The TolC-like protein HgdD of the cyanobacterium *Anabaena* sp. PCC 7120 is involved in secondary metabolite export and antibiotic resistance*

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Running head: HgdD has a TolC-like function in *Anabaena* sp.

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**Keywords:** Cyanobacteria, antibiotics and metabolite export, Omp85-independent OMP insertion, TolC, porin

**Background:** The metabolite and antibiotic export system of cyanobacteria is largely unexplored.

**Results:** Secondary metabolites uptake involves porin-type activity and their export across the outer membrane the TolC-like protein HgdD.

**Conclusions:** The antibiotic export of cyanobacteria involves a proton-gradient-driven TolC activity and MFS-type proteins.

**Significance:** TolC of *Anabaena* sp. is placed in the context of antibiotic uptake and export.

**SUMMARY**

The role of TolC has largely been explored in proteobacteria, where it functions as a metabolite and protein exporter. In contrast, little research has been carried out on the function of cyanobacterial homologues and as a consequence, not much is known about the mechanism of cyanobacterial antibiotic uptake and metabolite secretion in general. It has been suggested that the TolC-like homologue of the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, termed heterocyst glycolipid deposition protein D (HgdD), is involved in both protein and lipid secretion. To describe its function in secondary metabolite secretion we established a system to measure the uptake of antibiotics based on the fluorescent molecule ethidium bromide. We analyzed the rate of porin-dependent metabolite uptake and confirmed the functional relation between detoxification and the action of HgdD. Moreover, we identified two major facilitator superfamily proteins that are involved in this process. It appears that anaOmp85 (Alr2269) is not required for insertion or assembly of HgdD, since an *alr2269* mutant does not exhibit a phenotype similar to the *hgdD* mutant. Thus, we could assign components of the metabolite efflux system and describe parameters of detoxification by *Anabaena* sp. PCC 7120.

**INTRODUCTION**

Detoxification by secretion systems is essential for all Gram-negative bacteria. It involves an energizing machine in the plasma membrane and a TolC (tolerance to colicins) protein in the outer membrane (1-4). The latter is involved in the secretion across the outer membrane of a variety of essential factors. For example, it is required for the assembly of the outer layer of the cell wall (e.g. 5), for the uptake of iron (6-8), or for export of compounds that have entered the periplasm from the surrounding medium and would cause severe damage if not cleared (3).

The TolC-like proteins in general have a low sequence identity, but they appear to be highly conserved at the structural level (9). The biochemical and structural analysis revealed a homotrimer forming a single channel-tunnel with a membrane-inserted β-barrel and a periplasm-spanning α-helical barrel (e.g. 10). To secrete its various substrates to the extracellular space, TolC proteins are known to be involved in three different secretion systems. First, TolC is part of the type I secretion system where it interacts with different inner membrane ABC-transporters involved in the secretion of proteinaceous toxins (e.g. 11). Second, it functions as an outer...
membrane factor for different superfamilies of multidrug efflux (MDE) pumps such as the resistance-nodulation-cell division-type (RND), AcrAB or the major facilitator superfamily (MFS)-type (Fig. 1; e.g. 1, 12). Third, it has been described as part of cation efflux pumps for extrusion of toxic metal ions (13). Thus, the substrate specificity is determined by the periplasmic and the inner membrane components of the complexes that are engaged. The contact between TolC and the inner membrane permease is established in the periplasm with the help of adaptor proteins, also called membrane fusion proteins (MFP; 14). The complexes are transient and once substrate secretion has been completed, the complex disengages and reverts to the resting state (e.g. 15).

The best described model for TolC-secretion complex assembly is the RND-type tripartite AcrAB-TolC-system of E. coli (e.g. 3). The model that is currently favored suggests a transient recruitment of TolC by the transporter AcrB, which is enforced by the plasma membrane-anchored MFP AcrA upon substrate interaction (16, 17). Substrate recognition can occur in the cytoplasm as well as in the periplasmic space, which requires the secretion of the substrate by MFS proteins. However, although the understanding of complex formation has improved in the last few years, it is still unclear how the recruitment of TolC by the right transporter in the right situation is regulated.

In contrast to proteobacteria, little is known about the function of TolC and Type I secretion systems in cyanobacteria. A TolC homologue of Anabaena sp. PCC 7120 (in the following referred to as Anabaena sp.) has been identified by proteomic analysis (18, 19) and its relation to the TolC family was confirmed by homology modeling and analysis of the corresponding mutant (5, 7, 20). Anabaena sp. is a filamentous, photosynthetic cyanobacterium, which under nitrogen deficiency develops specialized nitrogen-fixing cells, the heterocysts (21-23). It was shown that the transcript of the TolC-like gene is enhanced upon nitrogen starvation. In line with this, the mutant resulting in a loss of function displays an impairment in heterocyst development due to the missing heterocyst-specific glycolipid layer and was therefore termed heterocyst glycolipid deposition (HgdD; 5). Preliminary secretome analysis demonstrated the correlation of this phenotype with an altered secretome, leading to the proposal that HgdD is involved in protein secretion (5). Subsequent studies demonstrated an impairment of the same mutant in siderophore and glycolipid secretion (7, 21). Although it has been proposed that three distinct substrate categories —proteins, lipids and siderophores — are secreted by HgdD, the molecular properties of HgdD and its involvement in secretion of secondary metabolites such as antibiotics and toxins in cyanobacteria has so far not been described. In this paper, we examine the secretion system in Anabaena sp. and demonstrate that the TolC-like protein HgdD is a major component of the metabolite export system of this cyanobacterium.

EXPERIMENTAL PROCEDURES

Growth of Anabaena sp., DNA isolation and PCR reaction - Anabaena sp. wild-type and mutants were grown photo-autotrophically at 30°C in liquid BG11 medium (24,25) with and without (in the case of BG11 0) 17.6 mM NaNO₃ (7). Mutant strains were grown in the presence of 3 μg streptomycin ml⁻¹ and 3 μg spectinomycin ml⁻¹. Isolation of DNA from Anabaena sp. was performed as described (26). Techniques for manipulation of plasmid DNA (27), PCR using the Triple master PCR System (Eppendorf, G), and conjugal transfer of plasmids into Anabaena sp. (28) have been described.

