The putative siderophore-dependent iron transport network in *Anabaena* sp. PCC 7120

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Table of contents

Table of contents

List of Abbrevations

ABC-transporter	ATP-binding cassette transporter
AFS-I	*A*nabaena Frankfurt Schleiff-Insertion in
All	*A*nabaena longer (than 100 codons) left (reading direction on the circular map)
Anabaena	*Anabena* sp. PCC 7120
Alr	*A*nabaena longer (than 100 codons) right (reading direction on the circular map)
Bfr	Bacterioferritin
BG11	Blue Green 11 medium
$BG11_0$	Blue Green 11 medium without nitrogen source
CAS	Chromeazurol S
Cit	Citric acid
C.S3	Sm/Sp resistance cassette
C-terminus	Carboxy-terminus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Dps	DNA-binding protein from starved cells
E.coli	Escherichia coli
Fec	Ferric citrate uptake
FeCit	Ferric citrate
Fhu	Ferric hydroxamate uptake
Ftn	Ferritins
Fur	Ferric uptake regulator
H_2O_2	Hydrogen peroxide
hgdD	heterocyst glycolipid deposition protein D
iacT	iron and copper transporter
IM	Inner membrane
isiA	iron stress induced gene A
MFS	Major facilitator superfamily
Mnt	manganese transport system
manR	manganese response regulator
manS	manganese sensor

N-terminus	Amino-terminus
Nucnui	nucA (sugar non-specific nuclease A) nuiA (sugar non-specific nuclease inhibitor A)
OM	Outer membrane
rnpB	RNase P subunit B
Rpm	rounds per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
schE	schizokinen exporter
schT	schizokinen transporter
Sm	Streptomycin
SodA	Superoxide dismutase A (manganese-containing)
SodB	Superoxide dismutase A (iron-containing)
Sp	Spectinomycin
TBDT	TonB-dependent transporter
TonB	Transport of iron protein B

Figure index

I. Zusammenfassung

Cyanobakterien gehören zu den vielfältigsten und am weitesten verbreiteten Mikroorganismen. Sie sind die einzigen Prokaryoten, die zur oxygenen Photosynthese fähig sind. Manche Cyanobakterien können auch atmosphärischen Stickstoff als direkte Stickstoffquelle nutzen (Stickstoff-Fixierung). Aufgrund ihrer Fähigkeit Photosynthese und Stickstoff-Fixierung zu betreiben, zählen Cyanobakterien hinsichtlich ihrer Ernährung zu den genügsamen Lebewesen, allerdings haben photo-diazotrophe Bakterien einen besonders hohen Eisenbedarf im Vergleich zu heterotrophen Bakterien. Das Spurenelement Eisen ist generell für fast alle Organismen essentiell, eine Ausnahme bilden hierbei nur die Laktobazillen. Als Bestandteil von Metalloproteinen spielen Eisenionen eine Schlüsselrolle bei Oxidations-, Hydrolyse- und Elektronenübertragungsprozessen. Die direkte Aufnahme von Eisen ist jedoch problematisch, da die Konzentration an freien Eisenionen, insbesondere in wässrigen Lösungen, sehr gering ist. Mikroorganismen haben meistens mehrere Mechanismen entwickelt um Eisen aufzunehmen. In Cyanobakterien scheint die Siderophor-abhängige Eisenaufnahme der bevorzugte Mechanismus zu sein.

Siderophore sind niedrigmolekulare, lösliche Chelatbildner mit sehr hoher Affinität für Eisen(III)-Ionen, welche die überwiegende Eisenform in wässrigen Lösungen darstellen. Diese Chelatbildner können von Pilzen, Bakterien und Pflanzen synthetisiert und sekretiert werden. Für eine diffusionsgesteuerte Aufnahme sind Eisen-Siderophor-Komplexe zu groß und liegen in zu geringer Konzentration vor, um eine ausreichende Versorgung zu gewährleisten. Die Aufnahme der Komplexe erfolgt über hoch-affine Transporter, genannt TonB-abhängige Transporter (TBDT), in der äußeren Membran Gram-negativer Bakterien. TBDTs durchspannen die äußere Membran mit einer Barrel-Domäne, die aus 22 antiparallelen β-Faltblättern besteht. Der N-Terminus der TBDTs

