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Alr0397 Is an Outer Membrane Transporter for the Siderophore Schizokinen in *Anabaena* sp. Strain PCC 7120▽

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Iron uptake in proteobacteria by TonB-dependent outer membrane transporters represents a well-explored subject. In contrast, the same process has been scarcely investigated in cyanobacteria. The heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 is known to secrete the siderophore schizokinen, but its transport system has remained unidentified. Inspection of the genome of strain PCC 7120 shows that only one gene encoding a putative TonB-dependent iron transporter, namely alr0397, is positioned close to genes encoding enzymes involved in the biosynthesis of a hydroxamate siderophore. The expression of alr0397, which encodes an outer membrane protein, was elevated under iron-limited conditions. Inactivation of this gene caused a moderate phenotype of iron starvation in the mutant cells. The characterization of the mutant strain showed that Alr0397 is a TonB-dependent schizokinen transporter (SchT) of the outer membrane and that alr0397 expression and schizokinen production are regulated by the iron homeostasis of the cell.

Filamentous cyanobacteria like *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 (herein named *Anabaena* sp.) form two different cell types under starvation of combined nitrogen: vegetative cells and heterocysts (1, 58). Vegetative cells carry out a plant-type oxygenic photosynthesis, and heterocysts contain the oxygen-labile nitrogenase and perform nitrogen fixation, which is dependent on respiration and photosystem I-dependent photosynthesis (55, 58). Many enzymes involved in these metabolic processes use cofactors like copper, magnesium, and iron (e.g., see references 28 and 50), and the level of iron found in cyanobacteria is generally 1 order of magnitude higher than that found in nonphotosynthetic bacteria (26). Even though these metals are required for the function of respiratory, photosynthetic, and nitrogen-assimilating complexes, their intracellular level and thereby their uptake have to be tightly controlled, as they pose a risk for oxidative stress (50). The presence of about 1 mM iron (29) or about 10 μM copper in medium (7, 33) largely impairs the growth of *Anabaena* sp. Not only intoxication but also starvation is a danger for cyanobacteria. Some physiological effects of iron deficiency are decreases of phycocyanins and chlorophyll (17), replacement of ferredoxin by *isiB*-encoded flavodoxin (21, 48), monomerization of photosystem I trimers (22), and oxidative stress (30).

To avoid iron starvation under iron-limiting conditions, several bacteria secrete low-molecular-weight iron chelators known as siderophores to complex iron present in the environment (41). The siderophore-iron complexes are bound by TonB-dependent transporters in the outer membrane and then passed into the cytoplasm by an ABC transporter present in the cytoplasmic membrane. Although many siderophores have been characterized for other bacteria (8, 41, 57), so far only the low-affinity dihydroxamate-type siderophore (15) schizokinen has been identified as being secreted by *Anabaena* sp. (51). It has been further observed that schizokinen also has a function in complexing toxic copper ions in the medium and thereby protecting the cells from copper intoxication (7).

Little is known about the iron uptake systems in cyanobacteria. Recently, open reading frames (ORFs) sll1206, sll1406, sll1409, and slr1490 encoding TonB-dependent transporters in *Synechocystis* sp. strain PCC 6803 have been identified; however, these transporters are not essential (25), indicating that other types of iron uptake systems might also exist. The expression of those genes is induced upon iron starvation, and for sll1406, a basal expression before starvation has been found (25). Microarray analysis has revealed that the expression of sll1406 is appreciably independent of the length of time cells were starved, whereas the expression of sll1409 is enhanced 3 h after initiation of starvation but decreased after 12 or 24 h (52). Based on these two reports, it can be proposed that the four genes are independently regulated and that their products might have different functions.

Recently, we reported the presence of a putatively TonB-dependent transporter (Alr0397) in the outer membranes of *Anabaena* vegetative cells and heterocysts (37, 38). Close to alr0397 are genes with similarity to the aerobactin biosynthesis genes. The alr0397 gene is not essential, but its inactivation results in a moderate phenotypic alteration with respect to iron supply. We show that the schizokinen-mediated iron uptake is indeed reduced by this mutation, confirming the function

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TABLE 1. *Anabaena* strains used in this study[a]

Anabaena strain	Resistance	Genotype	Relevant properties	Source or reference
PCC 7120			Wild type	C. P. Wolk
216		*hetR(S179N)*	*hetR* mutant	3
AFS-PDGF-alr0397	Sp^r Sm^r	$P_{alr0397}$-*gfp* in *nucA* region	N-terminal GFP fusion	This study
AFS-I-alr0397	Sp^r Sm^r	alr0397::pCSV3	Gene interruption	This study
NME-alr2887-GFP	Sp^r Sm^r	$P_{alr2887}$-*gfp* in *nucA* region	N-terminal GFP fusion	39

[a] AFS, Anabaena Frankfurt Schleiff; PDGF, promoter downstream GFP fusion; NME, Nostoc Munich Expression; Sp, spectinomycin; Sm, streptomycin.

of Alr0397 as a schizokinen-transporting outer membrane protein.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and general methods. The present study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 and mutant derivatives (Table 1). All strains were grown photoautotrophically under constant illumination from fluorescent lamps at 70 μmol photons m^{-2} s^{-1} at 30°C in liquid BG11 medium or BG11 medium without a source of nitrate ($BG11_0$ medium) (38, 44). Cultures of the mutant strains were grown in the presence of 2 μg ml^{-1} streptomycin and spectinomycin. Heterocyst formation was induced in liquid cultures by washing the cells three times in $BG11_0$ medium and by subsequent incubation in this medium for at least 48 h. For analysis of growth in media with reduced metal content, BG11 medium without the addition of $C_6H_8O_7 \cdot n\text{Fe} \cdot n\text{H}_3\text{N}$ (~18% Fe) ($BG11_{-Fe}$ medium), without the addition of $CuSO_4 \cdot 5H_2O$ ($BG11_{-Cu}$ medium), or without the addition of both metal sources ($BG11_{-Fe-Cu}$ medium) was used. Glassware used in experiments with iron-limited conditions was soaked with 6 M HCl or 1 mM EDTA to remove residual iron and rinsed with Milli-Q water.

