Functional Diversity of TonB-Like Proteins in the Heterocyst-Forming Cyanobacterium *Anabaena* sp. PCC 7120

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ABSTRACT The TonB-dependent transport of scarcely available substrates across the outer membrane is a conserved feature in Gram-negative bacteria. The plasma membrane-embedded TonB-ExbB-ExbD accomplishes complex functions as an energy transducer by physically interacting with TonB-dependent outer membrane transporters (TBDTs). TonB mediates structural rearrangements in the substrate-loaded TBDTs that are required for substrate translocation into the periplasm. In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, four TonB-like proteins have been identified. Out of these TonB3 accomplishes the transport of ferric schizokinen, the siderophore which is secreted by *Anabaena* to scavenge iron. In contrast, TonB1 (SjdR) is exceptionally short and not involved in schizokinen transport. The proposed function of SjdR in peptidoglycan structuring eliminates the protein from the list of TonB proteins in *Anabaena*. Compared with the well-characterized properties of SjdR and TonB3, the functions of TonB2 and TonB4 are yet unknown. Here, we examined *tonB2* and *tonB4* mutants for siderophore transport capacities and other specific phenotypic features. Both mutants were not or only slightly affected in schizokinen transport, whereas they showed decreased nitrogenase activity in apparently normal heterocysts. Moreover, the cellular metal concentrations and pigment contents were altered in the mutants, most pronouncedly in the *tonB2* mutant. This strain showed an altered susceptibility toward antibiotics and SDS and formed cell aggregates when grown in liquid culture, a phenotype associated with an elevated lipopolysaccharide (LPS) production. Thus, the TonB-like proteins in *Anabaena* appear to take over distinct functions, and the mutation of TonB2 strongly influences outer membrane integrity.

IMPORTANCE The genomes of many organisms encode more than one TonB protein, and their number does not necessarily correlate with that of TonB-dependent outer membrane transporters. Consequently, specific as well as redundant functions of the different TonB proteins have been identified. In addition to a role in uptake of scarcely available nutrients, including iron complexes, TonB proteins are related to virulence, flagellum assembly, pilus localization, or envelope integrity, including antibiotic resistance. The knowledge about the function of TonB proteins in cyanobacteria is limited. Here, we compare the four TonB proteins of *Anabaena* sp. strain PCC 7120, providing evidence that their functions are in part distinct, since mutants of these proteins exhibit specific features but also show some common impairments.

KEYWORDS *Anabaena*, TonB protein, cyanobacteria, metal transport, nitrogenase, outer membrane, siderophores
Cyanobacteria possess a Gram-negative type of cell envelope containing an outer membrane (OM), a peptidoglycan (PG) layer, and a plasma (cytoplasmic or inner) membrane (PM) (1). Macromolecular complexes that reside in the two membranes facilitate the assembly of the cell wall components as well as solute exchange and signaling (2). Among them, the OM-embedded TonB-dependent transport machinery is widely distributed in Gram-negative bacteria (3). The TonB-dependent transport system is important for growth under iron starvation conditions, since iron is an essential but scantily bioavailable nutrient (3–7). Iron-loaded proteins carry out functions in important cellular activities such as electron transport and DNA synthesis (8). This holds particularly true for cyanobacteria, in which iron is required for the synthesis of phycobiliproteins (9) and chlorophyll $\alpha$ (Chl) (10), as well as for photosynthetic complexes that in total require approximately 22 to 23 iron atoms (11). Moreover, in certain cyanobacterial species that are capable of nitrogen fixation, the nitrogenase enzyme is also dependent on iron (12).

Due to its low solubility under oxic conditions at neutral pH, iron rapidly forms insoluble aggregates that are inaccessible to many microorganisms (6, 13). Only a very small amount of dissolved iron exists as inorganic iron, whereas the largest proportion is bound to organic ligands such as siderophores (6). Siderophores are low-molecular-weight compounds that chelate ferric iron with high affinity. The production and secretion of siderophores is a widespread strategy of bacteria, fungi, and plants to cope with iron-limiting conditions (14). Siderophores are divided into three classes depending on the chemical nature of iron coordination, namely, catecholates, hydroxamates, or mixed types that contain another iron complexing group such as hydroxycarboxylate (15).

The TonB-dependent transport system involves a PM-localized energizing TonB-ExbB-ExbD complex and OM-localized TonB-dependent transporters (TBDTs). TBDTs constitute gated channels that facilitate the transport of substrates into the periplasm (16). The translocation process is energy dependent, as the substrates are typically large and rarely abundant (17, 18). Examples besides siderophores are carbohydrates, vitamin B$_12$ (cobalamin), and heme (16, 19). The energy for transport is derived from the proton motive force (pmf) across the PM (20, 21). ExbB and ExbD build up a proton channel that converts the pmf into energy for the translocation process (22). The TonB protein transfers the energy to the TBDT through direct interaction with both, ExbB/ExbD and the TBDT (23–26).

TonB proteins contain a transmembrane $\alpha$-helix and a conserved C-terminal motif that interacts with the so-called TonB box of the TBDTs (16, 27). Remarkably, more than 40% of the organisms that possess a TonB-dependent system have more than one tonB gene copy (3). For instance, *Pseudomonas aeruginosa* possesses three TonB proteins (28–30). Here, TonB1 and likely TonB2 facilitate the transport of iron-containing compounds and are required for growth under iron-limiting conditions, while TonB3 is crucial for motility and pilus assembly (28–32). In *Pseudomonas putida*, one of the two TonB proteins energizes the transport of siderophores, whereas the other TonB protein is important for maintaining the integrity of the cell envelope and flagellum localization (33–35). Also, *Vibrio* species typically contain multiple tonB copies in the genome. Here, distinct TonB proteins facilitate the transport of both common and individual substrates (36). Thus, multiple TonB proteins in one organism can take over redundant as well as unique functions. They can function in protein complex assembly, cell wall integrity regulation, or global or substrate-specific transport processes.