Generation of Anabaena sp. mutants - The present study was carried out with Anabaena sp. strain PCC 7120 and several mutant derivatives described in Table 1. For the generation of alr2215 and all5346 (hgdC) single-recombinant insertion mutants, an internal fragment of the corresponding coding region was amplified by PCR on Anabaena sp. genomic DNA (oligonucleotides in Table S1) introducing EcoRI/EcoRV restriction sites. PCR products were subsequently cloned into pCSEL24, replacing the nucA-nuiA region and producing plasmids pMS1 and pMS2 (Table 2). For the generation of mutant strains, DNA transfer from Escherichia coli Anabaena sp. was performed by conjugation as previously described (29).

mRNA isolation and RT-PCR – mRNA isolation from Anabaena sp. has been described (e.g. 5, 7). In brief: RNA was isolated from 15 ml cells at OD₆₀₀ = 1 by centrifugation (5 min / 3200 x g). The pellet was suspended in 200 ml PBS, mixed...
with 350 ml of 4 M guanidin thiocyanate, 0.1 mM DTT, 20 mM sodium acetate pH 5.2, 0.5% N-lauryl sarcosin, 10 ml ml-1 β-mercaptoethanol and two small spoons of glass beads. 300 ml phenol (65°C) was added and the mixture was incubated at 65°C / 10 min with vortexing, followed by centrifuging (5 min / 20 800 x g / 4°C). The aqueous layer was mixed with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), centrifuged (20 800 g / 5 min / 4°C) and the aqueous layer again mixed with an equal volume of chloroform: After centrifugation (5 min / 20 800 x g / 4°C) the aqueous layer was supplemented with 250 ml of 70% ethanol. Further RNA isolation was performed with the NucleoSpin RNA II isolation kit (Machery & Nagel, G) including the DNase treatment according to the instructions of the manufacturer. Digestion of residual DNA was performed for 1 hr at 37°C using 10 U RNase-free recombinant DNase I (Roche Diagnostics, G). cDNA was derived from 1 µg total RNA through reverse transcription (RT), which was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR, (Invitrogen, US) for 1h at 42 °C.

**Counting and surface estimation of Anabaena sp. cells** - For the estimation of Anabaena sp. cell count, cells were grown in BG11 to OD 750=1 and subsequently fragmented by sonification to an average length of 2-10 cells per filament. Cells were counted using a Fuchs-Rosenthal counting chamber (Blaubrang, G).

To calculate the surface of an Anabaena sp. cell, the approximation for an ellipsoid surface (S) and the equation for the volume (V) of an ellipsoid were used:

\[ S \sim 4 \times \pi \times ((2 \times r_a^{1.6} \times r_b^{1.6} + r_b^{1.6} \times r_f^{1.6})/3)^{0.625} \]
\[ V = 4/3 \times \pi \times r_a \times r_b \times r_f^2 \]

**Microscopy** – For microscopic analysis, a Zeiss Axiophot microscope (Zeiss, G) with a 63x /1,4 Plan – APOCHROMAT object lens was used combined with a Color View XS photo system (Soft Imaging System, G). Transmission light and autofluorescence microscopy images were taken 30 min after EB incubation.

**Homology search for MFS-type proteins -** Uniprot IDs assigned to the MFS were downloaded from PFAM (PF07690, PF13347, PF05977, v26.0). By a Python script using Biopython (www.biopython.org) the respective sequences and taxonomy information were downloaded from UniProt (www.uniprot.org) and only bacterial sequences were selected for further processing. Redundant sequences were removed with cd-hit (30) and the remaining ~55,000 MFS sequences were clustered with CLANS (31) for 100,000 iterations. Sequences from Anabaena sp. were assigned a putative function if they clustered closely with a known and functionally characterized MFS protein: AmpG (P0AE16), Bcr (P28246), EmrB (P0AEJ0), EmrD (P31442), EntS (P24077), SetA (P31675), ShiA (P76350), MelB (P02921), or MdfA (P0AEY8) of E. coli.

**Ethidium uptake measured by quantification in extracellular medium -** Anabaena sp. cells were grown in BG11 medium to late logarithmic phase based on the growth curve analysis (OD 750=1.5). To determine uptake, the cells were adjusted to OD 750=1, concentrated tenfold by centrifugation, and resuspended in BG11 medium containing 20 mM HEPES, pH 7. After addition of ethidium bromide (EB), cells were transferred into mini filter spin columns (Sigma-Aldrich, G) containing a PET membrane with a pore size of 0.45 µm. After 2.5, 5, 15 and 30 min, the growth medium was separated from cells by centrifugation (30 sec / 4000 x g).

For the measurement of ethidium bromide secretion, cells were incubated for 30 min in different concentrations of EB and washed twice with BG11 medium using mini filter spin columns. EB concentration in the flow-through was determined by fluorescence measurement (Ex: 316 nm, Em: 620 nm) after the addition of 100 µg of herring sperm DNA with an Infinite® 200 PRO multimode reader (Tecan, Sw). Standard deviation was calculated on the basis of at least three independent experiments. For the competition of EB with Erythromycin, both substances were mixed in a 10:1, 1:1 and 1:10 molar ratio before addition to the concentrated Anabaena sp. cell suspension.

**Ethidium uptake measured by DNA intercalation** – 190 µl of concentrated Anabaena sp. cell suspension was loaded into a 96-well microplate (Corning, US). After addition of EB, the increase of fluorescence due to the intercalation of ethidium into endogenous nucleic acids after uptake was monitored every minute for 30 min by (Ex: 316 nm, Em: 620 nm). The procedure was controlled by analysis of EB concentration by an independent method as outlined in Figure 2. For that the extraction was performed as follows:
Cells were incubated in different EB concentrations and applied to filter spin columns. After 15 min cells were separated from incubation media by centrifugation at (30 sec / 4000 x g). Cells were recovered from the column in an equal volume of EB free incubation media and mechanically opened by using glass beads and a cutting mill (Retsch MM 300, G). After centrifugation (2 min / 20.000 x g) supernatant was cleared from photosynthetic proteins by adding a small amount of DEAE-sepharose CL-6B beads (Sigma-Aldrich, G). EB concentration was determent as described earlier.

Measurement of ethidium uptake after CCCP or spermidine / spermine treatment - 10 min prior to a measurement, 10 mM CCCP in DMSO was added to a final concentration of 200 µM to a concentrated Anabaena sp. cell suspension and incubated at room temperature. A control sample was treated in parallel with an equal amount of DMSO. The same procedure was applied for incubation with spermidine / spermine at the concentrations indicated in the figure legend.