Agar plates were prepared by the addition of 1% Bacto agar (Becton Dickinson GmbH, Heidelberg, Germany) to the indicated media. To test the ability to grow in media with altered metal content, $BG11_{-Fe-Cu}$ medium with (control for mutant strains) and without antibiotics was supplemented with different combinations of concentrations of $CuSO_4 \cdot 5H_2O$ as the copper source and $FeCl_3 \cdot 6H_2O$ or $C_6H_8O_7 \cdot n\text{Fe} \cdot n\text{H}_3\text{N}$ (~18% Fe) as the iron source. Chromazurol S (CAS)-containing agar plates (49) were prepared by the addition of a 1/10 volume of a CAS stock solution to BG11 media. To prepare the CAS stock solution, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml Fe(III) solution (1 mM $FeCl_3 \cdot 6\ H_2O$, 10 mM HCl), and while this solution was stirred, 72.9 mg HDTMA (hexadecyltrimethylammonium) dissolved in 40 ml water was added.

Fractionation of *Anabaena* sp. or mutant cells (38), microscopic visualization of filaments, and visualization and quantification of green fluorescent protein (GFP) signals were previously described (39). For quantification, GFP fluorescence (excitation at 480 nm) of mutant and wild-type strains was recorded in a window between 500 and 570 nm (Perkin Elmer LS55; Germany). The integral of each spectrum was determined and corrected for the background value obtained using the wild-type strain. The results of three independent measurements are presented. The differential picture was created by subtracting the intensities using the GFP channel and the chlorophyll autofluorescence channel. To avoid background fluorescence, the GFP detection window was controlled and adjusted with wild-type *Anabaena* sp. RNA was isolated from whole filaments in the presence of a ribonucleoside-vanadyl complex as previously described (40). Reverse transcriptase (RT)-PCR to produce cDNA was performed with the Superscript III first-strand kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol for random hexamer primer usage. All RT-PCR experiments presented were made by performing a limited number of 30 PCR cycles to allow a comparison of the possible initial amounts of transcript in the different samples.

Genetic procedures. The procedures for transformation of *Escherichia coli* and isolation and manipulation of plasmid DNA were standard (46). PCR was done with the TripleMaster PCR system (Eppendorf, Hamburg, Germany). Total DNA from *Anabaena* sp. was isolated as described previously (4) from 50-ml shaking cultures (100 rpm) without additional air/CO_2 bubbling.

To generate AFS-I-alr0397 (Table 1), 600 bp of the coding region of alr0397 (gi 17227839 ref NP_484441.1) was amplified by PCR on genomic DNA of *Anabaena* sp. using oligonucleotides containing BamHI restriction sites (Table 2). The restricted PCR product was cloned into pCSV3 (a vector containing a Sp^r Sm^r gene cassette) (Table 3) producing plasmid pAFS-I-alr0397. The plasmid was amplified through transformation into *E. coli* DH5α and sequenced. Before conjugal transfer to *Anabaena* sp., the cargo plasmid pAFS-I-alr0397 was transformed into HB101(pRL623) (11). Triparental mating with J53-RP4 was performed as described previously (10), generating single-recombinant plasmid integration mutants (i.e., strains in which the plasmid has integrated into the genome by a single crossover event). Segregation of the mutant chromosomes was confirmed by Southern blotting of genomic DNA according to standard procedures (46). The probe was ^{32}P labeled with a Ready-To-Go (GE Healthcare, Freiburg, Germany) DNA labeling kit using [α-^{32}P]dCTP, and the internal fragment of the gene which was cloned to obtain pASF-I-alr0397 was used as a

TABLE 2. Deoxyoligonucleotide primers used for cloning and RT-PCR

Use	Primer name[a]	Oligonucleotide sequence
Cloning	AFS-PDGF-alr0397-F	ATCGATCGATGCATCGCTTCTATTGCTACTGG
	AFS-PDGF-alr0397-R	ATCGGATATCATTATGGCTAGTGACACAATCCATC
	AFS-I-alr0397-F	ATCGGGATCCAGCACTAACCTACAGCATTTTATCTC
	AFS-I-alr0397-R	ATCGGGATCCGGTATCTTCCTGGGAGTAATCTAC
Other	alr0397-F	TGCGTCGCGGGATTTGCGAAC
	alr0397-R	GGATAGTATTGACCCTGGGGTC
	isiA-F	GCCCGCTTCGCCAATCTCTC
	isiA-R	CCTGAGTTGTTGCGTCGTAT
	rnpB-F	AGGGAGAGAGTAGGCGTTGG
	rnpB-R	GGTTTACCGAGCCAGTACCTCT

[a] AFS, Anabaena Frankfurt Schleiff; PDGF, promoter downstream GFP fusion.

