7* in *Anabaena* sp. PCC 7120, we inserted the cassette C.K3 into the first gene downstream of the cassette promoter (*P_{psbA}*). Hence, mutant M0809F is presumably a KO mutant of the promoter, and their expression should not take place. In strain M0809R, the downstream genes presumably lack a *psbA* promoter but lacks a STOP signal. Two mutants were created with both orientations of the cassette with respect to the reading frame (M0809F and M0809R: F for forward and R for reverse orientation; Table 1). In strain M0809F, the downstream genes can still be expressed under the control of the strong *psbA* promoter. In strain M0809R, the downstream genes presumably lack a promoter, and their expression should not take place. Hence, mutant M0809R is presumably a KO mutant of the entire gene cluster. By mutating just one essential part, the MFP-encoding gene *all0809*, we prevented the possible assembly of a hypothetical tripartite All0809/8/7–TolC efflux pump. Independently of the orientation of the C.K3 cassette, both knockout mutants of *all0809* showed the same phenotype. The mutant strains grew on NH$_4^+$ like the wild-type with no visible differences (Fig. 1b, +N). However, neither mutant was able to grow without combined nitrogen (Fig. 1b, −N). Although the filaments bleached and fragmented after several days of combined nitrogen starvation, heterocysts and vegetative cells were indistinguishable from the wild-type with respect to their ultrastructure (Fig. 1d, e). Mutant heterocysts contained a laminated HGL layer (Fig. 1d, HGL) and a normal-looking HEP layer (Fig. 1d, HEP). The poles were reduced to the polar neck containing the cyanophycin granule (Fig. 1d) and the thylakoid membranes rearranged as in wild-type heterocysts (for wild-type, see e.g. Merino-Puerto et al., 2011).

Compared with the wild-type, heterocysts of the *all0809* mutant strains contained fewer insoluble formazan crystals, which are formed by reduction of added INT. This could be due to an impairment in the ability to provide a microoxic environment or a reduced uptake of INT (Fig. 1e). The microoxic environment is a prerequisite for diazotrophic growth. The mutant phenotype could be rescued by complementation of the mutant strain M0809R.

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**Fig. 1.** Phenotype of mutant M0809. (a) Genetic organization of the *devBCA* operon (also referred to as *alr3710–12*) and the *all0809/8/7* gene cluster. (b) Samples of liquid cultures of wild-type *Anabaena* sp. PCC 7120 (WT), the *all0809* knockout mutant (M0809) and the *devB* knockout mutant (DR74). Cultures were either grown in the presence of 5 mM NH$_4$Cl (+N) or starved for combined nitrogen for 1 week (−N). Cultures were either grown in the presence of 5 mM NH$_4$Cl (+N) or starved for combined nitrogen for 1 week (−N). (c) Cultures of wild-type *Anabaena* sp. PCC 7120 (WT), a complementation mutant of M0809 (C0809) and mutant M0809. Cultures were starved for combined nitrogen for 1 week. (d) Electron micrograph of a terminal heterocyst (H) and a vegetative cell (V) of M0809. (e) Light micrographs of wild-type *Anabaena* sp. PCC 7120 (WT) or the *all0809* knockout mutant (M0809) starved for combined nitrogen for ~40 h, after pre-incubation with INT. H, heterocysts; asterisks, crystals inside the heterocysts (reduced INT). The percentage and SD values refer to heterocysts containing formazan crystals. At least 100 heterocysts were counted from three different cultures of WT, M0809F and M0809R (each). Insets, ×3 magnifications of heterocysts.
with a wild-type copy of *all0809*, creating strain C0809 (Table 1). The putative operon *all0809/8/7* could be restored by single recombination of the wild-type gene *all0809* and its upstream region into a site downstream of the disrupting cassette in *all0809::C.K3*. The resulting strain contained a mutated version of gene *all0809*, followed by the inserted vector and an intact gene cluster. This strain, C0809, was able to grow in the absence of combined nitrogen (Fig. 1c).