befindet sich auf der periplasmatischen Seite und verschließt den Kanal als so genannte ‘Korkdomäne‘. Der Prozess der Eisen-Siderophor Aufnahme stellt einen aktiven Transport dar und wird über einen multimären Komplex in der inneren Membran, das so genannte TonB-system (TonB-ExbB-ExbD), energetisiert. Der genaue Mechanismus der Energieübertragung des TonB-Systems auf die TBDTs in der äußeren Membran ist noch nicht verstanden. Bekannt ist, dass die Energieübertragung zwischen der Cytoplasmamembran und der äußere Membran eine Reihe von Konformationsänderungen des TonB-Proteins voraussetzt. Es wird davon ausgegangen, dass der ExbB/ExbD-Komplex Energie aus dem Protonenpotenzial der Cytoplasmamembran auf das TonB-Protein überträgt. Das ‘energetisierte‘ TonB-Protein interagiert über seinen C-Terminus mit dem TBDT in der äußeren Membran. Diese Interaktion führt zu einer Konformationsänderung des TBDTs, die die Freilassung des gebundenen Substrats (Eisen-Siderophor-Komplex), die Verschiebung der Korkdomäne und somit die Öffnung des Kanals zur Folge hat.

Vom Periplasma aus wird das Eisen oder die Siderophor-Eisen-Komplexe über Substratbindeprotein-abhängige ABC-Transporter in das Innere der Zelle transportiert. Diese Transportsysteme bestehen aus einem membrandurchspannenden Teil, der sich aus zwei Permeasen und zwei cytoplasmatisch vorliegenden ATPasen zusammensetzt. Das Substratbindeprotein liegt frei im Periplasma vor und bindet die kompatiblen Substrate mit hoher Affinität. Diese Bindung führt zu einem Konformationswechsel des Substratbindeproteins und befähigt es, an den ABC-Transporter in der Cytoplasmamembran zu binden. Der ABC-Transporter transportiert das Substrat, angetrieben durch ATP-Hydrolyse, aktiv über die Cytoplasmamembran.

Trotz des besonders hohen Eisenbedarfs ist das Verständnis der Eisenaufnahmesysteme in Cyanobakterien noch weitgehend unverstanden. Cyanobakterielle Eisenaufnahmesysteme sind am besten untersucht in einzelligen, nicht Siderophore

produzierenden Cyanobakterien der Gattung *Synechococcus* und *Synechocystis*. *Anabaena* sp. PCC 7120 hingegen stellt als ein im Süßwasser lebendes, Siderophore-synthetisierendes und multizelluläres Cyanobakterium eine andere Art von Modelorganismus dar. Im *Anabaena* Genom konnten mittels bioinformatischer Analyse 22 TBDT kodierende Gene identifiziert werden. Da Gram-negative Bakterien üblicherweise nur 1-5 TBDTs im Genom tragen, stellt dies eine deutliche Überrepräsentation an TBDTs dar. Eine Expressionsanalyse vom Wildtyp *Anabaena* ergab, dass alle Gene exprimiert werden, wenn auch unter verschiedenen Bedingungen. Die hohe Anzahl an TBDTs deutet auf ein komplexes TonB-abhängiges Aufnahmesystem in *Anabaena* hin, welches im Hinblick auf die Expressionsanalyse möglicherweise nicht ausschließlich auf Eisen beschränkt ist. Für zwei der 22 TBDTs (Alr0397; All4026) konnte experimentell auch eine Rolle bei der Eisenaufnahme nachgewiesen werden. Um die Siderophor-abhängigen Eisenaufnahmesysteme in *Anabaena* zu vervollständigen, wurde die bioinformatische Analyse auf die fehlenden *tonB*, *exbB* und *exbD* kodierenden Gene erweitert. Die Analyse ergab vier hypothetische *tonB* kodierende Gene (*alr0248*, *all3585*, *all5036*, *alr5329*), zwei hypothetische *exbB-exbD* kodierende Gen-Kluster (*alr0643-alr0644*, *all5047-all5046*) und ein einzelnes putatives *exbB* kodierendes Gen (*alr4587*). Von den Bindeprotein-abhängigen ABC-Transportern trägt das Genom von *Anabaena* verschiedene Klassen, darunter Komponenten des Fec-, Fhu- und Fut-Systems.