Analysis of uptake results and mathematical concepts - The results were processed by Sigma Plot and, if not stated otherwise, analyzed by a least square fit analysis to the equations shown in Figure 1. For equation 2, the abbreviations used are listed in Figure 1 as well. Equation 2 is taken from (32).

RESULTS
Putative model for analysis of metabolite uptake - To describe the uptake and secretion of metabolites by Anabaena sp., we utilized the fluorescent dye ethidium bromide as a model substrate. We first defined a model for the analysis (Fig. 1 top), which is based on the established models for secretion by Gram-negative bacteria (1-4). We considered five independent reactions: the uptake by porins (k₁), the transfer into the cytoplasm (k₂), association and dissociation with and from DNA (k₃, k₋₃), the export into the periplasm by MFS proteins (k₋₂) and the export by TolC (k₋₁; 33). In general, the initial process across the outer membrane is defined by passive diffusion through porins (e.g. 34, 35). However, for simplicity we treated the reaction with a simple kinetic constant, which can be represented by k₁. In a first step, we determined uptake by analyzing the ethidium remaining in the growth medium. In this case we only considered the k₁ and k₋₁, which can be analyzed by the equation 1 ([E1] Fig. 1). Using this method we could determine the properties of the porins in the outer membrane and the rate of export catalyzed by the TolC-containing machinery as we do not discriminate between ethidium in the periplasm or cytosol.

In another step, we analyzed the uptake by measuring the intercalation of ethidium into intracellular DNA, which requires a two-state model. Given the dissociation constant of ethidium for DNA, about 4 µM (e.g. 36), and the high concentration of DNA and RNA in the cell, it is possible to combine k₂ and k₃ as well as k₋₂ and k₋₃. With this simplification, the reaction can be described analytically (Fig. 1, [E2], (32)). In this way, we were able to describe differences of the components involved in the export cycle between the wild type and mutants. However, the combination of k₂ and k₃ must be taken into account for the interpretation of the rate constants.

Uptake of ethidium by Anabaena sp. involves a porin type activity - We analyzed the uptake of ethidium by Anabaena sp. and AFS-I-hgdD (5, 7; Fig. 1, bottom, uptake). We incubated Anabaena sp. with various concentrations of ethidium bromide and analyzed the kinetics of uptake by least square fit analysis to [E1] (Fig. 3A). The used ethidium bromide concentration did not alter the morphology of the cells and the filament of wild-type Anabaena sp. or AFS-I-hgdD, respectively (Fig. 4). In the wild type, the equilibrium between uptake and export was reached after 500 sec while almost no export was detectable from the AFS-I-hgdD strain under the conditions used (Fig. 3A). The same result was observed for an independently generated hgdD deletion mutant, strain DR181 (5; Fig. S1). This supports the notion that the reduced export is the result of the loss of HgdD function. Consistent with this notion, none of the Anabaena sp. genes coding for putative porins (18, 19, 22) were altered in their expression in the hgdD mutant used in this study (Fig. 5).
which could be applied was 1 µM. However, even at this concentration a significant decrease of ethidium uptake was observed (Fig. 3B). Analyzing the uptake kinetics by [E1] revealed a decrease of k1 in relation to the SPM/SPD concentration, which led to half the maximal inhibition at a concentration of about 0.7 µM (Fig. 3C). This is in the same range as the SPM concentration at which the half-maximum of the SPM-dependent change of closing frequency of OmpC or OmpF from *E. coli* was observed (38).

To further describe the properties of the porins, we subsequently determined the initial rate V0. The uptake was analyzed by

\[ V_0 = P \times A \times (C_o - C_p) \]

describing the relation for a passive pore (e.g. 34), where V0 is the rate of uptake, P the permeability coefficient (cm sec⁻¹), A the surface area of a cell (cm² mg⁻¹) and C the concentrations in the extracellular space and the periplasm. To apply this equation we estimated the cell surface present in our assay as described below.

By counting cells we observed an average of 2.8*10⁷ cells ml⁻¹ at an OD 750nm=1. A cell has an average length of 2.5 µm and an average width of 2.2 µm as determined by electron microscopy (e.g. 5). This leads to a cell volume of about 6.3 µm³ and a cell surface of approximately 17 µm². The cell-cell contact is approximately 1.5 µm in diameter (leading to an area of ~2 µm²). Thus, the contact sites reduce the surface of a single cell by about 15%, which is in the range of the error for the determination of cell dimensions and thus can be neglected. Accordingly, we estimated a cell volume of 1.8 mm³ and a surface of 48 cm² * ml⁻¹ cell culture at OD 750nm=1. In addition, the cell density of OD 750nm=1 reflects a dry mass of about 0.7 mg * ml⁻¹, which at least in the case of the filamentous cyanobacterium *Gloeotrichia echinulata* was shown to be linearly related to the OD measured at this wavelength (e.g. 39). Thus, we had an estimated surface of 69 cm² * mg⁻¹ (DW) in our experiments.

Using the initial rate, the equation can be simplified to

\[ V_0 = P \times A \times C_o \]

reflecting a simple linear behavior (Fig. 2D). V0 was normalized to the dry weight of the cells used. As a result, we obtained for P * A (1.03±0.08)*10⁻⁴ cm² * sec⁻¹ * mg⁻¹ and thus a permeability coefficient of 1.5 * 10⁻⁶ cm * sec⁻¹ (Table 3). This value is comparable to the one obtained for porin-dependent transport of other, similarly sized compounds in proteobacterial systems (e.g. 40, 41).

**Ethidium export in Anabaena sp. is HgdD-dependent** - Next we aimed to confirm that HgdD is involved in metabolite export. We incubated *Anabaena* sp. and the *hgdD* mutant with ethidium bromide, calculated the intracellular concentration of ethidium for each strain and analyzed the export by its re-occurrence in the medium. We determined the initial rate (v0) as suggested for TolC-dependent efflux (34), which was analyzed with respect to the pre-incorporated concentration (Fig. 6). However, this method is error prone at multiple levels. The starting concentration calculated was normalized to the reaction volume and not to the intracellular volume. Furthermore, we could not quantify to which extent ethidium was distributed between the periplasmic space and the cytoplasm. Thus, the values determined are only for comparison between the two genotypes and do not reflect the exact kinetic parameters of the TolC system.