The putative tripartite efflux pump-encoding cluster *all0809/8/7* is essential for diazotrophic growth of *Anabaena* sp. PCC 7120. M0809 is not able to survive the absence of combined nitrogen under aerobic conditions, and this phenotype can be rescued by restoration of *all0809/8/7*. Although light or electron micrographs do not reveal abnormalities in cell morphology, heterocysts of M0809 seem to be impaired in providing the microoxic conditions necessary to fix N₂.

### The expression patterns of *all0809 and all0807* are different from that of *devBCA*

Genes of the *all0809/8/7* cluster are necessary for the function of heterocysts. Therefore, we analysed the expression pattern of the first gene, *all0809*, under different nitrogen supplies. The homologous heterocyst glycolipid ABC-exporter DevBCA was shown to be upregulated during nitrogen stepdown in the maturing heterocyst, showing maximal abundance at 9–12 h (Fiedler et al., 2001; Maldener et al., 1994; Staron et al., 2011). Transcripts of *all0809* could be detected by RT-PCR in comparable amounts in filaments grown on NH₄⁺ and after 24 h of nitrogen stepdown (Fig. 2a). However during the first 9 h of N deprivation the expression level decreased to a minimum at 6 h.

Furthermore, we could localize the nucleotide-binding protein All0807 within filaments of *Anabaena* sp. PCC 7120 by fusing it to GFP. GFP cannot fold to an active fluorescent protein outside the cell, so a translational fusion to the C terminus of All0807 does not seem to be appropriate to investigate the presence and differential expression of the efflux pump. Any modification of the N-terminal cytoplasmic tail of the homologous protein DevB resulted in a loss of function, as did tagging both cytoplasmic ends of the IMF DevC (P. Staron and I. Maldener, unpublished data). Therefore, these proteins were not considered to be a good choice for a GFP fusion. However, we have previously shown that a C-terminal fusion to the nucleotide-binding protein DevA does not lead to a loss of function (Staron et al., 2011). Hence a GFP fusion with the ATP-binding cassette All0807 was created and introduced into the chromosome of the wild-type *Anabaena* sp. PCC 7120. In the homologous IMFs MacB and HlyB from *E. coli*, the cytoplasmic nucleotide-binding proteins are C-terminally fused to their respective substrate-binding proteins (Blight & Holland, 1990; Kobayashi et al., 2001). In contrast, nucleotide-binding proteins such as DevA and MJ0796, or as predicted for All0807, are single polypeptides located in the cytoplasm (Yuan et al., 2001). Therefore, a cytoplasmic localization of All0807–GFP and DevA–GFP was expected.

Since *Anabaena* shows weak green fluorescence, in all experiments settings for GFP detection were chosen in which the wild-type did not show any background, as seen for the DevA_GFP strain when grown on ammonia (Fig. 2a, 0 h, GFP). Before depletion of combined nitrogen, All0807_GFP was localized in all cells of the filament (Fig. 2a). 0 h refers to NH₄Cl-grown filaments, 12 h to N-starved filaments. BF, bright-field; AF, autofluorescence; GFP, GFP fluorescence.

![Fig. 2. Expression of the DevBCA-like efflux pump-encoding genes *all0809/8/7*. (a) Time-dependent expression pattern of *devB* (taken from Staron et al., 2011) and *all0809* analysed by RT-PCR. *rnpB* refers to the loading control used for RT-PCR of *all0809*. RNA was obtained from a wild-type culture before (0) and after indicated time points of nitrogen starvation. (b) Micrographs of filaments bearing translational fusions of DevA_GFP and All0807_GFP. 0 h refers to NH₄Cl-grown filaments, 12 h to N-starved filaments. BF, bright-field; AF, autofluorescence; GFP, GFP fluorescence.](image-url)
2b, All0807_GFP, 0 h). In agreement with the expression data for all0809, green fluorescence from All0807_GFP decreased after 12 h of combined nitrogen stepdown, and was hardly detectable (Fig. 2b, All0807_GFP, 12 h). After 24 h, when heterocysts have usually completed maturation, All0807_GFP was detectable in all cell types (Fig. 2b, All0807_GFP, 24 h). In contrast, GFP was barely detectable before nitrogen stepdown (Fig. 2b, DevA_GFP, 0 h). In agreement with devBCA expression analyses performed previously (Fiedler et al., 2001; Maldener et al., 1994; Staron et al., 2011), the fluorescence of DevA_GFP localized to single cells, presumptive proheterocysts (Fig. 2b, DevA_GFP, 12 h).