In dieser Arbeit wurde die Funktion der bioinformatisch vorhergesagten Siderophor-abhängigen Eisenaufnahmesysteme in *Anabaena* untersucht. Die meisten bakteriellen Gene, die an der Eisenaufnahme beteiligt sind, werden erst bei Eisenmangel transkribiert. Um einen Überblick über die Gene zu erhalten, die an der Eisenaufnahme in *Anabaena* beteiligt sind, wurde der Wildtyp unter Eisenmangel kultiviert und eine Expressionsanalyse der vorhergesagten Gene mittels quantitativer real time PCR (qRT-

PCR) durchgeführt. Da Cyanobakterien ihren Stoffwechsel an Eisenmangelbedingungen anpassen können, indem sie unter anderem auch eisenhaltige Proteine durch kupferhaltige Proteine ersetzen, siehe Ersatz von Cytochrome c_6 durch Plastocyanin, und dadurch die Folgen des Eisenmangels mindern, wurde bei der Expressionsanalyse auch eine Kombination aus Eisen- und Kupfermangel getestet. Als Kontrollbedingungen dienten hohe Eisen- und/oder Kupferkonzentrationen, sowie Medium ohne Stickstoffquelle ($BG11_0$). Die Expressionsanalyse ergab unter Eisenmangel eine verstärkte Transkription der folgenden Gene: *all5036* (*tonB3*), *all5047* (*exbB3*), *all5046* (*exbD3*) und *all0387* (*fhuB*). Das Transkriptionsprofil dieser Gene erfüllte auch unter den Kontrollbedingungen die erwartete Regulation eines spezifisch unter Eisenlimitation induzierten Systems, wie z.B. eine Verminderung der Gen-Transkription bei erhöhten Eisenkonzentrationen.

Um die *in vivo*-Relevanz dieser Gene zu überprüfen wurden Insertionsmutanten von *tonB3*, *exbB3*, *fhuB*, *fhuC* und *fhuD* erstellt. Hierbei wurde mittels Insertion eines Vektors das jeweilige Gen unterbrochen (Homologe Rekombination). Da das Genom von Cyanobakterien mehrere Kopien desselben Gens trägt, muss bei Insertionsmutanten getestet werden, ob noch Wildtyp-Kopien des entsprechenden Gens vorhanden sind. Die Analyse der Insertionsmutanten zeigte, dass ausschließlich die *exbB3*-Mutante keine Wildtyp-Kopien des Gens mehr aufwies und somit vollständig segregiert war. Die *tonB3* Insertionsmutante hingegen musste zunächst in Medium mit erhöhter Eisenkonzentration kultiviert werden, um eine vollständige Segregation zu erreichen. Die Überprüfung der *tonB3*-Mutante in BG11 Medium resultierte jedoch in einem schnellen Wiederauftauchen der *tonB3* Wildtyp-Kopien. Das beobachtete Segregationsverhalten der *tonB3* Mutante entspricht dem beobachteten Transkriptionsprofil von *tonB3* und deutet letztendlich auf eine essentielle Funktion des Proteins bei der Eisenaufnahme hin. Zur Untersuchung des Eisenhaushaltes der