Comparing the Michaelis-Menten constant determined for the wild type and for AFS-I-hgdD, we observed a difference of 5 orders of magnitude. As the concentration of ethidium in the *Anabaena* sp. cells was underestimated while considering the concentration of the entire reaction volume for the calculation, we could conclude that the TolC-independent export has a Michaelis-Menten constant in a high Molar range. The maximal rate is higher in the mutant than in the wild type as well (Fig. 6, Table 3). This suggests that TolC-independent export from the mutant strain occurs by a process – most likely passive diffusion.

**HgdD is involved in general antibiotic export in Anabaena sp.** - Based on the observations described above, one may assume that HgdD is involved in a general antibiotic- and toxin-export pathway. To test this hypothesis we used erythromycin to compete for both uptake and export of ethidium. Increasing concentrations of erythromycin were added to the measurement of the uptake kinetics of ethidium (Fig. 7A). The analysis by E1 (Fig. 1) revealed the import and export rates in the absence, the presence, and tenfold excess of erythromycin compared to 12.5 µM ethidium. We analyzed the erythromycin concentration-dependent rates using a hyperbolic equation (Fig. 7B) and determined an IC₅₀ value of 8.6 µM and 12.9 µM for k₁ and k₋₁. This reflects
an ethidium/erythromycin ratio of 0.7 for uptake and 1.0 for secretion for the applied concentration of ethidium. Considering the IC\textsubscript{50} values that were obtained, we can conclude that the porin system and the TolC system can transport both ethidium (394 Da) and erythromycin (734 Da). However, the ratio between the uptake rates of ethidium and erythromycin might be concentration dependent as the two compounds are structurally distinct. Nevertheless, by this observation we can generalize the conclusion that HgdD is involved in export of secondary metabolites such as antibiotics.

**Intercalation of ethidium by Anabaena sp. and AFS-I-hgdD** - Next we analyzed the uptake of 12.5 µM ethidium by Anabaena sp. and AFS-I-hgdD using the intercalation assay (Fig. 1). We observed a significant intercalation of ethidium by AFS-I-hgdD, but not by the wild type (Fig. 8). This confirms the observation made while measuring the uptake by the two strains (Fig. 3-7). Analyzing the intercalation by E2 (Fig. 1; Fig. 8A, lines) we observed a 100-fold lower rate constant k\textsubscript{1} for AFS-I-hgdD than for wild-type, which reflects that HgdD is indeed involved in export (Table 3). All other rate constants were in the same range.

Since proteins of the TolC family usually represent the outer membrane factor of a tripartite secretion system, they have to interact with a plasma membrane-localized permease. Hence, we analyzed whether such putative HgdD-charging permease is proton gradient-dependent. For this we abolished the proton gradient across membranes in the absence of the protonophore. Thus, the proton gradient, which is most likely produced by HgdD-dependent transport indeed requires the presence of CCCP. Measuring the uptake of ethidium by the wild type and AFS-I-hgdD mutant in the presence of CCCP we observed a similar intercalation by both strains (Fig. 8). Furthermore, the intercalation in the presence of CCCP was comparable to the one observed for AFS-I-hgdD in the absence of the protonophore. Thus, the HgdD-dependent transport indeed requires the proton gradient, which is most likely produced by a proton pump-type permease (42, 43).

**Identification of putative MSF-type proteins** - We have demonstrated that HgdD of Anabaena sp. is involved in detoxification. As depicted in Fig 1, additional plasma membrane-inserted proteins of different families are involved in the process of uptake and export (33). To obtain further insights into putative factors involved in metabolite export, we used a bioinformatics approach to identify candidates for MFS proteins in the genome of *Anabaena* sp. Within the three MFS families annotated in the PFAM database (MFS 1, MFS 2, MFS 3), we found in total 18 sequences from *Anabaena* sp. We performed a CLANs analysis (Fig. 9A; e.g. 31) to assign the identified sequences to the different MFS families.

Two of these sequences are similar to the permease for GlcNAc-1,6-anhydromuropeptides (AFS-I-hgdD) and AmpG (MFS 1; e.g. 44), namely the schizokinon exporter SchE (Alr4025; 7) and Alr4533. Furthermore, a single sequence is related to sugar transporters described by the MFS 2 family (Air3705), which is similar to e.g. MelB (45), UidB (46), YihP (47) and YihO (48).

Among sequences of the MFS 3 family, which contains transporters involved in enterobactin (49) or antibiotic efflux (50), seven proteins are from *Anabaena* sp. From the remaining eight proteins, which do not cluster with any known protein, only Alr0391 was previously identified as part of the putative schizokinin synthesis cluster (51).

To analyze whether such proteins are involved in the TolC-dependent export cycle, we analyzed the mutant strain CSCW2, which carries an insertion in *schE*, as an example of an MSF 1-type permease. This mutant has been previously described as defective in secretion of the siderophore schizokinin (7). We also generated an insertion mutant of the MFS 3-type permease gene *all2215* (Fig. 9B; strain AFS-I-all2215). We excluded the MFS 2-type permease from subsequent studies since they are known to be involved in sugar transport (45-48). A mutant of *hgdC* (MFS-I-hgdC), a plasma membrane ABC transporter involved in heterocyst development (*all5346*; 52), was used as a negative control. The strains generated were fully segregated, as no wild-type gene was detectable (Fig. 9B, 1/3). In contrast to AFS-I-hgdD (5) and AFS-I-hgdC (52), AFS-I-all2215 was able to grow on the medium without a fixed nitrogen source (Fig. 9C). Testing the mutants with respect to their sensitivity to EB or erythromycin, we observed a growth reduction of AFS-I-hgdC, AFS-I-hgdD and CSCW2, but not of AFS-I-all2215 or AFS-I-anaOmp85. This observation supports the notion that the TolC-like protein HgdD performs multiple
functions in concert with distinct plasma membrane permeases (7).

The expression of hgdD in different strains was probed by RT-PCR. Using identical amounts of mRNA, as controlled by amplification of RNase P RNA (mpB) cDNA, we observed an enhanced expression of hgdD in AFS-I-alr2215 and CSCW2, while the transcript level did not change in AFS-I-hgdC or AFS-I-anaOmp85, suggesting a functional relation between HgdD and Alr2215 and SchE.