From these data we could conclude that the DevBCA homologue All0809/8/7 is not restricted to heterocysts. The expression pattern of all0809/8/7 contrasts with that of devBCA and is not specifically localized to heterocysts. Unfortunately, no data are available from other studies of global expression analysis of the all0809/8/7 gene cluster.

The transcriptional start point (TSP) for the whole cluster and TolC act in vitro stoichiometry as the DevBCA–TolC system from Anabaena sp. PCC 7120 and the MacAB macrolide exporter from E. coli (Kim et al., 2010; Xu et al., 2010, 2011). However, in contrast to DeV (pH 6.2), the highest response of the chip surface-bound OMF TolC towards the free MFP All0809 was observed at a pH of 6.5. As with the interaction of DevB with TolC and for several E. coli systems (Tikhonova et al., 2009), higher pH values impaired the binding of All0809 to TolC to a remarkable extent (Fig. 3a).

To summarize, all proteins encoded by the all0809/8/7 cluster and TolC act in vitro with each other in the same stoichiometry as the DevBCA–TolC system from Anabaena sp. PCC 7120 and the MacAB macrolide exporter from E. coli (Xu et al., 2011). Hence they can form a typical ATP-driven efflux pump, in which a hexameric MFP connects (Blight & Holland, 1990; Kobayashi et al., 2001). This approach worked well in a previous study of protein–protein binding of the proteins of the DevBCA–TolC system (Staron et al., 2011). In a concentration-dependent manner, immobilized TolC and All0808/7 (the fusion protein of All0808 and All0807) bound to free All0809 (Figs 3b and S2). By SPR, a strong pH-dependency of the binding of the periplasmic MFP and TolC was also demonstrated (Figs 3a and S1). A small change in pH resulted in a remarkable change in binding. Decreasing the pH by 0.2 units resulted in weaker binding of TolC to All0809 and to DeV (about one-fifth). Increasing the pH by 0.1 units (All0809) or 0.2 units (DevB) also resulted in weaker binding of TolC to the MFP (All0809 about one-third, DevB about one quarter) (Fig. 3a).

These in vitro binding studies showed that All0809/8/7–TolC can form a typical type I secretion system with a calculated stoichiometry of 3:6:2. This means that a trimeric TolC in the outer membrane is connected to the dimeric IMF All0808/7 by a hexameric All0809 fusion protein (Fig. 3b). The same ratio was previously obtained for the DevBCA–TolC system of Anabaena (Staron et al., 2011) and for MacAB–TolC in E. coli (Kim et al., 2010; Xu et al., 2010, 2011).

**All0809/8/7 and TolC interact with each other**

From their sequence homology, the products of all0809/8/7 were predicted to encode a DevBCA-like ABC exporter, which could form an ATP-driven efflux pump with TolC. To prove this, the interaction of the MFP All0809 with the OMF TolC, and the interaction of All0809 with an All0807/8 fusion protein as IMF were measured via SPR. For a better handling of the interaction partners, a fusion protein of All0809 and All0808 was created in analogy to MacB and HlyB from E. coli. Both IMFs comprise the nucleotide-binding and substrate-binding proteins in one polypeptide.