Mutanten wurden verschiedene Experimente durchgeführt. So wurde die Expression der Gene *furA* und *isiA* getestet, da beide Gene als Indikatoren für Eisenmangel dienen. Die Analyse ergab eine erhöhte Transkription beider Gene in allen Mutanten und das bereits im mit Eisen versehenen BG11 Medium. Da Siderophorsynthese ein starkes Anzeichen für Eisenmangel ist, wurde die Siderophorsekretion der Mutanten untersucht. Zur Detektion der sekretierten Siderophore wurden Chromazurol S (CAS)-Platten verwendet. Das Wachstum der Mutanten auf BG11 sowie eisen- und kupferlimitierten CAS-Platten ergab eine besonders starke Siderophorsekretion der *tonB3* und *fhuC* Mutante im Vergleich zum Wildtyp und den restlichen Mutanten. Insgesamt kann anhand der Ergebnisse ein Modell zur Eisenaufnahme in *Anabaena* entworfen werden. Hierbei sind das TonB3-System, bestehend aus TonB3-ExbB3-ExbD3, und das Fhu-System Komponenten der Siderophor-abhängigen Eisenaufnahme in *Anabaena*.

Zur Untersuchung der Funktion von TonB1 und TonB2 wurden Insertionsmutanten von diesen Genen erstellt. Die Anzucht der *tonB1*, *tonB2* und *tonB3* Mutanten im flüssigem BG11 Medium ohne Zugabe von Eisen, führte nur zum verzögertem Wachstum der *tonB3* Mutante. Interessanterweise war das Wachstum der *tonB2* Mutante beeinflusst, wenn das Medium weder Eisen noch den Eisenchelator Zitronensäure enthielt. Da die Zugabe von Eisenchlorid das Wachstum der *tonB2* Mutante wiederherstellen kann, scheint es sich hierbei auch um einen Eisen-abhängigen Phänotyp zu handeln. Unterstützt wird diese Annahme auch durch den verstärkten oxidativen Stress in der Mutante, welcher anhand der verstärkten Transkription des *sodB* kodierenden Genes ersichtlich ist.

Die *tonB1* Mutante wies nach mehrmaligem Überführen in BG11 Medium ein verzögertes Wachstum auf. Dieser Phänotyp ist jedoch unabhängig von der Eisenkonzentration, da die Zugabe von unterschiedlichen Eisenquellen das Wachstum der Mutante nicht wiederherstellen kann. Wachstumstests auf Agarplatten mit

unterschiedlichen Metallkonzentrationen (Zink, Mangan, Eisen, Kobalt, Kupfer) zeigen eine erhöhte Resistenz der *tonB1* Mutante gegenüber hohen Mangan- und Kupferkonzentrationen. Da in Gram-negativen Bakterien für beide Metalle eine Porin-abhängige Aufnahme postuliert wird, wurde die Expression von sieben hypothetischen Porin kodierenden Genen in der *tonB1* Mutante untersucht. Die Expressionsanalyse ergab eine reduzierte Expression aller sieben getesteten Gene. Eine veränderte Permeabilität der äußeren Membran, die möglicherweise von einer reduzierten Porin-Konzentration herrührt, unterstützt auch die beobachtete Resistenz der *tonB1* Mutante gegenüber unterschiedlichen Antibiotika auf Agarplatten. Zusammenfassend deutet die Charakterisierung der *tonB1* Mutante auf eine regulatorische Rolle des Proteins in der Porin-Expression hin.