Little is known about the functionality of TonB proteins in cyanobacteria, which are photoautotrophic organisms that can be found in terrestrial, marine, or freshwater habitats. The number of putative TBDT, TonB, or ExbB/D proteins in the genomes of analyzed cyanobacteria is highly variable (37, 38). For example, in the genome of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (*Anabaena* hereafter) 22 different TBDTs were predicted (37). In contrast, only four genes with a tonB signature were assigned by bioinformatics methods (38). TonB1 contains an exceptionally short
periplasmic domain that is likely not sufficient in size to reach OM-embedded factors. In contrast, TonB3 is supposed to be a central component of the ferric siderophore transport system (38). The tonB3 mRNA abundance increases under iron-limited conditions, and a single recombination mutant can be generated only in the presence of enhanced iron concentrations (38). The growth of this mutant in the absence of iron is reduced, and siderophore synthesis genes are upregulated in this genetic background (38). Further, we could show recently that the transport of ferric schizokinen, the siderophore secreted by *Anabaena*, was abolished in a tonB3 mutant (39). In contrast, the tonB1 mutant retained the siderophore transport capacity but was severely impacted in diazotrophic growth. This could be traced back to an abnormal peptidoglycan morphology in the heterocyst septa of the mutant, and therefore, TonB1 was renamed septal junction disc regulator (SjdR) (39).

TonB2 encoded by *all3585* or TonB4 encoded by *alr5329* has a domain structure comparable to the TonB proteins from *Escherichia coli* or TonB3 from *Anabaena* (38). However, the distance between the transmembrane domain and the TonB-box binding domain is smaller than in TonB3 (Fig. 1). Estimation of the size assuming an extended helix suggests a dimension of 12 nm for TonB4 and 22 nm for TonB2, while for TonB3 a 32-nm size is estimated. The latter fits the determined distance between OM and PM in *Anabaena*, as well as the estimated size of the TolC system (40, 41). Both *tonB2* and *tonB4* are expressed at highest levels in low-density cultures and lowest levels in the stationary phase (42). Their expression is enhanced at all growth stages in the presence of elevated iron (38, 42), while the expression of *tonB4* is also enhanced in the presence of elevated copper concentrations (38, 42).

Considering the essential role of TonB3 in siderophore transport, opposed to the novel functionality of SjdR, which is not related to TonB-dependent transport, we now aimed to characterize the TonB-like proteins TonB2 and TonB4, since the role of those proteins in *Anabaena* is still unclear (38, 39). Insertion mutants for *tonB2*, *tonB3*, and *tonB4* demonstrated alterations in cellular metal levels as well as in carotenoid (Car) or chlorophyll *a* concentrations compared to the wild type, although to different extents. Moreover, the *tonB2* mutant filaments aggregated in liquid cultures, which might be related to an enhanced production of lipopolysaccharide in this strain. Also, the outer membrane integrity as well as the expression of porins was affected in the *tonB2* mutant. On the other hand, the *tonB2* mutant as well as the *tonB4* mutant retains the siderophore transport capacity, which suggests a functional diversity of *Anabaena* TonB proteins.

**RESULTS**

The *Anabaena tonB* mutants bear pigment alterations. To analyze putative functions of the individual TonB proteins, the growth behavior of mutants of the corresponding genes was examined. The mutant strains, I-sjdR, I-tonB2, I-tonB3, and I-tonB4,
were generated through single recombination insertion of a plasmid in the gene of interest (Fig. 2A), as described before (38, 39). In accordance with previous reports, I-\textit{sjdR} and I-\textit{tonB2} were segregated, as no wild-type copy of the respective genes was detectable in the mutants (Fig. 2B) (39). In contrast, I-\textit{tonB3} and I-\textit{tonB4} could not be segregated, as even after repeated dilution on plates with antibiotics, the wild-type genes were detectable in the corresponding genomic DNA (gDNA) by PCR (Fig. 2A) (38). For I-\textit{tonB3} this is consistent with the previous report, where full segregation was obtained only in the presence of enhanced iron (38). This suggests that TonB3 and TonB4 are important for viability under the conditions used in this study.

None of the \textit{tonB} mutants exhibited an altered growth behavior compared to wild type under standard conditions (YBG11 medium, Fig. 2C). However, I-\textit{tonB2} cells frequently formed aggregates in liquid medium (Fig. 2D). The enhanced tendency of I-\textit{tonB2} to aggregate was also verified by sedimentation analysis (see Fig. S1 in the supplemental material). In addition, the color of I-\textit{tonB2} was considerably different from that of the wild type (Fig. 2E), suggesting a modification in the cellular pigment content.

The synthesis of carotenoids and Chl in \textit{Anabaena} is differentially regulated in response to growth temperature and light intensity (43–46). Therefore, the concentrations of these pigments in the mutants and the wild type were determined after growth of the cultures.
for 7 days under ambient light (70 μmol photons m⁻² s⁻¹) as well as under high-light or low-light conditions (140 and 15 μmol photons m⁻² s⁻¹, respectively).