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**Fig. 3.** Binding of the All0809/8/7–TolC complex. (a) Summary of SPR analysis of the interaction of immobilized TolC with free DeV or with free All0809 at the indicated pH values. The raw data were taken from Staron et al. (2011) for DeV. The raw data for All0809 are shown in Fig. S1. (b) Summary of SPR analysis of the interaction of immobilized TolC or immobilized All0808/7 with free All0809 at the indicated MFP concentrations. The raw data are shown in Fig. S2.
the OMF TolC with the IMF. The binding affinity of the periplasmic proteins of this secretion system is strongly affected by the pH value.

**All0809/8/7 do not react towards heterocyst glycolipids**

The phenotype of the *all0809* mutants M0809F/R did not allow a conclusion to be drawn about the precise function and nature of the substrate of the putative efflux pump All0809/8/7–TolC. To identify possible substrates, All0809/8/7 were exposed to different cell fractions, and the ATPase activity of All0807 was recorded.

All0809/8/7 did not react to any fraction via changes in ATPase activity (Fig. 4). No remarkable differences could be recorded when All0809/8/7 were assayed for basal activity or in the presence of soluble or any membrane fractions from NH$_4$Cl-grown filaments (Fig. 4, V fractions) or 12 h N-starved filaments (Fig. 4, H fractions). All0809/8/7 did not respond to the presence of HGLs, even when All0809 was replaced with DevB, or when All0808/7 was replaced with DevAC.

Assaying All0809/8/7 for a substrate did not provide any information about the function of All0809/8/7–TolC, and therefore no further indications of the reason for the loss of diazotrophy of *Anabaena* sp. PCC 7120. The subunits of the DevBCA glycolipid transporter were not able to provoke recognition of the glycolipids.

**DISCUSSION**

**All0809/8/7–TolC appear to form a typical ATP-driven efflux pump for an as-yet-unknown substrate**

DevBCA was the first ABC exporter described for cyanobacteria. It was shown to form an ATP-driven efflux pump with TolC and to export glycolipids necessary for the formation of an additional layer of the heterocyst cell wall (Staron et al., 2011). In this work, we investigated All0809/8/7, another predicted ABC exporter from *Anabaena* sp. PCC 7120, and demonstrated that it is also needed for proper heterocyst function.

In our protein-binding studies by SPR we could unquestionably show that All0809/8/7 and TolC assemble to form a typical ATP-driven efflux pump. Since the molar ratio of the OMF TolC to the MFP All0809 to the IMF All0808/7 was determined to be 3 : 6 : 2, it exactly resembles the molar ratio of TolC : DevB : DevAC, or the (theoretically combined) molar ratio of TolC : MacA : MacB (Staron et al., 2011; Yum et al., 2009; Zgurskaya et al., 2011). So, similarly to other reported ATP-driven efflux pumps, a central MFP All0809 hexamer seems to play a key role in the physiological function of All0809/8/7–TolC. Consequently, a tip-to-tip cogwheel-like interaction of the MFP with the OMF in ATP-driven efflux pumps also seems to be true for All0809 and TolC (Yum et al., 2009).