II. Abstract

Cyanobacteria belong to the most widely distributed microorganisms in the biosphere and contribute significantly to global primary production. Their metabolism is based on oxygenic photosynthesis and some cyanobacteria can fix elemental nitrogen. Obligate photosynthetic diazotrophs have a particularly high iron demand in comparison to heterotrophic bacteria. Nevertheless the understanding of iron acquisition in cyanobacteria is just beginning to emerge. Iron acquisition in bacteria comprises highly specific transport of siderophore-iron complexes over the outer membrane by TonB-dependent transporter (TBDT). The transport itself is active and energized by a multi-complex localized to the inner membrane termed the TonB-system (TonB-ExbB-ExbD). The siderophore-iron complexes are further transported into the cytosol by a binding protein dependent ABC-transporter. Cyanobacterial iron acquisition response has most extensively been studied in unicellular, non-siderophore synthesizing cyanobacteria in the genus *Synechococcus* and *Synechocystis*. *Anabaena* sp. PCC 7120, however, is a different model organism as it is a freshwater living, siderophore synthesizing and, truly multicellular microorganism. It can be assumed that siderophore synthesis and siderophore-dependent iron uptake are tightly coordinated processes, therefore *Anabaena* represents a different model organism as compared to non-siderophore producing cyanobacteria. Moreover the surprisingly abundant protein family of 22 putative TBDTs in *Anabaena* indicates a high complexity of TonB-dependent uptake systems. Sequence similarity analysis revealed 4 putative *tonB* encoding genes (*alr0248*, *all3585*, *all5036*, *alr5329*), 2 putative *exbB-exbD* encoding gene cluster (*alr0643-alr0644*, *all5047-all5046*), one single standing putative *exbB* encoding gene (*alr4587*) and several hypothetical binding-protein-dependent ATP

binding cassette (ABC)-type transporter encoding genes (*fhu*-, *fec*- and *fut*-type transporter).

In this study the respond of the predeicted systems to iron-limiting conditions was analysed by qRT-PCR. The expression analysis revealed on the one hand an enhanced transcription of *all5036* (*tonB3*), *all5047-all5046* (*exbB3-exbD3*) and the *fhu*-like encoding genes (*all0387-all0389*) under iron-limitation and at the same time down-regulation of expression under enhanced iron concentrations. Summerizing the transcription profile of the *tonB3*- and the *fhu*-system showed an expression regulated by iron-availability. To further characterize the role of TonB3-, ExbB3- and the Fhu-system, mutants thereof were generated. None of the generated mutants, except for the *exbB3* mutant, could be fully segregated, suggesting an essential character of the genes. Characterization of the mutants revealed enhanced expression of iron-starvatrion indicator genes (*isiA*, *fhuA*) and altered growth of the *tonB3* mutant under iron-limiting conditions. The iron starvation phenotype was further strengthened by enhanced siderophore secretion in the *tonB3, exbB3* and *fhuC* mutants. Taken as a whole the results strongly indicate involvement of the *tonB3*- and the fhu-system in siderophore-dependnet iron uptake in *Anabaena*.

Investigation of the tonB2 (*all3585*) mutant under iron and citric acid limitation resultated in altered growth of the mutant. However, growth could be restored by addition of iron chlorid. Therefore a connection of the TonB2 protein to iron uptake is implied and further supported by ressitance to toxic iron concentrations. Lastly, mutation of *tonB1* (*alr0248*) reuslted in insensibility to toxic manganese and copper concentrations and macrolid antibiotics. The altered permeability of the outer membrane may be a result of decreased expression of seven putative porin encoding genes in the mutant. A possible role in transcriptional regulation of porin expression is discussed.

1. Introduction

1.1 Cyanobacteria

Anabaena sp. strain PCC 7120 (*Anabaena* hereinafter) is a filamentous, freshwater living, and heterocyst-forming cyanobacterium. Cyanobacteria, also known as "blue-green algae", belong to the kingdom of eubacteria (Castenholz and Waterbury, 1989). They represent a large and morphologically diverse group of Gram-negative bacteria, common in aquatic and terrestrial ecosystems. Cyanobacteria can be found even in the most extreme environments on Earth, ranging from hot springs, Antarctic ice shelves to deserts (Castenholz, 1973; Dor and Danin, 1996; Laamanen, 1996). Their impressive ability to colonize such a wide range of habitats is a result of a combination of effective metabolic pathways and highly effective eco-physiological adaptations (Paul et al., 2007). In general, cyanobacteria have contrasting roles in natural environments, as they can form productive symbiotic associations with other organisms, the most notable of these is the *Anabaena-Azolla* symbiosis (reviewed by Peters and Meeks, 1989). At the same time, many species form harmful ‘blooms’ by massive proliferations in freshwater, brackish and coastal marine ecosystems. Cyanobacterial blooms have massive impacts on ecosystem functioning, due to changes of light conditions and oxygen concentrations, furthermore, many cyanobacteria are able to synthesize toxins (reviewed in Sivonen and Jones, 1999; Pearl et al., 2001). All of these factors in turn lead to changes of biodiversity and can in addition reinforce cyanobacterial growth.