TABLE 3. Plasmids used in this study

Plasmid	Marker[a]	Properties	Source or reference
pCSEL21	Ap^r	pIC20R with gene-GFP insertion	42
pCSV3	Sp^r Sm^r	pRL500 with substituted Ap^r gene	42
pCSEL24	Ap^r Sp^r Sm^r	pBR322 containing *Anabaena* sp. 2-kb *nucA-nuiA* fragment and C.S3 cassette	42
pAFS-PDGF-alr0397	Sp^r Sm^r	pCSEL24 with alr0397 promoter-*gfp* fusion	This study
pAFS-I-alr0397	Sp^r Sm^r	pCSV3 with fragment of alr0397	This study
pRL623	Cm^r	Mobilization helper and methylases for AvaI-AvaII and AvaIII sites	11

[a] Ap, ampicillin; Sp, spectinomycin; Sm, streptomycin; Cm, chloramphenicol.

template. Images were obtained with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

To generate AFS-PDGF-alr0397 (Table 1), 800 bp of the upstream region of alr0397, including the first eight codons of the coding region, was amplified by PCR on genomic DNA using primers with ClaI/EcoRV restriction sites (Table 2). Restricted PCR product was cloned into pCSEL21 (Table 3) in frame with the *gfp* ORF. The fusion fragment was excised by digestion with PstI/EcoRI and ligated into vector pCSEL24 (Table 3), producing plasmid pAFS-PDGF-alr0397. Conjugation to *Anabaena* sp. (10) resulted in single recombinants whose genomic structures were confirmed by PCR.

Determination of Chl concentration and growth rates. To determine chlorophyll *a* (Chl) concentration, 100 μl of a 50-ml shaking culture of *Anabaena* sp. without additional air/CO_2 bubbling (Table 1) was mixed with 1 ml of methanol and vortexed vigorously. Cell debris was pelleted, and the absorbance of the clear supernatant was measured at 665 nm. Chl concentration was calculated according to the following formula: μg Chl/ml = 13.43 × OD_{665} × dilution factor, where OD_{665} is the optical density at 665 nm. To determine the growth on plates, a concentration of 1 μg/ml chlorophyll was used for 5-μl spots.

To determine growth rates, 50-ml shaking cultures (100 rpm), without additional air/CO_2 bubbling, of the wild type and of the AFS-I-alr0397 mutant were grown in the standard BG11 medium for 1 week. The cells were washed three times with the indicated medium, and a volume was reinoculated in the same medium to produce a suspension with 0.4 μg Chl/ml. Samples of 200 μl were taken immediately after the reinoculation and afterwards regularly every 12 hours for 5 or 6 days. The 50-ml shaking cultures were thoroughly resuspended by six to eight passages through a 0.8-mm needle with the help of a syringe every time before the 200-μl samples were taken. Collected samples were frozen and stored at −20°C. The protein content of the samples was determined, and the data are expressed as described previously (35).

Chl fluorescence measurements. Chl fluorescence measurements utilizing the pulse amplitude technique were determined using a Maxi-Imaging-pulse amplitude technique chlorophyll fluorimeter (Heinz Walz GmbH, Effeltrich, Germany) according to reference 22. The intensity of actinic (photosynthetically active) light used for saturation pulses was 185 μmol/m^2s. Pulses were 2 s long in intervals of 20 s. The variable fluorescence as an indicator of the ability of the photosystem II to perform photochemistry calculated from the difference of the maximal fluorescence (F_m') at each time point minus the minimal fluorescence (F_0') at each time point was expressed as the ratio to the maximal variable fluorescence at the beginning of the measurement ($F_m - F_0$).

Fifty-milliliter shaking cultures (100 rpm), without additional air/CO_2 bubbling, of *Anabaena* sp. and AFS-I-alr0397 were washed three times in $BG11_{-Fe-Cu}$ medium prior to reinoculation in $BG11_{-Fe-Cu}$ or $BG11_{-Fe}$ medium (supplemented with antibiotics in the case of mutants). Cultures grown in normal BG11 medium were used as a control. All glassware used in experiments was carefully washed with 6 M HCl and Milli-Q water to diminish possible traces of metals.

Atom absorption spectroscopy. The copper, iron, and, as a control, magnesium contents were measured for *Anabaena* sp. and the AFS-I-alr0397 mutant. Cells were grown for 2 weeks in 50-ml BG11 shaking cultures (100 rpm) without additional air/CO_2 bubbling (supplemented with antibiotics in the case of mutants), washed three times in $BG11_{-Fe-Cu}$ medium, and reinoculated to grow for the next 3 days in $BG11_{-Fe-Cu}$ or $BG11_{-Fe}$ medium with the addition of antibiotics when necessary. BG11 medium-grown cultures were used as a control. Cells were collected by centrifugation at 4,000 rpm for 5 min, washed in fresh medium, and lyophilized.

(i) Sample preparation for element determination: pressure digestion. The samples were properly weighed into quartz vessels. Subsequently, 1 ml suprapure, subboiling distilled HNO_3 (Merck, Darmstadt, Germany) was added. The vessels were closed and introduced into a pressure digestion system (Seif, Unterschleissheim, Germany) for 10 h at 170°C, and the resulting clear solution was filled up exactly to 5 ml with Milli-Q H_2O and used for element determination.

(ii) Element determination. An inductively coupled plasma atomic emission spectrometer, the Spectro Ciros Vision system (Spectro Analytical Instruments GmbH & Co. KG, Kleve, Germany), was used for Fe, Cu, and Mg determination in samples. Sample introduction was carried out using a peristaltic pump (Spetec, Erding, Germany) connected to a Meinhard nebulizer with a cyclone spray chamber. The measured spectral element lines were as follows: Fe, 259.940 nm; Cu, 324.754 nm; and Mg, 279.553 nm. The RF power was set to 1,000 W, the plasma gas was 15 liters Ar/min, and the nebulizer gas was 600 ml Ar/min. Every 10 measurements, three blank determinations and a control determination of a certified Mn standard were performed. Results were calculated on a computerized laboratory data management system, relating the sample measurements to calibration curves, blank determinations, control standards, and the weight of the digested sample. The iron, copper, and magnesium metal contents were subsequently expressed in grams per kilogram or milligrams per kilogram of dry cell mass.