Interception of ethidium by different mutant strains - Having generated and confirmed the mutant strains, we analyzed their behavior based on the interception of ethidium. Additionally, we used AFS-I-anaOmp85, in which the outer membrane protein assembly was most likely affected (53, 54). However, it has to mentioned that this mutant is not fully segregated although a drastic phenotype can be observed (54).

At a low concentration of ethidium bromide (12.5 µM; Fig. 10A) we did not observe a significant interception by the strains with the exception of AFS-I-hgdD. Notably, the result obtained for the strain AFS-I-anaOmp85 suggests that in this mutant the proper targeting and assembly of HgdD is not affected.

However, we observed a concentration-dependent increase in the interception of ethidium by the wild type (25 - 125 µM; Fig. 10B, C; black circles). Consistent with this phenotype (Fig. 9B) interception by AFS-I-hgdC (Fig. 10B, C; open circles) was largely similar to that in the wild type. In contrast, interception by AFS-I-all2215 (grey squares), CSCW2 (black squares) and AFS-I-hgdD (grey circles) exceeded the one of wild-type, whereas interception by AFS-I-anaOmp85 was reduced (open square).

We next described the data by least square fit analysis by E2 (Fig. 1; lines in Fig. 10A, C) and compared the determined rate constants. The rate constant reflecting the interception across the outer membrane (k1) was only affected in AFS-I-anaOmp85, which is consistent with its function in the assembly of outer membrane proteins (Table 3). Similarly, the rate constant reflecting the export across the outer membrane (k3) was strongly reduced in AFS-I-hgdD, while only slight alterations were observed for other mutants. Here, the slight increase of the rate observed for AFS-I-alr2215 or CSCW2 can be explained by the enhanced expression of hgdD in these two strains (Fig. 9).

The rate for interception across the plasma membrane (k2) was not affected in any of these mutants, although slight variations existed (Table 3). The rate for export across the plasma membrane (k3) was of the same order of magnitude as the rate of transport across the plasma membrane (k2). CSCW2 and AFS-I-all2215, but not AFS-I-hgdC (Table 3), showed a reduced rate for export across the plasma membrane, which was consistent with the enhanced sensitivity of these two strains against erythromycin (Fig. 9C). However, having two exporters explains the lower sensitivity against this antibiotic seen for each of the corresponding mutants than for AFS-I-hgdD and explains why only a reduction, but not a complete loss of export across the plasma membrane was observed.

Uptake of ethidium by CSCW2 and AFS-I-all2215 - The observed enhanced interception by CSCW2 and AFS-I-all2215 at high ethidium bromide concentrations prompted us to analyze the uptake behavior by measuring the ethidium bromide remaining in the solution. At 12.5 µM no significant difference in uptake by CSCW2 and AFS-I-all2215 when compared to the wild type was observed (Fig. 11A). However, the uptake of ethidium at 125 µM was reduced in both strains (Fig. 11B). This is noteworthy considering the higher interception in comparison to the wild type at 125 µM ethidium bromide (Fig. 10C). Analysis by E1 confirmed the slight increase of the TolC-dependent export rate (k1) when compared to the wild type (Table 3). Thus, we suggest that the reduced uptake of both MFS mutants is a consequence of an enhanced export activity across the outer membrane (Fig. 9D). At the same time, the reduction in export across the plasma membrane (k3) led to an accumulation of ethidium bromide in the cytosol, which was therefore no longer accessible for secretion by HgdD.

We next compared the ratio of interception and total uptake of ethidium for hgdD and both MFS mutants with the one obtained for the wild type. It became obvious that at low ethidium concentrations the accumulation in the cytosol of AFS-I-hgdD was 25 fold higher when compared to the wild type, while at high concentration the ratios became comparable (Fig. 11C). In contrast, for CSCW2 and AFS-I-all2215 the ethidium retention in the cytosol was about 6 fold higher compared to the wild type and at high
ethidium concentration even to \textit{hgdD} (Fig. 11C). These observations show that at low concentrations \textit{TolC} is sufficient to export ethidium from the periplasm, while at high concentrations the process of secretion is rate limiting by the export efficiency of ethidium across the plasma membrane.

\textbf{DISCUSSION}

\textit{An anaOmp85-independent insertion of HgdD into the outer membrane? –} Omp85 is thought to be a general factor for the insertion of outer membrane proteins in gram-negative bacteria, mitochondria and chloroplasts (e.g. 53, 55). Consistent with its global function, Omp85 (BamA) of \textit{E. coli} is essential for the assembly and subsequently for the function of \textit{TolC} (56). Interestingly, in \textit{Anabaena} sp. Omp85 encoded by \textit{alr2269} influences the uptake activity (\(k_{1}\), Fig. 10, Table 3), but not the secretion efficiency (\(k_{-1}\), Table 3). Thus suggests that in AFS-I-Omp85 the assembly of HgdD is not disturbed. Furthermore, the mutants of the other two Omp85-like genes (\textit{alr0075} and \textit{alr4893}; 54, 57, 58) do not show an overlapping phenotype with the \textit{hgdD} mutant (59) in respect to heterocyst development (5) or the sensitivity of AFS-I-\textit{hgdD} to 30 mM lysozyme (59). A function of \textit{Alr0075} in HgdD insertion and assembly is further unlikely as this Omp85-like protein only exists in vegetative cells (54), while the expression of \textit{hgdD} is enhanced in heterocysts (5). This could suggest that in \textit{Anabaena} sp. the function of the Omp85 is not required for the assembly of HgdD, which however, has to be further investigated.

\textit{Porin-mediated permeability of the outer membrane of \textit{Anabaena} sp. PCC 7120} – The existence of classical porins in cyanobacteria is largely under debate. On the one hand, the porins found to be encoded in the genomes of different cyanobacteria or by proteomic approaches are generally larger than those found in proteobacteria (e.g. 18, 19, 60). The extension is thought to serve as a link of the proteins to the peptidoglycan layer (61), which has a defined distance to the outer membrane (e.g. 62). On the other hand, the only experimentally approached porin-like proteins, SomA and SomB from \textit{Synechococcus} (63-65), have a conductance of about 0.5 nS (66), which parallels the observed conductance of cell-envelope fractions of \textit{Anabaena variabilis} (67), but is about 10 fold smaller than that found for typical proteobacterial porins (e.g. 68). The reduced size could be interpreted as remnant of the pore activity, which is no longer required as cyanobacterial “porins” if the hypothesis is made that they are involved in anchoring to the peptidoglycan rather than in transporting molecules. Alternatively, if a function in transport is considered, the pore activity could be regulated in a different manner than that found for proteobacterial porins. One could envision that such regulation includes a periplasmic section functioning as a “plug”. Both ideas would be consistent with the hypothesis that the periplasm of \textit{Anabaena} sp. is involved in long distance transport of solutes in the filament (69).