The capability of cyanobacteria to dominate primary production in diverse habitats is also attributable to their photoautotrophic lifestyle. In fact, cyanobacteria are thought to be the most ancient oxygenic phototrophic organisms (Brock, 1973). It is widely assumed that the ability of ancient cyanobacteria to perform oxygenic photosynthesis led to the oxidation of the ancient atmosphere and resulted in the ‘Great Oxidation

Event' around 2,3 billion years ago (Holland, 2002; Guo et al., 2009). Additionally, cyanobacteria also strongly influenced the evolution of phototrophic eukaryotes as according to the 'Endosymbiosis theory', plastids in red algae, green algae, and plants are believed to have originated from cyanobacterial ancestors (Margulis, 1970; reviewed by Keeling, 2010). These days, cyanobacteria are one of the most abundant photoautotrophic organisms in the ocean and therefore among the major primary producers (Garcia-Pichel et al., 2003).

Another advantageous feature of some cyanobacterial species is their ability to fix elemental nitrogen. Diazotrophic cyanobacteria are interesting research objects for many reasons, one of them is the fact that cyanobacteria that spatially separate between oxygenic photosynthesis and N_2-fixation (heterocystous cyanobacteria *Nostoc* and *Anabaena*) are considered as truly multicellular organisms (Lehner et al., 2011). Moreover, the ability of cyanobacteria to fix elemental nitrogen enables them to provide a substantial source of nitrogen to terrestrial ecosystems, which also has been exploited for fertilising purposes in agriculture (Roger, 1991). However, the two metabolic features that mark cyanobacteria, come at a price, which is a high demand for iron as essential cofactor for many catalytic proteins in these bioenergetic pathways. This demand is most notably derived from the 23-24 iron atoms needed for the functional photosynthetic apparatus, and the 19 iron atoms per heterodimeric protein complex moiety required for nitrogenase function (Shi et al., 2007).

1.2 Role and properties of iron

Iron is not only for cyanobacteria an essential micronutrient, but for virtually all organisms, except for the genus Lactobacilli (Archibald, 1983). The biological functionality of iron depends upon its incorporation into proteins. Iron is incorporated either as a mono- or binuclear species, or in some cases in a more complex form as part of iron-sulphur clusters or haem groups (Andrews et al., 2003). The redox potential of iron (ranges from -300 to +700 mV) makes it extremely versatile as a biocatalyst or an electron carrier in proteins (Andrews et al., 2003). Besides photosynthesis and nitrogen fixation, iron is also essential for many other major biological processes, such as respiration, the trichloroacetic acid (TCA) cycle, oxygen transport, DNA biosynthesis, and gene regulation (Boyer et al., 1987; Straus, 1994; reviewed by Krewulak and Vogel, 2008).

Iron is the fourth most abundant element in the Earth's crust and approximately one-third of Earth's mass is comprised of iron (Fox, 1988). Its abundance and the two readily convertible redox states have made it the ideal choice for incorporation into proteins during the evolution of early life. Therefore, iron was probably the key constituent in the first prosthetic moieties (Beinert et al., 1997; Cody et al., 2000; Andrews et al., 2003). However, once the oxygenic photosynthesis, performed by ancient cyanobacteria, began to 'pollute' the atmosphere with molecular oxygen, the prevailing iron chemistry altered significantly. Oxygenation of Earth's atmosphere led to a switch from the relatively soluble (0.1 M at pH 7.0) ferrous state (Fe^{2+}) to the extremely insoluble (10^{-18} M at pH 7.0) ferric form (Fe^{3+}) of iron (Andrews et al., 2003). Therefore the essential minor nutrient became severely restricted and growth-limiting. The bioavailability of iron is particularly low in aquatic systems, where roughly 30 % of surface waters in the open ocean are described as high nutrient low chlorophyll (HNLC) regions (Boyd et al., 2007; Gledhill and Buck, 2012). Nowadays it is accepted that the