Analysis of siderophore secretion and iron uptake. Fifty-milliliter shaking cultures of *Anabaena* sp. and mutants (100 rpm), without additional air/CO_2 bubbling, grown in BG11 or $BG11_{-Fe-Cu}$ medium (supplemented with antibiotics in the case of the mutant) to an OD_{750} of ~0.5 were pelleted for 10 min at 4,000 rpm. To analyze secreted siderophores, the supernatant was acidified to pH 3.5 and passed through an XAD16 column. Siderophores were eluted with methanol and further analyzed by high-pressure liquid chromatography as described previously (2). Schizokinen used as a reference was obtained from EMC microcollections (Tübingen, Germany).

To analyze the secretion of siderophores on CAS plates, a white surfactant-free filter (Millipore, Schwalbach, Germany) was placed on top of the medium. The cells were spotted onto the filter to prevent an alteration of the light spectrum by the dark-blue background. The plates were photographed after 14 days from above and below.

To determine the uptake rates of schizokinen-bound iron, the cells were grown in 50 ml BG11 medium without additional air/CO_2 bubbling and shaken at 100 rpm (supplemented with antibiotics in the case of the mutant), pelleted, washed with $BG11_{-Fe-Cu}$ medium, reinoculated to an OD_{750} of 0.5 in either BG11 medium or $BG11_{-Fe-Cu}$ medium, and further grown for 19 h. The cells were pelleted and washed again and resuspended in 7 ml $BG11_{-Fe-Cu}$ medium with 100 μM nitrilotriacetate to an OD_{750} of 0.5. The cells were shaken at 28°C and illuminated. ^{55}Fe-schizokinen (EMC microcollections, Tübingen, Germany) was added to a concentration of 1 μM (18.5 kBq/ml), and 0.7-ml samples were taken at the indicated times, filtered on mixed-cellulose GN-6 Metricel membrane filters (Pall, Dreieich, Germany), washed twice with 2 ml 0.1 M LiCl solution, dried, and counted after the addition of a scintillation cocktail.

RESULTS

Iron limitation induces expression of outer membrane protein Alr0397. The orientation of the coding sequence of the alr0397 gene in the genome of *Anabaena* sp. is opposite to that of adjacent genes (Fig. 1A) (24). The deduced amino acid sequence of alr0397 shows similarity to TonB-dependent transporters of ferric aerobactin, and the highest similarity was found to IutA from *Escherichia coli* (E value, $3e^{-72}$) and RhtA from *Sinorhizobium meliloti* 1021 (E value, $2e^{-69}$). Closely located genes all0394, all0393, all0390, and all0392 show similarity to the *iuc* genes for the biosynthesis of the siderophore aerobactin in *E. coli* (9, 16) and to *rhb* rhizobactin biosynthesis genes (32). Another gene in this genomic region, all0391, shares similarity with *pvsC*, which encodes the cytoplasmic membrane exporter for the siderophore vibrioferrin in *Vibrio parahaemolyticus* (53). Finally, genes all0387, all0388, and all0389 show similarity to the *fhu* genes encoding the ABC-type ferric hydroxamate transporter. This genomic context, together with the detection of Alr0397 in the outer membrane proteome of *Anabaena* sp. (37, 38), suggests that Alr0397 might be the transporter of a hydroxamate-type siderophore like the *Anabaena* schizokinen (15, 29). We therefore investigated the expression of alr0397 under normal and iron-limiting conditions.

A translational fusion of the GFP to the promoter and eight N-terminal amino acids of Alr0397 (AFS-PDGF-alr0397) was generated in a construct comprising about 800 noncoding base pairs upstream of alr0397 and the first eight codons of the gene cloned in front of the *gfp* gene. This construct will report protein synthesis directed by the alr0397 promoter and translation signals. The fusion product was transferred to *Anabaena* sp. and integrated into the α-megaplasmid by homologous recombination. GFP fluorescence was observed in filaments of *Anabaena* sp. carrying the AFS-PDGF-alr0397 construct