In general, the pigment concentrations under ambient light and low light were comparable. When grown under ambient or low-light conditions, the Chl concentration in the wild type was 9 ± 2 μg ml⁻¹ at an optical density at 750 nm (OD₇₅₀) of 1, the carotenoid concentration was 2.5 ± 0.3 μg ml⁻¹ at an OD₇₅₀ of 1, and the phycoerythrin (PC) concentration was 26 ± 7 μg ml⁻¹. Compared to that, all mutant strains were diminished in their cellular Chl content under both conditions (Table 1) even though a significant difference was found only for l-sjdR (low light) and l-tonB₄ (ambient and low light). TonB₃ is involved in siderophore transport (38, 39), and because a reduction in Chl is an indicator of iron starvation in Anabaena, the decrease in Chl possibly mirrors a fast iron starvation (47, 48). SjdR, however, is not involved in TonB-dependent schizokinen transport (39), and therefore, the observed reduction of the Chl content should not be related to iron uptake. Likewise, TonB₄ is not involved in iron uptake, and the cause of Chl decrease remains elusive.

Notably, l-tonB₂, with 2.6 ± 0.8 μg ml⁻¹, exhibited an elevated carotenoid concentration under ambient light compared to wild type. Under low light, the l-tonB₂ carotenoid concentration was enhanced. In contrast to that, l-tonB₄ had a significantly lower carotenoid level under both ambient and low-light conditions, and l-sjdR under low light. There were no significant differences of chlorophyll-to-carotenoid ratio under ambient or low-light conditions with the exception of a small decrease in l-tonB₃ (ambient light). Under both conditions, l-tonB₂ and l-tonB₃ had lower ratios than the wild type and the other mutants (Table 1).

The concentrations of phycocyanin were similar in all strains except l-tonB₂, in which it was significantly increased by a factor of 1.5 and 2 (low and ambient light, respectively).

The Chl concentration was higher in all strains under normal (ambient) light or low-light conditions compared to high light (Table 1). For the wild-type strain, an average Chl concentration per OD₇₅₀ of 7 ± 1 μg ml⁻¹ was determined under high light. Overall, the carotenoid level tended to be lower under high light than under ambient light as well (Table 1), but the difference between these conditions was not as drastic as in the case of Chl. This resulted in a lowered ratio of Chl to Car. These data are consistent with previous observations (49–51). Only in l-sjdR was the carotenoid content significantly decreased compared to the wild type (Table 1). This reduction did not result in compromised growth (Fig. 1). Phycocyanin content did not differ significantly between the strains under high light. While the PC content of l-tonB₂ increased relative to ambient light, the PC content of the other strains did not differ significantly between light conditions.

In summary, l-tonB₂ contained a strongly lowered level of phycocyanin under ambient and low-light conditions and all mutants showed a mildly lower chlorophyll content compared to wild type under the same condition. Therefore, the color alterations observed for l-tonB₂ likely result from the observed alterations in cellular pigmentation.

The cellular metal content is altered in tonB mutants. The carotenoid concentration in cyanobacteria is affected by metal availability. Elevated Cu, Zn, or Co concentrations result in an elevation of the carotenoid content in Anabaena oryzae (52). Similarly, Ca supplementation enhances the level of pigments in Anabaena (53, 54). Thus, the cellular metal concentrations in l-tonB₂, l-tonB₃, and l-tonB₄ were determined by inductively coupled plasma mass spectrometry (ICP-MS) analyses and compared to the wild-type concentrations that were described before (39) since SjdR is functionally not related to TonB-dependent transport (39), this strain was excluded from the further studies. Remarkably, alterations in metal concentrations were observed for all tonB mutants compared to the wild type. (i) l-tonB₃ and l-tonB₄ exhibited a decrease in cellular Mg and Co concentrations compared to the wild type (Table 2). (ii) In l-tonB₂ and l-tonB₃ the Mn concentration was decreased. In l-tonB₄ the level of Mn showed a large
**TABLE 1** Chlorophyll a, carotenoid, and phycocyanin concentrations in the wild type and the tonB mutants

| Strain   | Chl/OD750 (µg ml⁻¹) | Car/OD750 (µg ml⁻¹) | PC/OD750 (µg ml⁻¹) | Ratio, Chl/Car | Chl/OD750 (µg ml⁻¹) | Car/OD750 (µg ml⁻¹) | PC/OD750 (µg ml⁻¹) | Ratio, Chl/Car | Chl/OD750 (µg ml⁻¹) | Car/OD750 (µg ml⁻¹) | PC/OD750 (µg ml⁻¹) | Ratio, Chl/Car |
|----------|---------------------|---------------------|---------------------|-----------------|---------------------|---------------------|---------------------|-----------------|---------------------|---------------------|---------------------|-----------------|
| WT       | 7 ± 2               | 2.2 ± 0.2           | 3 ± 1               | 27 ± 3          | 9 ± 2               | 2.4 ± 0.3           | 3.6 ± 0.3           | 27 ± 6          | 9 ± 1               | 2.5 ± 0.4           | 3.7 ± 0.3           | 25 ± 3          |
| I-sjdR   | 5 ± 2               | 1.6 ± 0.4           | 3 ± 1               | 22 ± 5          | 7 ± 2               | 2.0 ± 0.7           | 3.6 ± 0.5           | 21 ± 7          | 7 ± 2               | 1.8 ± 0.6           | 3.9 ± 0.8           | 24 ± 5          |
| I-tonB2  | 4.4 ± 0.8           | 2 ± 1               | 2 ± 1               | 21 ± 3          | 7 ± 3               | 2 ± 1               | 3.0 ± 0.6           | 12 ± 2          | 7 ± 3               | 3 ± 1               | 3.2 ± 0.8           | 15 ± 4          |
| I-tonB3  | 6 ± 2               | 2.1 ± 0.5           | 2.7 ± 0.9           | 30 ± 3          | 7 ± 2               | 2.6 ± 0.8           | **2.9 ± 0.5**       | 24 ± 7          | 8 ± 2               | 2.3 ± 0.4           | 3.4 ± 0.9           | 28 ± 5          |
| I-tonB4  | 5 ± 2               | 2.0 ± 0.3           | 3 ± 1               | 27 ± 9          | **7 ± 1**           | **2.0 ± 0.4**       | 3.6 ± 0.5           | 24 ± 9          | **7 ± 1**           | **1.8 ± 0.5**       | 4.2 ± 0.5           | 21 ± 6          |