However, our results put the previous notion into question that porins of cyanobacteria are smaller because of “the photoautotrophic lifestyle” of cyanobacteria, which require porins that are only large enough to facilitate the uptake of small solutes, such as ions, from their environment, whereas biopolymers are synthesized by the bacteria themselves (61). We observed uptake of ethidium (394 Da) and erythromycin (734 Da; Fig. 3-11), and a permeability coefficient for \textit{Anabaena} sp. (Fig. 3, Table 3) comparable to the one found for proteobacteria (e.g. 40, 41). Further, uptake of ethidium is reduced in the Omp85 mutant (Table 3), the latter being involved in the insertion and assembly of porins (e.g. 53) and by adding spermine/spermidine, well-established inhibitors of porin activity (e.g. 37, 38). The effective concentration of the poly-amines is in the same range as found to alter the closing probability of OmpC or OmpF from \textit{E. coli} (Fig. 3, 38), which is consistent with the interpretation that a porin-like activity accounts for the uptake of ethidium.

Thus, we provide evidence that a porin-like permeability exists in \textit{Anabaena} sp. and future work will be needed to dissect which of the proposed \(\beta\)-barrel proteins (22) might serve this function.

\textit{The antibiotic resistance system} – HgdD is the only TolC-like protein encoded in the \textit{Anabaena} sp. genome (5). Including our results, four distinct functions have been assigned to it: protein secretion (5) and glycolipid (20), siderophore (7) and antibiotic export (Fig. 7). The \(K_{M}\) for secretion of ethidium by the wild type is more than 5 orders of magnitude lower than in the strain with
abolished HgdD function (Fig. 6). Interestingly, the metabolite export by HgdD described here is a proton gradient-dependent process (Fig. 8). The latter suggests that metabolite efflux is likely catalyzed by a proton gradient-dependent efflux pump protein, similar to the AcrAB system in E. coli (e.g. 3, 16). Thus, we conclude that HgdD engages different energizing complexes to perform the different functions observed. This notion is consistent with the phenotype (Fig. 9B) and the wild-type-like ethidium uptake behavior of AFS-I-hgdC (Fig. 10B, C; open circle). Thus, HgdC is important for glycolipid export, but not relevant for the drug-efflux cycle.

We observed that metabolite export in Anabaena sp. occurs in at least two separate stages, which become apparent at different toxin concentrations (Fig. 12). At low concentrations of toxins the export from the periplasm by the action of HgdD, charged by a proton pump, is sufficient for secretion since equilibrium between uptake and secretion is observed after a few minutes (Fig. 3, 11), while intercalation of ethidium into cellular DNA is very low (Fig. 8). Under these conditions, the function of HgdD seems to be essential, while the two MFS-like proteins (SchE and All2215) that were analyzed seem to play only a minor role as reflected by the very low intercalation of ethidium in the corresponding mutants (Fig. 3, 8, 10, 11). Therefore, under these conditions the periplasm serves as a threshold reservoir from which toxins and antibiotics are efficiently exported before crossing the plasma membrane (Fig. 12 center).

At high extracellular concentrations, the “buffer capacity” of the periplasm is exceeded, which is reflected by an enhanced uptake (Fig. 11) and intercalation (Fig. 10) by the wild type. At this stage, the function of the MFS proteins becomes relevant (Fig. 12 right). At high ethidium bromide concentrations, we observed an enhanced intercalation for the mutants the MFS genes alr2215 and schE, which even exceeds the intercalation of HgdD. The ratio between intercalation and uptake in the MFS mutants is at least 5 fold higher than in the wild type and the AFS-I-hgdD mutant. This suggests that ethidium is effectively retained in the cytoplasm, most likely by the impaired transport to the periplasm in these mutants (Fig. 11C). However, the ethidium uptake (Fig. 12) is still lower compared to wild-type activity, which might be explained by the slightly enhanced export activity across the outer membrane as reflected by k_0. This parallels the enhanced expression of hgdD in both MFS mutant strains (Fig. 9; Table 3), which most likely is a response to the impaired MFS system. This upregulation shifts the buffer capacity of the periplasm to higher concentrations and prevents toxins to reach the cytosol. Interestingly, the two MFS proteins act in parallel and cannot complement each other, as both mutants show an impaired, but not abolished export activity (Table 3).

Thus, we have documented that HgdD is involved in the export of toxins in a proton gradient-dependent manner. This underlines the importance of this protein previously found to modulate the protein secretion pattern (5) and the lipid export activity (20) as well.

ACKNOWLEDGMENTS

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REFERENCES:


FOOTNOTES
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8 The on-line version of this article contains supplemental data.
### Tables

#### Table 1. *Anabaena* sp. strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resist.</th>
<th>Genotype</th>
<th>Properties</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td>wild-type</td>
<td></td>
</tr>
<tr>
<td>PCC 7120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSR10</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>alr4167:: Sp\textsuperscript{R} Sm\textsuperscript{R}</td>
<td>gene interruption by plasmid pCSV3</td>
<td>70</td>
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<td>AFS-I-alr2887</td>
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<td>alr2887:: Sp\textsuperscript{R} Sm\textsuperscript{R}</td>
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<tr>
<td>AFS-I-alr2215</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>alr2215:: pMS1</td>
<td>gene interruption by plasmid pMS1</td>
<td>TS</td>
</tr>
<tr>
<td>CSCW2</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>all4025::pCSW11</td>
<td>gene interruption by gene cassette C.S3 (with partial gene deletion)</td>
<td>7</td>
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<tr>
<td>AFS-I-HgdC</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>alr5346::pMS2</td>
<td>gene interruption by plasmid pMS2</td>
<td>TS</td>
</tr>
<tr>
<td>AFS-I-alr2269</td>
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<tr>
<td>DR181</td>
<td>Nm\textsuperscript{R}</td>
<td>(\Delta\text{alr2887::C.K3})</td>
<td>partial replacement of <em>alr2887</em> by C.K3 resistance cassette</td>
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</table>