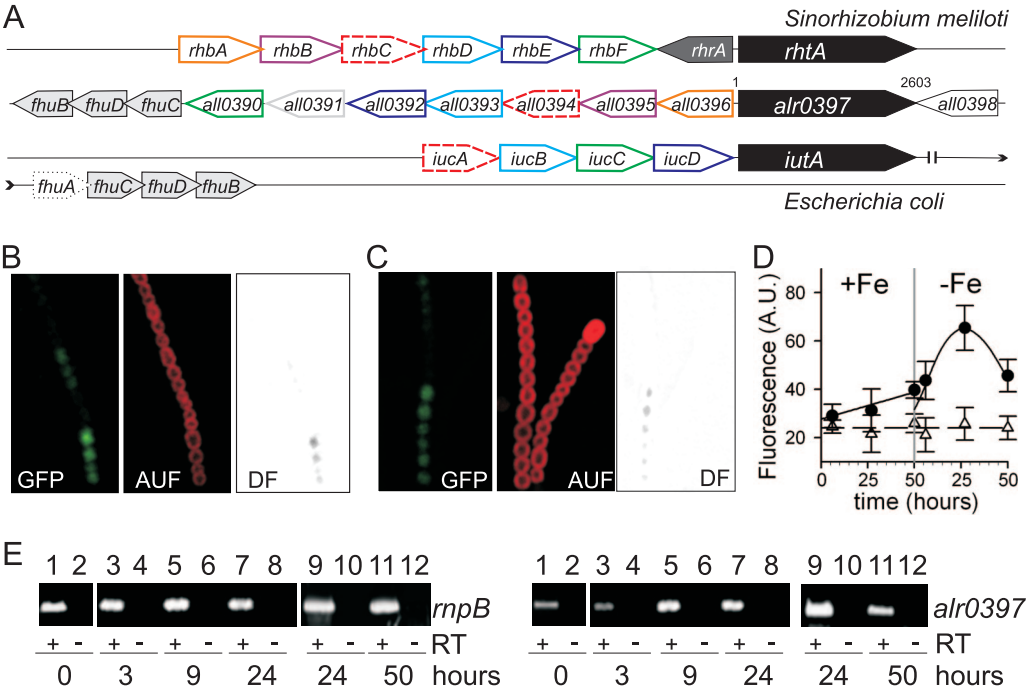

FIG. 1. Genomic organization and expression of alr0397. (A) The genomic organization of alr0397 and upstream genes is compared to that of the *rhb*, *rhr*, and *rht* genes in *S. meliloti* and the *iuc*, *iut*, and *fhu* genes in *E. coli*. White pentagons outlined by the same line indicate similar genes. The black pentagons indicate genes encoding the outer membrane iron transporters. The unique genes indicated by gray pentagons are discussed above. (B and C) The autofluorescence (AUF) and GFP fluorescence and the difference between the signals of the two channels (DF) of strain AFS-PDGF-alr0397 grown for about 40 h in BG11 medium (B) or in $BG11_{-Fe}$ medium (C) are shown. (D) The fluorescence of AFS-PDGF-alr0397 (circles) or the control strain NME-alr2887-GFP (triangles) was determined, and the difference from the background of the wild-type strain of three independent cultures is shown for the indicated times before (left) and after (right) transfer to $BG11_{-Fe}$ medium. The fluorescence (excitation at 480 nm and emission at 500 nm to 570 nm) is given in arbitrary units (A.U.). (E) RT-PCR was performed in the presence (+) or absence (−) of the reverse transcriptase using oligonucleotides for amplification of *rnpB* (left) or alr0397 (right) on RNA isolated from *Anabaena* sp. at time zero (lanes 1 and 2), or at 3 (lanes 3 and 4), 9 (lanes 5 and 6), 24 (lanes 7, 8, 9, and 10), or 50 (lanes 11 and 12) hours after transfer to $BG11_{-Fe-Cu}$ medium.

grown under normal conditions (no limitation of iron or nitrogen) (Fig. 1B). To confirm the specificity of the fluorescence signal, the signal detected using wild-type *Anabaena* sp. was determined (not shown), and additionally, the difference of the GFP and autofluorescence signal in *Anabaena* sp. carrying AFS-PDGF-alr0397 was calculated (Fig. 1B, panel DF). Both results suggested a specific GFP signal. Interestingly, the GFP fluorescence signal was not uniformly distributed along the filament (Fig. 1B), but the basis for this uneven distribution of the signal remains unknown. The same pattern was obtained when the expression was analyzed in filaments grown in $BG11_{-Fe}$ medium (Fig. 1C), a medium inducing *isiA* expression and lipid peroxidation (not shown), indicating the iron starvation (e.g., see references 13, 19, 20, 30, and 31). Hence, the expression of alr0397 is only moderately affected by iron limitation. Analyzing the time-dependent GFP fluorescence of entire cultures showed a basal expression under normal growth conditions (Fig. 1D) as previously observed for NME-alr2887-GFP (39) used as a control (Fig. 1D). When cells were shifted to $BG11_{-Fe}$ medium, the expression of AFS-PDGF-alr0397 initially increased but returned to the level before iron limitation after 2 days (Fig. 1D). The enhanced expression of alr0397 after 24 h and the subsequent decay of the expression level were confirmed by RT-PCR analyzing RNA levels in wild-type filaments after transfer to $BG11_{-Fe-Cu}$ medium (Fig. 1E). Therefore, consistent with the proteomic results, expression of alr0397 takes place in regular BG11 medium (37, 38) but is transiently enhanced under iron-limiting conditions.

Alr0397 is not essential for growth of *Anabaena* sp. To analyze the function of Alr0397, the pCSV3 plasmid was inserted into the chromosome at the alr0397 locus by single homologous recombination (see Materials and Methods). Clones with completely segregated mutant chromosomes could be isolated (Fig. 2A), indicating that this gene is not essential in *Anabaena* sp. under laboratory conditions. We designated the mutant strain AFS-I-alr0397. This strain also grew on $BG11_0$ medium (Fig. 2B), confirming that alr0397 is not essential for heterocyst development. In contrast, strain 216 carrying a *hetR* mutation (3), which was used as a control, did not grow on this medium (Fig. 2B). Consistent with diazotrophic growth of AFS-I-alr0397, synthesis of the heterocyst-specific glycolipid was not impaired and heterocyst morphologies in the mutant and the wild type were similar (not shown). Inactivation of the gene had no significant effect on amino acid uptake by cells grown in medium with or without combined nitrogen (performed according to reference 43; not shown). This shows that the mutation does not generally affect the outer membrane permeability. However, a significant reduction of the growth rate of the mutant was observed in liquid BG11 medium (Fig. 2C, BG11). When the medium was not supplemented with iron