Given are the concentrations in cultures grown for 7 days under high, ambient, or low light (140, 70, or 15 µmol photons m⁻² s⁻¹, respectively) in YBG11 medium. The average from 4 to 10 biological replicates and the standard deviation are given normalized to an OD₇₅₀ of 1. Values in the mutants that significantly differ from wild-type values are indicated in bold (P < 0.05, Student’s t test with Bonferroni correction). Chl, chlorophyll a; Car, carotenoid; PC, phycocyanin.
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**TABLE 2** Metal concentration in wild-type *Anabaena* and the mutants I-tonB2, I-tonB3, and I-tonB4 expressed as atoms per OD$_{750}$

| Metal | WT | I-tonB2 | I-tonB3 | I-tonB4 | I-tonB2 | I-tonB3 | I-tonB4 |
|-------|----|---------|---------|---------|---------|---------|---------|
| Mg    | 500 ± 40 | 410 ± 20 | 350 ± 40 | 280 ± 10 | 0.82    | 0.70    | 0.56    |
| Ca    | 61 ± 7   | 70 ± 50  | 60 ± 20  | 40 ± 20  | 1.15    | 0.98    | 0.66    |
| Mn    | 51 ± 2   | 17 ± 1   | 11 ± 1   | 30 ± 20  | 0.33    | 0.22    | 0.59    |
| Fe    | 34 ± 2   | 35 ± 5   | 29 ± 3   | 30 ± 8   | 1.03    | 0.85    | 0.88    |
| Co    | 1.8 ± 0.1| 1.8 ± 0.1| 1.2 ± 0.1| 1.3 ± 0.1| 1.00    | 0.66    | 0.72    |
| Cu    | 5.1 ± 0.5| 6.7 ± 0.6| 5 ± 2    | 6 ± 1    | 1.31    | 0.98    | 1.18    |
| Zn    | 5.4 ± 0.3| 6.1 ± 0.4| 5 ± 4    | 7 ± 3    | 1.13    | 0.93    | 1.29    |
| Mo    | 6.4 ± 0.2| 9.8 ± 0.7| 7.3 ± 0.6| 4.8 ± 0.4| 1.53    | 1.14    | 0.75    |

$^a$The ratio of the metal content in wild type and mutants is shown. The values represent averages and standard deviation from three biological measurements. The bold letters indicate significant changes in the ratio column ($P < 0.05$, Student’s t test).

variation, but Mn was always at a lower level than in the wild type. The Mo concentration was (iii) enhanced in I-tonB2 and (iv) reduced in I-tonB4. (v) In I-tonB2 cells an elevated Cu concentration was observed compared to the wild type. Notably, after 7 days of growth in YBG11 medium an alteration in the Fe concentration was not observed, although TonB3 is supposed to be involved in ferric siderophore transport.

Ca and Zn levels were not drastically altered in the mutants. In contrast, Cu, which influences the carotenoid content in cyanobacteria (52), was enhanced in I-tonB2, in which the carotenoid level was found to be enhanced as well (Tables 1 and 2). In turn, in I-tonB3 and I-tonB4 the Co levels were reduced, which in the case of I-tonB4 could be related to the reduced carotenoid level (Tables 1 and 2). In summary all tonB mutants exhibited alterations in the cellular metal levels compared to wild type to different extents. Whereas I-tonB3 and I-tonB4 were reduced in Co, Mn (I-tonB3), or Mo (I-tonB4), only I-tonB2 did, besides the observed reduction in Mn, significantly enrich metals, namely, Cu and Mo.

**Membrane properties and transcriptional alterations in I-tonB2.** Considering the relative accumulation of Cu and Mo in I-tonB2 and the tendency of this strain to form aggregates in solution, a modification in the cell surface could cause the mentioned effects. Therefore, lipopolysaccharide (LPS) was extracted from the wild-type and I-tonB2 strains and separated by SDS-PAGE. Reproducibly enhanced signals for the O-antigen ladder were observed for the tonB2 mutant compared to wild type, in which the O-chain was barely visible when similar amounts of LPS extracts were loaded (Fig. 3A and Fig. S2). This confirms an increased synthesis of LPS in the mutant strain that could result from an aberrant regulation.

LPS structure as well as carotenoids are known to influence membrane properties (55–59). In cyanobacteria, carotenoids are found in all membranes including the outer membrane (60–62). Therefore, the integrity of the OM in the tonB mutants was tested by spotting suspensions of the strains on plates containing antibiotics or SDS. Here, I-sjdR was utilized as an unrelated control strain bearing a plasmid insertion.

Interestingly, I-tonB2 exhibited an increased resistance toward SDS, erythromycin, and neomycin compared to the wild type (Fig. 3A). In addition, I-tonB3 and I-tonB4 were more resistant toward the selected antibiotics, but the effect was not as pronounced as for I-tonB2. Moreover, I-sjdR grew in a similar manner as I-tonB2 in the presence of SDS but was as sensitive toward the tested antibiotics as the wild type.

The reduced susceptibility of I-tonB2 suggests a limited uptake of the selected compounds into the cell and confirms an alteration to the cell envelope. Typically, porins mediate the transport of certain antibiotics across the OM, and thus, porin mutants often display hyperresistance toward those compounds (63). Although it has been discussed that lipophilic macrolide antibiotics likely enter the cell through diffusion across the membranes and not through porins (17), for *Anabaena* a relation to porin function...
has been proposed (64). Thus, the transcript abundance of nine genes coding for porin-like proteins (1, 65) was examined in I-tomB2.