Sp (Spectinomycin), Sm (Streptomycin), Nm (Neomycin), TS (this study)

#### Table 2. Plasmids used in this study.

<table>
<thead>
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<th>Plasmid</th>
<th>Marker</th>
<th>Properties</th>
<th>Ref.</th>
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<td>pCSV3</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>pRL500 with substituted Ap\textsuperscript{R} gene</td>
<td>28, 71</td>
</tr>
<tr>
<td>pCESEL24</td>
<td>Ap\textsuperscript{R} Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>pBR322 containing <em>Anabaena</em> sp. 2 kb <em>nucA-nuiA</em> fragment and C.S3 cassette</td>
<td>72</td>
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<tr>
<td>pMS1</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>pCESEL24 where <em>nucA-nuiA</em> was replaced by an internal fragment of <em>alr2215</em></td>
<td>TS</td>
</tr>
<tr>
<td>pMS2</td>
<td>Sp\textsuperscript{R} / Sm\textsuperscript{R}</td>
<td>pCESEL24 where <em>nucA-nuiA</em> was replaced by an internal fragment of <em>hgdC (alr5346)</em></td>
<td>TS</td>
</tr>
</tbody>
</table>

Ap (ampicillin), Sp (Spectinomycin), Sm (Streptomycin)
Table 3. Kinetic parameter determined in this study.

<table>
<thead>
<tr>
<th>strain</th>
<th>porin*</th>
<th>secretion**</th>
<th>E1**</th>
<th>E2**</th>
<th>K_M (M)</th>
<th>V_max (nM/sec)</th>
<th>k_1 (sec^-1)</th>
<th>k_1 (sec^-1)</th>
<th>k_1 (sec^-1)</th>
<th>k_1 (sec^-1)</th>
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<tbody>
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<td>Wild-type</td>
<td>1.5 x 10^-6</td>
<td>170±10 µM</td>
<td>420±20 nM *sec^-1</td>
<td>(12±1)*10^-4</td>
<td>(20±5)*10^-4</td>
<td>(15±2)*10^-4</td>
<td>(9.7±0.6)*10^-4</td>
<td>(18±3)*10^-4</td>
<td>(34±3)*10^-4</td>
<td></td>
</tr>
<tr>
<td>AFS-I-hgdD</td>
<td>n.d.</td>
<td>40±8 M</td>
<td>25±5 mM *sec^-1</td>
<td>(14±4)*10^-4</td>
<td>(0.8±0.2)*10^-4</td>
<td>(19±2)*10^-4</td>
<td>(6±1)*10^-4</td>
<td>(15±5)*10^-4</td>
<td>(27±5)*10^-4</td>
<td></td>
</tr>
<tr>
<td>AFS-I-hgdC</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(18±2)*10^-4</td>
<td>(6.9±0.9)*10^-4</td>
<td>(13±2)*10^-4</td>
<td>(35±3)*10^-4</td>
<td>(5±1)*10^-4</td>
<td>(5±1)*10^-4</td>
<td></td>
</tr>
<tr>
<td>AFS-I-omp85</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>(3.7±0.6)*10^-4</td>
<td>(7±2)*10^-4</td>
<td>(7±2)*10^-4</td>
<td>(7±2)*10^-4</td>
<td>(7±2)*10^-4</td>
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</table>

a...Figure 3; b...Figure 6; c...Figure 3&11, d...Figure 8&10; e...significance of change compared to wild-type with p<0.005 or f...p<0.01
FIGURE LEGENDS

FIGURE 1. Description of the uptake system used throughout the study.
Shown is the scheme of the uptake and export cycle for metabolites (top) based on the current information for Gram-negative bacteria in general (1-4). Please note, that parts thereof are only a model which has to be confirmed in future by the identification of the components. The mathematical equation for the analysis of the transport across the outer membrane only or including the entire system as used to analyze results throughout the manuscript are given.

FIGURE 2. Justification of the reliability of the analysis by intercalation.
(A) The uptake of EB by wild-type (left) or AFS-I-hgdD (right) incubated at the indicated EB concentration was measured by intercalation (black) and extraction of ethidium (white). The line represents the least square fit analysis of both data sets in combination by a linear function. (B) The ethidium remaining in the solution (supematant) and intercalated was determined for wild-type and AFS-I-hgdD. The line represents the x=y. For (A) and (B) the average of at least three independent results are shown with the respective standard deviation.

FIGURE 3. Ethidium uptake by Anabaena sp. and the hgdD mutant by analysis of the extracellular ethidium.
(A) The ethidium bromide (EB) remaining in solution after incubation of wild-type (black) or AFS-I-hgdD (white) with 12.5µM EB was quantified after the indicated time periods. Lines indicate the analysis according to [E1]. (B) The ethidium bromide (EB) remaining in solution after incubation of AFS-I-hgdD with 2.5µM EB in the presence of 0 (circle), 0.1 (square) or 1.0 µM spermine/spermidine (diamond) was quantified after the indicated time periods. Lines indicate the analysis according to [E1]. (C) k₁, determined in the presence of indicated concentrations of spermine/spermidine that were set in relation to k₁ in the absence of the poly-amine. The line represents the least square fit analysis to a four parameter logistic equation to determine the EC₅₀ value. The dashed line represents the extrapolated result according to the parameters determined. (D) The initial uptake rate (v₀) was determined from measurements as in A for wild-type and plotted against the EB concentration. The line represents the least square fit analysis to the equation described (34).

FIGURE 4. Cellular morphology after ethidium bromide incubation.
Shown are representative images of filaments of Anabaena wild-type (left) or AFS-I-hgdD (right) incubated for 30 min with the indicated concentration of ethidium bromide. No obvious alteration of filament structure or length, as well as no obvious alteration of the cellular integrity was realized. BF…bright field, AF…auto-fluorescence, scale bare = 10 µm.