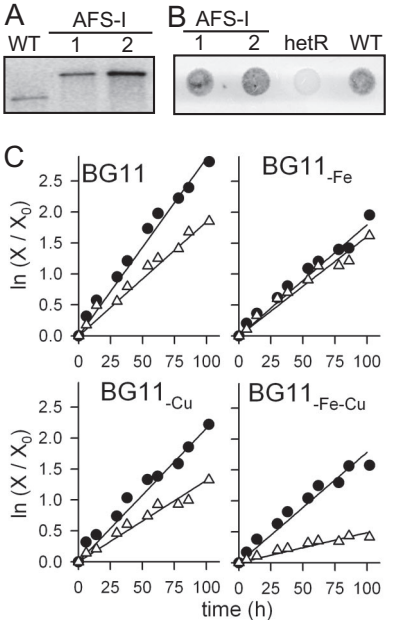

FIG. 2. Analysis of an alr0397 insertion mutant. (A) Southern blot analysis of DNA from wild-type *Anabaena* sp. (WT) and of two independent AFS-I-alr0397 clones (lanes 1 and 2) using a ^{32}P-labeled probe for the gene. (B) Wild-type *Anabaena* sp., strain 216 (*hetR*), and two independent clones of AFS-I-alr0397 (lanes 1 and 2) were incubated on solid $BG11_0$ medium. (C) The growth of *Anabaena* sp. (filled circles) or AFS-I-alr0397 (open triangles) was analyzed in BG11, $BG11_{-Fe}$, $BG11_{-Cu}$, and $BG11_{-Fe-Cu}$ media and is expressed as a natural logarithm of the ratio of the protein content at the indicated times and at time zero.

(Fig. 2C, $BG11_{-Fe}$), the mutant and the wild-type *Anabaena* sp. showed similar growth rates. Because schizokinen has been described as complexing copper ions in the medium and as protecting cells from a surplus of copper (7), we analyzed the influence of copper on the growth rate. When grown in $BG11_{-Cu}$ medium, the mutant strain showed a reduced growth rate in comparison to that of the wild-type *Anabaena* sp., and the difference was comparable to that of the strains grown in BG11 medium. Nevertheless, the strongest reduction of growth rate of wild-type *Anabaena* sp. was observed when it was grown in $BG11_{-Fe-Cu}$ medium. Under these conditions, the mutant showed a much greater growth retardation.

The ability of the mutant to grow on different iron sources and concentrations was also studied. When wild-type *Anabaena* sp. and the AFS-I-alr0397 mutant were grown on media with ferric chloride, the mutant was strongly affected independently of whether copper was present (Fig. 3, lanes 1 and 2) or not (not shown). However, this was not a general inhibition of growth by the addition of ferric chloride, because the levels of growth of the AFS-I-alr0397 mutant on BG11 medium without or with 0.01 mM ferric chloride were indistinguishable (not shown). This suggests that Alr0397 is important for the uptake of iron when provided as ferric chloride. In contrast, when iron was provided as ammonium citrate, the mutant exhibited a growth similar to that of the wild type when nontoxic iron concentrations are provided (Fig. 3, lanes 3 and 4). At iron concentrations of 1 mM, the growth of wild-type *Anabaena* sp. was significantly reduced in comparison to the

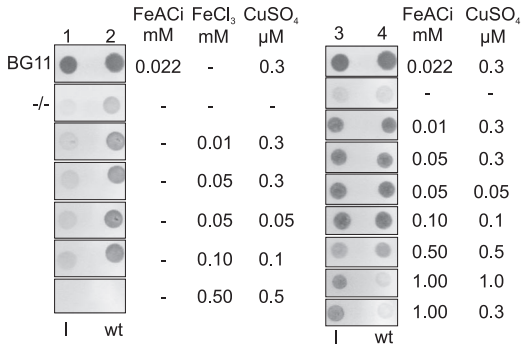

FIG. 3. Response of *Anabaena* sp. to metal variations. Wild-type *Anabaena* sp. (wt; lanes 2 and 4) and AFS-I-alr0397 (I; lanes 1 and 3) were spotted at a concentration of 1 μg/ml Chl on agar plates composed of BG11, $BG11_{-Fe-Cu}$ (−/−), and $BG11_{-Fe-Cu}$ media supplemented with the metal salts listed to the right of each panel. Cells were grown for 7 days, and the plates were photographed.

growth of the mutant. This suggests that the uptake of iron citrate is lower in the mutant, so that the cells are not poisoned by the metals as observed for the wild type.

Analysis of other indicators like *isiA* expression or lipid peroxidation, previously linked to iron starvation (13, 19, 20, 30, 31), indicated an enhanced iron stress in strain AFS-I-alr0397 (data not shown). Iron stress in the insertion mutant could also be confirmed by analysis of Chl fluorescence (Fig. 4). As previously described (22), the quenching of photosystem II activity in dark-adapted *Anabaena* sp. was released upon activation with actinic white light and increased with respect to the maximal fluorescence level (Fig. 4). This behavior is characteristic of cells that undergo a transition from state II of the dark-adapted cells to state I (12, 22). In contrast, cells under iron stress exhibited only minor changes of the Fm′ with respect to the fluorescence maximum after dark adaptation (Fm) (Fig. 4), which suggests that these cells are locked in state I (12). AFS-I-alr0397 grown in normal medium (Fig. 4), however, exhibited the same behavior of Chl fluorescence as the wild type in $BG11_{-Fe-Cu}$ medium, consistent with a defect of this strain in iron uptake. This parallels a

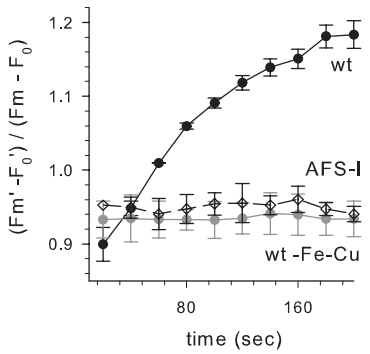

FIG. 4. Response of chlorophyll fluorescence to metal limitation. The average values of at least three independent measurements of the chlorophyll fluorescence of AFS-I-alr0397 (AFS-I; diamonds) and of wild-type *Anabaena* sp. (wt; filled circles) grown in BG11 medium or of the wild type grown in $BG11_{-Fe-Cu}$ medium (wt -Fe-Cu; gray circles) were determined as described in reference 22. The ratio between $F_m' - F_0'$ and the maximal variable fluorescence at the beginning of the measurement ($F_m - F_0$) is shown.