Remarkably, the transcript abundance of six genes coding for putative porins was reduced in I-tomB2 compared to the wild type after 7 days of growth in YBG11 medium (Fig. 3B). Among those, the transcript abundance of all7614, all4499, and alr4550 was most drastically reduced by 54-, 37-, and 36-fold, respectively. The transcript level of the three genes alr4741, alr3608, and alr3917 was higher in the mutant than in the wild type. The maximum increase was, however, 5-fold (alr3917), which appears to be only moderate compared to the drastic downregulation of other putative porin-encoding genes (Fig. 3B).

In addition to their impact on membranes, carotenoids are involved in the protection of the photosynthetic apparatus from reactive oxygen species (ROS). Thus, an increased carotenoid concentration might result from an elevated level of oxidative stress. To test whether I-tomB2 exhibits a higher oxidative stress level, the expression of superoxide dismutase A (SodA, MnSOD) and B (SodB, FeSOD) was analyzed. Both enzymes confer resistance to oxidative stress under distinct nitrogen regimes (66). The abundance of both transcripts was reduced in I-tomB2 in comparison to the wild type after 7 days of cultivation in YBG11 medium (Fig. 3C). This suggests that the tonB2 mutant does not suffer from increased oxidative stress. The assessment of reactive oxygen (ROS) production in I-tomB2 and wild type by the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) confirmed this finding, as no difference in fluorescence that represents cellular ROS content was detected (Fig. S3).

**I-tomB2 and I-tomB4 are impaired in nitrogenase activity.** The nitrogenase enzyme in Anabaena has Mo as a cofactor (12). Notably, I-tomB2 and I-tomB4 were altered in the cellular Mo concentration compared to wild type (Table 2), and the abundance of the tonB4 transcript was found to be enhanced in the absence of combined nitrogen (38). Therefore, the nitrogenase activity was determined for I-tomB2 and I-tomB4 by means of
the acetylene reduction assay. Under oxic conditions, a nitrogenase activity of 0.8 ± 0.7 or 1.3 ± 0.9 nmol ethylene/μg Chl · h was measured in I-tonB2 and I-tonB4, respectively (Fig. 4A), which was significantly lower than in the wild type (4.7 ± 2.6 nmol ethylene/μg Chl · h). Under anoxic conditions, the nitrogenase activity in both mutants was enhanced compared to the oxic conditions. However, the average values of 2 ± 2 and 3 ± 2 nmol ethylene/μg Chl · h for I-tonB2 and I-tonB4, respectively, were again significantly lower than the nitrogenase activity of the wild type. Hence, both strains showed a reduced but not abolished nitrogen fixation capacity.

Considering the alterations in nitrogenase activity, the heterocysts of the tonB mutants were analyzed under the light microscope and compared to those of the wild type. I-tonB2 and I-tonB4 mutants differentiated wild-type-like heterocysts as judged from light microscopy (Fig. 4B), and the formation of the constricted heterocyst pole was not altered as determined with fluorescently labeled vancomycin (Fig. 4C). Therefore, TonB2 and TonB4 are not essential for heterocyst differentiation, although tonB4 expression is upregulated under nitrogen starvation (38) and nitrogenase activity in the mutants is somewhat reduced compared to the wild type (Fig. 4A).

Iron starvation and siderophore transport capacities of tonB mutants. Next, we addressed the question whether TonB2 and TonB4 are involved in the transport of ferric siderophores, as described for TonB3 (39). First, a potential complementation of single tonB insertions through enhanced expression of other tonB genes was analyzed by
quantitative reverse transcription-PCR (qRT-PCR). SjdR was excluded from this analysis, as it is no longer considered a TonB candidate (39). The transcript abundance of the nonmutated tonB genes was determined after 7 days of iron starvation and normalized against the expression of the housekeeping gene rnpB. Starvation was applied since the expression of genes involved in TonB-dependent transport in *Anabaena* is triggered in the absence of iron (38, 42, 48, 67).

The abundance of tonB2 transcripts in I-tonB3 and I-tonB4 mutants was not significantly different from the wild-type level, both under standard conditions and after iron starvation (Fig. 5A, left). Further, no significant alteration of tonB2 expression was observed in response to iron starvation in any of the strains tested. Similarly, the expression of tonB4 was not significantly affected by iron starvation (Fig. 5A, right) but was enhanced in I-tonB2 compared to the wild type when strains were grown in YBG11 medium.

The tonB3 transcript was increased under iron starvation in all strains (Fig. 5A, middle), although the change in I-tonB4 was not significant (Fig. 5A, middle). Moreover, tonB3 expression in I-tonB2 was enhanced compared to the wild type when cells were grown in YBG11 medium but not after iron starvation. This may suggest an early level of starvation in I-tonB2, since tonB3 expression is triggered upon iron depletion.
However, no changes in Chl (as indicator of iron starvation) and no significant alteration in the cellular iron content were observed in I-tonB2 compared to the wild type (Tables 1 and 2), which does not support iron starvation in I-tonB2.

In contrast, tonB3 expression was not as drastically enhanced after iron starvation in I-tonB4 as observed in the wild type or I-tonB2. This suggests a comparatively lower level of starvation in the tonB4 mutant. To test this, wild-type and mutant cultures were grown for 25 days in iron-free medium and the Chl level was determined as an indicator for the degree of starvation. Indeed, the Chl content was more drastically decreased in the wild type (0.38 µg ml⁻¹ at an OD₇₅₀ of 1) than in I-tonB4 (1.4 µg ml⁻¹ at an OD₇₅₀ of 1) (Fig. 5B). Thus, the tonB4 mutant shows an unusually weak iron starvation phenotype, which explains the reduced induction of tonB3 expression during starvation.