FIGURE 5. Expression of genes coding for putative porin like proteins of Anabaena sp.
RNA from Anabaena sp., AFS-I-hgdD, AFS-I-all2215, CSCW2, AFS-I-hgdC and AFS-I-anaOmp85 grown in BG11, as well as genomic DNA from Anabaena sp. grown in BG11 was isolated and analyzed. RT-PCR / PCR was performed using oligonucleotides for the indicated genes listed in Table S1. The lowest panel shows the result in the absence of the reverse transcriptase. The genes for analysis were selected according to (18, 19, 22, 24).

FIGURE 6. Ethidium release by Anabaena sp. and the hgdD mutant.
The ethidium released from preloaded wild-type (top) or AFS-I-hgdD cells (bottom) was analyzed as described in experimental procedures. Michaelis-Menten constants were determined by least square fit analysis and confirmed by Lineweaver-Burk analysis (inset). Values are listed in Table 3.

FIGURE 7. Competition of ethidium uptake by Anabaena sp. and the hgdD mutant by analysis of the extracellular ethidium.
(A) Comparison of ethidium uptake in the absence (white) and presence (grey) of 125µM erythromycin (ERY) as measured in Figure 2. Lines represent least square fit analysis by [E1]. The k₁ (B) and k⁻¹ rate (C) for different ratios between erythromycin and EB is plotted and lines represent the least square fit to determine the IC₅₀ value indicated.

FIGURE 8. Analysis of ethidium uptake by Anabaena sp. and the hgdD mutant by intercalation.
(A) Ethidium uptake (12.5µM) in the absence (black) and presence (grey) of CCCP by wild-type (circle) or AFS-I-hgdD cells (diamond) was measured as described in experimental procedures. Lines represent least square fit analysis by [E2].
FIGURE 9. **Putative MFSs involved in antibiotic resistance.**

(A) A CLANS clustering of ~55,000 non-redundant MFS proteins of the PFAM families MFS_1, MFS_2, and MFS_3 is shown. Clusters containing functionally characterized transporters (marked as red dots) are encircled and are listed below the clustering. Sequences from *Anabaena* sp. are marked with green dots. (B) AFS-I-*alr2215* and AFS-I-*hgdC* were generated via insertion of pMS1 and pMS2, respectively, by single homologues recombination (primer combination 1/2). Both strains are fully segregated since no amplification of wild-type genomic DNA could be observed under standard PCR conditions (primer combination 1/3). Wild-type genomic DNA was used as a control (lane 1). (C) *Anabaena* sp., AFS-I-*hgdD*, AFS-I-*all2215*, CSCW2, AFS-I-*hgdC* and AFS-I-*anaOmp85* were grown on indicated media. Images are taken after 7 days. (EB, ethidium bromide; Ery, erythromycin) (D) RNA from *Anabaena* sp., AFS-I-*hgdD*, AFS-I-*all2215*, CSCW2, AFS-I-*hgdC* and AFS-I-*anaOmp85* grown in BG11, as well as genomic DNA from *Anabaena* sp. grown in BG11 was isolated and analyzed. RT-PCR / PCR was performed using oligonucleotides for *hgdD* (top) or *rnpB* (middle and bottom). The lowest panel shows the result in the absence of the reverse transcriptase.

FIGURE 10. **Analysis of ethidium uptake by wild-type and different mutants of *Anabaena* sp. by intercalation.**

(A) Ethidium uptake at 12.5 µM EB by wild-type (filled circle), AFS-I-*hgdD* (grey circle), AFS-I-*hgdC* (white circle), CSCW2 (filled square), AFS-I-*all2215* (grey square) and AFS-I-*anaOmp85* (white square) was measured as in Figure 5. Lines represent least square fit analysis by [E2]. (B) Ethidium uptake in the presence of 25, 50, 75 and 100 µM EB was determined and presented as in A. (C) Ethidium uptake at 125 µM EB was determined and presented as in A. The determinant rate constants are listed in Table 3.

FIGURE 11. **Ethidium uptake by *Anabaena* sp. and mutants by analysis of the extracellular EB.**

The free ethidium bromide was quantified as described after incubation of wild-type (filled circle), AFS-I-*hgdD* (grey circle), CSCW2 (filled square) and AFS-I-*all2215* (filled square) with 12.5 µM EB (A) or 125 µM EB (B) for the indicated time periods. Lines indicate the analysis according to [E1]. (C) The ratio between uptake (U) determined by intercalation (IN) and determined by measuring the EB remaining in solution (FR) was calculated and placed in relation to the value observed for the wild-type strain (WT). Uptake values after 900 sec were used.

FIGURE 12. **The model of secondary metabolite export.**

On the left the characterized components in the outer (top) and plasma membrane (bottom) are depicted. In the middle the components involved in export at low toxins/antibiotic concentrations and on the right the components involved in export at high concentrations are shown in grey and the path of metabolites/antibiotics is given by arrows.
\[ [A]_t = [A]_0 e^{-k_1 t} \]

\[ [I]_t = [A]_0 e^{-k_1 t} \]

\[ a = k_1 \]
\[ b = (k_1 + k_2 + k_3 + k_4 + k_5 + k_6)^2 \]
\[ c = (k_1 + k_2)^2 k_3 (k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6 + (k_3 + k_4 + k_5 + k_6)^2)^2 \]
\[ d = (k_1 + k_2 + k_3 + k_4 + k_5 + k_6)^2 \]
\[ e = (k_1 + k_2 + k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6 + (k_3 + k_4 + k_5 + k_6)^2)^2 \]
\[ f = (k_1 + k_2 + k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6 + (k_3 + k_4 + k_5 + k_6)^2)^2 \]
\[ g = (k_1 + k_2 + k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6)^2 (k_3 + k_4 + k_5 + k_6 + (k_3 + k_4 + k_5 + k_6)^2)^2 \]

Fig. 1
Fig. 2

A) wild type

EB [µM]

signal [RFU]

SIG=136+85*EB

intercalation extraction

B) AFS-I-hgdD

EB [µM]

signal AFS-I-hgdD (intercalation + supernatant) [RFU]

SIG=358+190*EB

signal wild-type (intercalation + supernatant) [RFU]
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
Fig. 10
Fig. 11
Fig. 12
The TolC-like protein HgdD of the cyanobacterium Anabaena sp. PCC 7120 is involved in secondary metabolite export and antibiotic resistance

Alexender Hahn, Mara Stevanovic, Oliver Mairus and Enrico Schleiff

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