The interaction of All0809 with TolC had a higher pH optimum than the DevB interaction with TolC (Figs 3 and S1). Since the gene *alr2887* predicted from the genome of *Anabaena* sp. PCC 7120 is the only gene encoding a TolC-like outer membrane protein, several TolC-dependent exporters have to compete with each other for the binding of the respective MFP to the OMF. Different pH optima for the affinity of the MFP to TolC could determine the selection of the MFP needed for a specific purpose. Tikhonova and co-workers demonstrated that the pH optima of MFP–OMF interactions of three very different efflux pumps in *E. coli* were exactly the same (5.8; namely MacAB–TolC, AcrAB–TolC and EmrAB–TolC as representatives of ABC-type, RND-type and MF-type systems, respectively; Tikhonova et al., 2009). All of them were heavily impaired in efflux pump formation at higher pH. Given the different lifestyles of *E. coli* and *Anabaena* sp. PCC 7120, even minimal differences in the pH optima of closely homologous but spatially and temporally nonredundant systems could reflect an adaptation to the ability to form different cell types and therefore to multicellularity. If during heterocyst maturation different MFPs have to compete with each other for TolC, the different pH optima for binding could facilitate the replacement process. The expression pattern of *all0809*...
(Fig. 2a) and the localization of All0807>GFP (Fig. 2b) would not contradict this possible pH-dependent affinity regulation. During heterocyst maturation DevB has to outcompete at least All0809 in binding to TolC due to its pivotal role. Increasing the amount of DevB and decreasing that of All0809 (and other MFPs) may be not enough, and pH-dependent affinity could add an additional level of rapid regulation. This concept could also be true for other heterocyst-related functions: among adjustments of the efflux pump repertoire, heterocyst maturation involves many adjustments in the outer membrane/periplasmic proteome (Moslavc et al., 2007b; Nicolaisen et al., 2009a) or remarkable modifications of the cell wall (e.g. the murein layer; Lázaro et al., 2001; Lehner et al., 2011; Zhu et al., 2001). It remains to be shown in the future whether developing heterocysts spatially and temporally change their periplasmic pH values (or the pH value of the continuous periplasm of the whole filament) to discriminate between heterocyst-specific and heterocyst-nonspecific functions. Homologues of DevBCA are crucial for aerobic diazotrophic growth of Anabaena sp. PCC 7120 Several mutants have been described which cannot grow on N₂ as a nitrogen source, because their specific heterocyst envelope layers are missing or aberrant (Nicolaisen et al., 2009a; Zhao & Wolk, 2008). Heterocysts of M0809 show a wild-type-like laminated HGL layer (Fig. 1d). In addition, as measured by ATP hydrolysis rate, All0809/8/7 did not react towards the presence of HGLs (Fig. 4). So, a specific contribution of All0809/8/7–TolC to the export and/or formation of the laminated layer appears to be unlikely. A functional relationship with the nearby hglK gene (all0813; Black et al., 1995) seems unlikely, since a hglK mutant is not able to deposit a laminated layer.

The reason for the inability of mutants in All0809 to carry out diazotrophic growth could be impairment in providing a microoxic environment for N₂ fixation. Indications for this came from the observation of a remarkably decreased frequency of formazan crystals inside the mutant heterocysts. However, since we could not identify a substrate for the All0809/8/7–TolC efflux pump, the reason for this is unclear at present. All0809/8/7–TolC seems either to indirectly affect heterocyst maturation by an unknown mechanism or to adopt an as-yet-unknown heterocyst-specific function. An indirect effect on heterocyst formation could be explained in the same manner as the proposed function of an H⁺-driven efflux pump in E. coli: during cell division, AcrEF–TolC are thought to act as a ‘cleaner’ for the periplasm to remove proteins and products of murein and membrane recycling. Overproduction of a periplasmic protein in an acrEF mutant results in filamentous E. coli (Lau & Zgurskaya, 2005). Similarly, All0809/8/7–TolC could play a role in maintaining the periplasm of Anabaena sp. PCC 7120. The absence of this function could introduce unforeseen, and maybe unseen, artefacts into the cell wall of developing heterocysts, and therefore somehow allow the entrance of O₂ into the heterocyst or impede the supply of reductants to the mature heterocyst by periplasmic diffusion (Flores & Herrero, 2010; Flores et al., 2006; Mariscal & Flores, 2010; Nicolaisen et al., 2009b).

All0809/8/7–TolC is the second ABC-type efflux pump characterized so far in cyanobacteria and, like its homologues DevBCA–TolC and HgdBC–TolC, is essential for diazotrophic growth of Anabaena sp. PCC 7120. Future studies on the other homologues will underline the importance of this broadly distributed class of exporter systems in cyanobacteria and in Gram-negative bacteria in general.

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