In conclusion, no drastic alterations of tonB transcript abundance were observed between the mutants and the wild type after iron starvation. Thus, complementation effects between the tonB genes appear not to take place according to the analysis of the phenotypes under iron starvation. Then, the growth behavior in iron-free medium was analyzed for I-tonB2 and I-tonB4 (Fig. 5C). The cultures were prestarved prior to monitoring growth, since iron starvation in Anabaena which involves the expression of relevant uptake systems requires initiation time (67). We did not observe a compromised growth of the mutants compared to the wild type, which argues against a direct function of TonB2 and TonB4 in iron uptake. To support this conclusion, the transport rates of schizokinen loaded with ⁵⁵Fe were determined. Although the normalized uptake rates of 4.9 × 10⁻⁹ mol Fe/liter · h for I-tonB2 and 7.0 × 10⁻⁹ mol Fe/liter · h for I-tonB4 were slightly lower than the wild-type rates (8.5 × 10⁻⁹ mol Fe/liter · h), no significant difference could be established (Fig. 5D). Thus, TonB2 and TonB4 do not seem to function in schizokinen transport in Anabaena.

**DISCUSSION**

The TonB-ExbB-ExbD system is conserved among Gram-negative bacteria, as approximately two-thirds of these bacteria have at least one TonB-encoding gene (3). Notably, many species encode multiple TonB copies in the genome (3), and diverse functions have been assigned to the different genes in one species (30, 31, 36). In Anabaena four genes have been annotated as encoding possible TonB proteins. For SjdR (formerly annotated as TonB1), a function distinct from TonB-dependent transport was described (39). With respect to the three TonB-like proteins of Anabaena that exhibit a conserved domain architecture (Fig. 1), TonB3 represents the central component of the siderophore-dependent iron uptake system (38, 39). In contrast, TonB2 and TonB4 are likely not related to iron uptake, since schizokinen uptake is not impaired in their mutants (Fig. 5). The abnormal iron starvation behavior of I-tonB4 requires further investigation and cannot be explained at this stage. Notably, Anabaena is capable of transporting other siderophores besides the endogenously synthesized schizokinen, such as aerobactin, ferrioxamine B (48, 67, 68), or ferrichrome (unpublished data). Aerobactin penetrates through the same TBDT as schizokinen does (67); therefore, it is likely that aerobactin transport is TonB3 dependent as well (38). That TonB2 or TonB4 is involved in the TonB-dependent transport of other iron-containing substrates cannot be excluded. However, because the growth of the mutant cultures is not affected in the absence of iron, a relation to ferric siderophore transport seems unlikely. Besides ferric siderophores, also other substrates such as cobalamin, nickel, or sugars are transported in a TonB-dependent manner in some organisms (16, 19). This further broadens the spectrum of possible functions for TonB2 and TonB4 that will need to be investigated, especially considering the high number of 22 TBDTs that are predicted from the Anabaena genome (37).

The phenotypes of I-tonB2 and I-tonB4 were investigated in order to figure out possible functions. Differential characteristics of the strains were unveiled, including alterations of the cellular metal concentrations that were observed, albeit to different
extents, for all tonB mutants. Both I-tonB3 and I-tonB4 show decreased cellular contents of Mg and Co, and I-tonB4 also shows a decreased content of Mo (Table 2). Molybdenum is the cofactor of the nitrogenase enzyme (12), which might contribute to the reduced nitrogenase activity in I-tonB4 (Fig. 4). Moreover, in I-tonB4 the Chl level is comparatively decreased (Table 1), which in turn might be a consequence of the reduced Mg content in this strain. Notably, in I-tonB4 the regulation of Chl synthesis seems to be generally affected, considering the remarkably high Chl concentration after iron starvation.

Besides a decrease in Mn in I-tonB2 and I-tonB3, an accumulation of Cu and Mo was observed in I-tonB2 that might be caused by altered outer membrane properties. The anionic LPS surface is involved in metal binding in bacteria (69–72), and it was reported previously that cyanobacterial negatively charged exopolysaccharides are capable of binding metals (73) and might even accumulate trace metals under starvation conditions. Thus, the enhanced LPS production in I-tonB2 might (i) lead to an enhanced adsorption of certain metals that subsequently diffuse into the cell and (ii) cause the formation cell aggregates. LPS is thought to have an important role in porin trimerization, stability, and conductance (74–76). In the so-called deep rough mutants, strains that are compromised in LPS synthesis and thus produce only truncated LPS, a smaller amount of protein is present in the OM (17). Additionally, those mutants are increasingly susceptible to SDS or hydrophobic antibiotics (77, 78). An opposite effect might take place in I-tonB2, in which the excessive LPS production might result in the monitored decrease in susceptibility toward drugs, possibly reinforced by the reduction of the expression of genes encoding porins observed in this strain. Although it has been speculated that aminoglycoside antibiotics cross the OM through a diffusion-based self-promoted pathway in which the LPS surface is involved (17, 79), for Anabaena a relation of erythromycin uptake to porin function has been established (64). In the I-tonB2 mutant most of the genes coding for porins are downregulated, among them the porins described to be most abundant in Anabaena (40, 65). The decreased porin expression could display a feedback transcriptional response to a putatively enhanced substrate (metal) diffusion into the cell, reflected by a higher metal adsorption in I-tonB2. Hence, considering these characteristics a TolA-like function could be proposed for TonB2 rather than a TonB-like function.

The TolA-TolQ-TolR system embedded in the PM (Tol system here) (80) is structurally related to the TonB system. The C-terminal domains of TonB and TolA are structurally analogous (81), and it is assumed that the Tol system is involved in maintaining OM integrity (82–84). TolA interacts with trimeric porins of E. coli (85, 86), and the Tol-Pal system constitutes a component of the divisome involved in cell constriction and peptidoglycan remodeling (87, 88). Moreover, TolA might modulate the expression of LPS components in E. coli (89, 90), which also might be the case for TonB2.