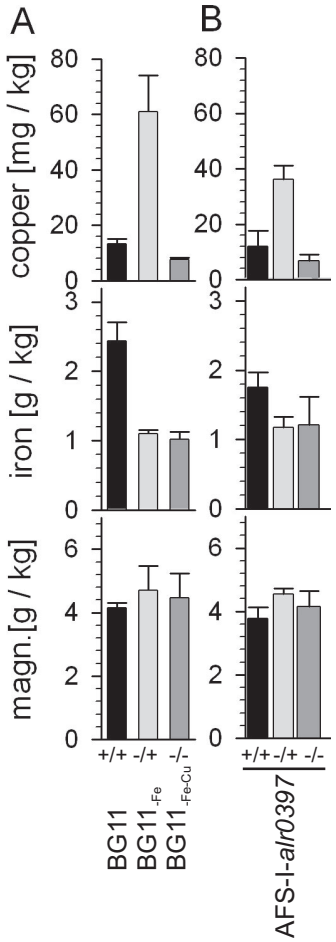

FIG. 5. Metal contents of *Anabaena* sp. and mutant strain AFS-I-alr0397. The amounts of copper (top), iron (middle), and magnesium (bottom) found in *Anabaena* sp. (A) or AFS-I-alr0397 (B) grown in BG11 (black; +Fe/+Cu), $BG11_{-Fe}$ (gray; −Fe/+Cu), or $BG11_{-Fe-Cu}$ (dark gray; −Fe/−Cu) media were quantified and are expressed relative to the dry weight of cells. Error bars are derived from the analysis of three independently grown cultures.

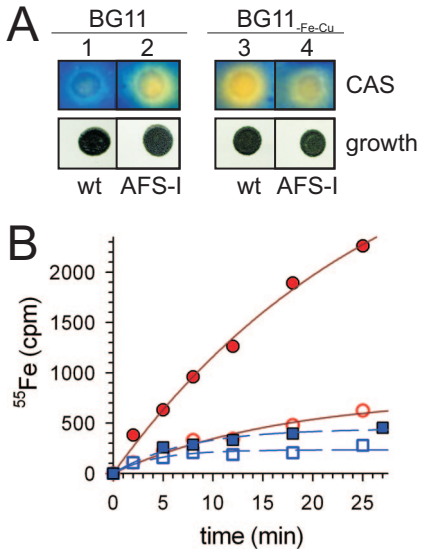

FIG. 6. Schizokinen-mediated iron uptake. (A) *Anabaena* sp. (wt; lanes 1 and 3) and AFS-I-alr0397 (AFS-I; lanes 2 and 4) were grown on CAS plates prepared with BG11 (lanes 1 and 2) or with $BG11_{-Fe-Cu}$ (lanes 3 and 4) media for 14 days as described in the text. The secretion of the siderophores (top) and the growth density of the colony (bottom) are shown. (B) The uptake of iron complexed with schizokinen by *Anabaena* sp. (circles) or AFS-I-alr0397 (squares) grown in BG11 (open symbols) or $BG11_{-Fe-Cu}$ (closed symbols) media was determined and is expressed in counts per minute retained by the cells (the same amount of cells was used in the different assays [see Materials and Methods]). Results of a representative experiment are shown.

blue shift of Chl fluorescence observed in AFS-I-alr0397 grown in BG11 medium (not shown), which has previously been determined as a sign of iron starvation (17).

Alr0397 is involved in metal uptake. To further define the role of Alr0397 in metal homeostasis, the influence of inactivation of alr0397 on the cellular levels of copper, iron, and magnesium was analyzed. For wild-type cells grown under our laboratory conditions, we obtained about 13 mg copper per kg (dry weight) of cells. This agrees with earlier data (45). For iron, we obtained about 2.5 g/kg (dry weight) and for magnesium, 4.1 g/kg (dry weight). Again, these values agree with earlier data (14, 45, 47). When *Anabaena* sp. was grown in $BG11_{-Fe}$ medium, the concentration of magnesium was not affected (Fig. 5A), whereas the iron content decreased by a factor of 1.5 (Fig. 5A, middle panel). In contrast, the copper content increased three- to fourfold (Fig. 5A). This suggests that in an iron-limited environment, copper is taken up by *Anabaena* sp., a phenomenon that will merit further research in the future. When iron and copper were omitted from the media, the cellular copper level dropped by about 50% compared to that of the BG11 medium-grown cells (Fig. 5A), whereas the magnesium content again was not affected.

Under all tested conditions, the cellular magnesium levels were similar in the wild type and strain AFS-I-alr0397 (Fig. 5B). The copper levels were not different from those in the wild type when the mutant was grown in BG11 medium or $BG11_{-Fe-Cu}$ medium (Fig. 5B). However, when the cells were grown in medium without added iron, the amount of copper was lower in the mutant than in the wild type. Analysis of the cellular iron content indicated that in BG11 medium, the metal content of strain AFS-I-alr0397 was reduced in comparison to that of the wild type (Fig. 5B). In $BG11_{-Fe}$ or $BG11_{-Fe-Cu}$ medium, the iron content of the mutant was similar to that of the wild type (Fig. 5B). These observations suggest that Alr0397 participates in iron transport and that its function, when inactivated, can partially be taken over by other as yet unknown iron transporters.