The lack or low dosage of any TonB-like protein induces pigment alterations in Anabaena (Table 1). The reduction of the phycocyanin level in I-tonB2 is also reflected in the comparatively bright color of its cultures (Fig. 2). Phycocyanin-containing cyanobilins are accessory pigments that harvest light and transfer energy to photosystem II. A possible explanation for the modifications in pigment content is the observed alterations in cellular metal contents because different metal treatments are known to affect cyanobacterial pigment concentrations. For example, the treatment of Anabaena oryzae with 1 to 100 ppm of Cu resulted in an increased carotenoid concentration after 6 to 8 days of incubation (52).

In summary, the four TonB-like proteins found in Anabaena apparently take over distinct functions. Neither I-toB2 nor I-tonB4 is drastically reduced in schizokinen uptake capacity, which suggests that TonB3 exclusively mediates schizokinen transport in Anabaena. On the other hand, both tonB2 and tonB4 mutants are compromised in the production of full nitrogenase activity, especially under oxic conditions, although
heterocyst differentiation seems not affected. TonB2 influences OM properties, including LPS synthesis, with an effect on susceptibility toward antibiotics and porin abundance (as deduced from porin gene expression), and its role might be related to that of the Tol system as discussed above. The role of TonB4 is elusive, consistent with its peculiar predicted structure (Fig. 1), although we note that its mutant is affected in the cellular levels of several metals, including Mo, that may result in the observed low nitrogenase levels.

MATERIALS AND METHODS

Anabaena culture conditions. Anabaena (also known as Nostoc) sp. strain PCC 7120 was stored on plates of BG11 medium with 1% (wt/vol) Bacto agar (BD Biosciences) (91). For liquid culturing either BG11 (91) or YBG11 medium (92) was used. Anabaena cultures were grown under constant shaking at 90 to 100 rpm and constant illumination (ambient light, 70 μmol photons m⁻² s⁻¹; Osram L 58 W/954-154 Lumilux de Luxe, daylight) at 28°C. In the case of mutant strains 5 μg ml⁻¹ of both spectinomycin dihydrochloride pentahydrate (Sp; Duchefa Biochemie) and streptomycin sulfate (Sm; Roth) was added. The growth was monitored spectrophotometrically in terms of optical density at 750 nm (OD750). For growth analysis on plates, 5 μl of cell suspensions with an OD750 of 1 was spotted in a dilution series (1, 1:10, and 1:100), and representative results are shown.

DNA extraction, molecular cloning, and generation of Anabaena mutants. Transformation of E. coli and isolation and manipulation of plasmid DNA were performed according to standard protocols (93). Anabaena genomic DNA (gDNA) was isolated as described previously (94) with modifications: sodium dodecyl sulfate was not added, and the phenol extraction was done once followed by two washing steps with 400 μl of chloroform.

The Anabaena tonB mutants AFS-I-sjdR, AFS-I-tonB2, AFS-I-tonB3, and AFS-I-tonB4 were utilized in this study and have been introduced previously (38, 39). The annotation stands for Anabaena mutant generated in Frankfurt, Germany by the Schleiff Lab by plasmid insertion; for better readability “AFS-” is omitted throughout the text. In brief, internal fragments of the single genes were ligated into vector pCSV3 (95, 96) in the case of I-tonB2, I-tonB3, and I-tonB4 or pCSEL24 (97) in the case of I-sjdR, both carrying spectinomycin and streptomycin resistance markers. The oligonucleotides and the plasmids used in this study are listed in Tables S1 and S2 in the supplemental material, respectively. Plasmids were transferred to Anabaena with the triparental mating method as previously described (40, 97–99). The Anabaena strains analyzed in this study are listed in Table S3. The genotype of the exconjugants was tested by PCR, in which an oligonucleotide specific for the plasmid in combination with an oligonucleotide specific for the gene region was used.

Short-term siderophore transport measurements. The transport rates of ferric schizokinen were determined as described earlier (67, 100). Schizokinen was ordered from EMC Microcollections, and 55FeCl₃ was purchased from Perkin-Elmer. A single measurement of 15 nM 55Fe-schizokinen was used in single measurements, and the cell suspension utilized for measuring was inoculated at an OD₃₅₀ of 0.05. Cultures were prestarved in iron-free YBG11 before the measurements were conducted, and the degree of starvation was estimated by the chlorophyll a (Chl) concentration at an OD₇₅₀ of 1, as previous studies indicated that the Chl concentration per cell decreases in Anabaena with ongoing iron starvation (48). The Chl concentrations of experimental cultures are given in Table S4.

Inductively coupled plasma mass spectrometry (ICP-MS). The cellular metal concentrations in Anabaena were determined as described earlier (103). In brief, cultures grown for 7 days in YBG11 were harvested by centrifugation and washed twice with 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 5), 10 mM EDTA. Cells were resuspended in 5 ml double-distilled water, and 1 ml was subjected to inductively coupled plasma mass spectrometry (ICP-MS) measurement. The OD₇₅₀ of the suspension was measured, and cells were counted for normalization. Samples were digested overnight at 120°C in 7 M HNO₃, and then material was diluted to 5% HNO₃, for measurement.

Pigment extraction. The measurements of Chl and carotenoid (Car) concentrations were performed with methanolic extracts as previously described (102, 103). The Chl concentration was determined with the formula Chl (μg/ml) = 12.9447(ΔA₇₅₀ − ΔA₆₄₅) and the carotenoid concentration was calculated with the formula Car (μg/ml) = (1,000ΔA₅₇₄ − 2.86 × (Chl (μg/ml))) / 221.

To estimate the Chl concentration for experimental cultures, a simplified equation was utilized, as follows: Chl (mg/ml) = A₆₄₅/74.5 (104).

Determination of phycocyanin content was carried out as described by Horvath et al. (105). In brief, cultures were harvested by centrifugation (1 min, 4,000 × g) and concentrated to a volume of 0.3 to 2 ml. OD₇₅₀ of the concentrates was determined in 1:100 dilution, and the remaining material was frozen at −20°C. After thawing at room temperature (RT), a defined volume was diluted to 4 ml with phosphate-buffered saline (PBS) and subjected to sonication with a sonication probe at full power for 90 s. Cell debris was removed by centrifugation (1 min, 20,000 × g). PC concentration was calculated from UV-visible (UV-Vis) absorption spectra as described previously (106). PC (μg/ml) = 154(ΔA₆₅₅ − ΔA₆₃₅).

Van-FL staining and microscopy. For visualization of peptidoglycan, the filaments were stained with BODIPY FL vancomycin (Van-FL) (Invitrogen) as previously described (107). For microscopy a piece of agar was excised and reversedly placed onto a coverslip that was utilized as a microscope slide. Images were recorded with a Zeiss LSM 780 using 63× or 40× objectives with immersion oil. Diameter of the pinhole was set to 69.4 μm, and a 488-nm laser source was used for excitation.
Specific Van-FL fluorescence was recorded between 500 and 550 nm, Chl autofluorescence was recorded between 630 and 700 nm. Light microscopy images were recorded with a Thorlabs DCC1645G-HQ camera.

RNA extraction, RT-PCR, and qRT-PCR. RNA was isolated either from strains that had been grown for 7 days in YBGI1 medium or, in the case of iron starvation, from samples incubated for 7 days under culture conditions in iron-free YBGI1 medium. RNA was extracted with TRIzol (Thermo Fisher Scientific) according to previously described protocols (42). After DNase I digestion the absence of DNA in RNA samples was verified by PCR. Revert Aid reverse transcriptase was used for first strand cDNA synthesis (Thermo Fisher Scientific). qPCR was performed with a StepOnePlus cycler from Thermo Fisher Scientific, and the cycling conditions were set as 50°C, 2 min, and 95°C, 2 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Experiments were performed with PowerUp SYBR green Master Mix from Applied Biosystems. rnpB served as control gene. The threshold cycle (CT) value of the gene of interest was normalized to the rnpB CT value (ΔCT) and, if indicated, further normalized to the corresponding ΔΔCT of the wild-type (ΔΔCT) (108).

Extraction and analysis of lipopolysaccharide. Lipopolysaccharide (LPS) was extracted from wild type and I-tonB2, and the cultures were grown in flasks with constant shaking for 8 days or in tubes with supplementation of 1% CO2 in YBGI1 medium for 7 days (Fig. S2). Cells corresponding to an OD600 of 3 in 1 ml were harvested for LPS extraction. LPS was extracted as described previously (109) with modifications. In brief, the cells were harvested by centrifugation (6,000 × g, 5 min), washed with 1 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4), and resuspended in 50 μl lysing buffer (2% sodium dodecyl sulfate, 4% β-mercaptoethanol, 10% glycerol, 1 M Tris-HCl, pH 6.8, bromphenol blue). After the samples were heated at 100°C for 10 min, 10 μl of lysing buffer containing 100 μg proteinase K was added, followed by 60 min of incubation at 60°C.

The LPS was subjected to denaturing SDS-polyacrylamide electrophoresis utilizing a 12% acrylamide gel; afterward the gel was silver stained (110). For the loading control (large subunit of Rubisco is shown in figures), cells were treated as described for LPS extraction omitting proteinase K.

Nitrogenase activity. Nitrogenase activity was determined by the acetylene reduction assay (111) carried out under both oxic and anoxic conditions. Cells grown in 25 ml of liquid BG11 medium (supplemented with Sp/Sm when necessary) were harvested by centrifugation, washed with liquid BG11 medium, and incubated at 1 μg Chl ml−1 (without antibiotics) in 25 ml of liquid BG11 medium for 48 h. Cell suspensions of 2 ml at 6°C were incubated for 12 h at 30°C and 150 rpm. After incubation at 60°C for 30 min (30°C and shaking) before taking 1-ml samples for determination of ethylene by gas chromatography. For anoxic conditions, the sealed flasks were supplemented with 10 μM 3-(3,4-dichlorophenyl)-1,1-imethylurea (DCMU; from Sigma), bubbled with argon for 4 min, and incubated for 60 min (30°C and shaking) before acetylene injections, and then 1-ml samples were taken for ethylene determination. Under both conditions, samples for ethylene determination were taken for up to 2 h.

Determination of ROS production. Intracellular conversion of dichlorodihydrofluorescein (DCHF) to dichlorofluorescein (DCF) was measured as established previously (112). Cultures were harvested by centrifugation (1 min, 4,000 × g) and rediluted to an OD600 of 1 with PBS. For comparison, ascorbic acid (100 μM final concentration) was added to some samples. Samples of 10 ml were subjected to the respective light treatment for 20 min. UV treatment was carried out by placing the samples in the dark, and ambient light treatment was carried out under cultivation conditions. After addition of 2,7-dichlorodihydrofluorescein (final concentration, 5 μM), samples were incubated for 1 h in the dark. Finally, cells were harvested and transferred to 96-well plates, and fluorescence and OD600 were determined using a microplate reader.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 1.3 MB.
FIG S2, PDF file, 1.6 MB.
FIG S3, PDF file, 0.2 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.1 MB.
TABLE S4, DOCX file, 0.01 MB.

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We declare that we have no conflicts of interest.

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