Alr0397 is the transporter for schizokinen. The similarity of Alr0397 to IutA and RhtA (Fig. 1) suggested that Alr0397 might be involved in the transport of the hydroxamate-type siderophore schizokinen, which is secreted by *Anabaena* sp. (15, 51). To test a possible relationship between Alr0397 and siderophores, the secretion of siderophores on CAS agar was checked (Fig. 6A) (49). On BG11 medium, only the mutant, not the wild type, secreted a siderophore(s), indicating induction of siderophore synthesis in the mutant strain. A clear corona surrounding wild-type colonies could be observed on $BG11_{-Fe-Cu}$ medium, indicating that a siderophore(s) is secreted. Compared to the wild type, strain AFS-I-alr0397

secreted less of the siderophore(s) under these conditions. To further analyze whether schizokinen is secreted by the wild-type and mutant strains, the concentration of schizokinen in the supernatant of liquid cultures was determined. A culture of *Anabaena* sp. in BG11 medium (OD_{750} of 0.65) contained 30 μM schizokinen, and a culture of strain AFS-I-alr0397 contained about 10 μM (at an OD_{750} of 0.63). High-pressure liquid chromatography analysis showed an additional siderophore in the media of strain AFS-I-alr0397 compared to that of the wild type (not shown), which explains the larger halo around the AFS-I-alr0397 colony than that of the wild type (Fig. 6). This observation is consistent with a recent report of an additional siderophore synthesis cluster in *Anabaena* sp. (23), which will merit further analysis in the future.

Finally, we tested whether Alr0397 could be involved in the schizokinen-based iron uptake. No significant difference in ^{55}Fe-schizokinen uptake was observed between wild-type cells from cultures grown in BG11 (Fig. 6B), $BG11_0$, or $BG11_{-Fe}$ medium (not shown). However, when *Anabaena* sp. was grown in $BG11_{-Fe-Cu}$ medium, the uptake was significantly enhanced (Fig. 6B). The differential capacity of iron uptake by *Anabaena* sp. grown in BG11 or $BG11_{-Fe-Cu}$ media might be explained by the enhanced expression of alr0397 (Fig. 1). Compared to the wild-type strain, mutant AFS-I-alr0397 showed a slightly diminished schizokinen-mediated iron uptake when grown in BG11 medium (Fig. 6B), which was only moderately enhanced when it was grown in $BG11_{-Fe-Cu}$ (Fig. 6B) or $BG11_{-Fe}$ (not shown) medium. Hence, we conclude that Alr0397 is involved in iron-schizokinen uptake.

DISCUSSION

ORF alr0397, encoding a putative TonB-dependent transporter, is present in the vicinity of predicted hydroxamate biosynthesis genes in the *Anabaena* genome (Fig. 1). Consistent with data from proteomic studies, the gene is expressed during growth in BG11 medium. Additionally, expression is somewhat enhanced in response to iron limitation, but it decays again when starvation is continued (Fig. 1). A similar observation has been reported for sll1409 in *Synechocystis* sp. strain PCC 6803 (52). The IutA (27) homologue in *Synechocystis* sp. strain PCC 6803 (sll1206), however, is not expressed in BG11 medium but is induced by iron starvation (25), which might suggest different iron uptake regimens in different cyanobacteria.

The *Anabaena* gene alr0397 encodes a transporter for schizokinen. The significantly reduced ability of strain AFS-I-alr0397 to transport iron-schizokinen and the loss of adaptation of the amount of iron transported after growth in $BG11_{-Fe-Cu}$ medium (Fig. 6) are direct evidence for this conclusion. This is consistent with the phenotype of the alr0397 insertion mutant (Fig. 2 to 5) documented by a mild, but real, iron starvation of the cells. Nevertheless, inactivation of alr0397 results only in partially reduced growth depending on the medium composition (Fig. 2 and 3), similar to the findings for the TonB-dependent transporters in *Synechocystis* sp. strain PCC 6803 (25). However, Alr0397 is not the only iron transporter present in the outer membrane of *Anabaena* sp. strain PCC 7120 (38, 39). Although the specificity and regulation of the other transporter(s) remain unknown, they possibly mask to a certain extent the phenotype of the alr0397 insertion mutant. Thus, the mutation causes only growth arrest when iron is provided as iron chloride, not when provided in the form of iron ammonium citrate (Fig. 3). This suggests the existence of an additional iron-citrate transporter. In addition, the existence of a second siderophore synthesis cluster has been recently described (23). Hence, uptake of iron complexed with schizokinen is only one mode of iron uptake explaining the mild phenotype reported.

We have observed a connection between iron and copper homeostasis. Such a relation is of interest because iron and copper are both essential for photosynthetic activity (5, 23, 50). In addition, there are copper-containing ferroxidase-dependent iron uptake systems in bacteria (5) and eukaryotes (56) that might also exist in cyanobacteria. In search of a characteristic phenotype for the mutant strain, it was observed that the inactivation of alr0397 led to a higher sensitivity to depletion of both copper and iron ions in the medium (Fig. 2). However, the thermoluminescence of *Anabaena* sp. is reduced when copper as well as iron was removed (not shown). In the absence of iron only, copper was massively incorporated into *Anabaena* sp. in an Alr0397-independent manner (Fig. 5), and the transport of iron-schizokinen was significantly reduced compared to the rate in the absence of both iron and copper (not shown). Therefore, whereas on one hand schizokinen is involved in detoxification of copper (7), on the other hand one or more uptake systems that can transport iron and/or copper appear to be induced under iron deprivation.

To summarize, Alr0397 is a schizokinen transporter of *Anabaena* sp. strain PCC 7120, and we propose to designate this protein SchT. However, consistent with the importance of iron for cyanobacterial growth, alternative routes for iron uptake and additional siderophores (e.g., see reference 23) secreted by *Anabaena* sp. appear to permit the growth of the mutant lacking Alr0397.

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