lower phytoplankton biomass, determined as chlorophyll concentration, in the world's oceans is a result of iron limitation (Martin and Fitzwater, 1988; Boyd et al., 2007). Moreover, it is becoming apparent that the availability of iron also determines phytoplankton growth in coastal regions and some lakes (Hutchins et al., 1998; Twiss, 2000). In an endeavour to reduce the amount of carbon dioxide from the atmosphere, the idea of increasing phytoplankton production by addition of iron has renewed interest in the topic of iron bioavailability and phytoplankton growth-limitation (Tortell et al., 1999).

Under aerobic conditions and physiological pH the thermodynamically favoured state of iron is ferric iron. Ferric ion, however, aggregates into ferric hydroxides, which tend to precipitate and are considered the biologically unavailable form of iron (Ratledge and Dover, 2000). So the first step in iron transport is the mobilization of iron which can be accomplished by three strategies: i) reduction, ii) acidification, and iii) chelation.

Some species reduce ferric iron to ferrous iron prior to uptake by a surface reductase (Schroder et al., 2003). Reduction has some obvious advantages, as ferrous iron is significantly more soluble and binds with lower affinity to most chelators than ferric iron. Ferrous iron, though, is under aerobic conditions readily oxidized to ferric iron. For this reason, the reduction has to occur close to the site of transport or in excess amount. Reduction is accomplished by cell surface reductases and used for instance by fungi (Lesuisse et al., 1987).

Acidification of the extracellular environment is a strategy used by non-graminaceous plants (de Vos et al., 1986; Robinson et al., 1999). Under iron deficiency protons are secreted by H^+-ATPases from the roots into the soil and by this reducing the pH of the soil from seven to three or even lower. Low pH has in terms of iron uptake three main advantages. The concentration of free ferric ions is increased by inhibition of formation of hydrolysis products, ferrous ions are stabilized relative to ferric ions, thereby

disfavouring reoxidation by dioxygen. Furthermore the increased [H^+] competitively inhibits binding of both ferric and ferrous ions to extracellular chelators (reviewed by Lyons and Eide, 2007).

Soluble ferric ions exist at much higher concentrations in aquatic environments, if they are bound to organic chelators as for instance siderophores (Gledhill and van den Berg 1994). Siderophores are small organic molecules (<1000 Da) that are synthesized and secreted by many microorganisms, such as bacteria, fungi, and monocotyledonous plants (Romheld and Marschner, 1986; Miethke and Marahiel, 2007). They exhibit high affinity and selectivity for ferric iron, with binding constants (K_a) ranging from 10^{30} M^{-1} to 10^{52} M^{-1} (Albrecht-Gary and Crumbliss, 1998; Crumbliss and Harrington, 2009). Over 500 species of these molecules have been discovered, but approximately only one half of them are structurally characterized (Hider and Kong, 2010). Siderophores are classified according to the three putative functional groups that are used to bind ferric iron. These groups employ hydroxamates, α-hydroxycarboxylates, and catechols as ferric ligands (Miethke and Marahiel, 2007). Though siderophores keep the ferric iron soluble, the bioavailability of these iron forms is species dependent, as the siderophore-iron complex is taken up by transporters with a very stringent siderophore selectivity.

1.3 Siderophore-dependent iron uptake

Following the mobilization of iron by siderophores, as described above, the resulting complexes need to be transported across the outer membrane (OM) of Gram-negative bacteria. Siderophore-iron complexes, however, cannot be taken up by simple diffusion, as they need on one hand to be taken up into the periplasm against their concentration gradients and exceed, on the other hand, the pore-diameters of porins in the OM (Nikaido, 1992). Therefore the siderophore-iron complexes are taken up in an energy-dependent process by specific OM receptors termed TBDTs (TonB-dependent transporter; Braun and Endriss, 2007). TBDTs exhibit structural similarities to porins, as they consist of a 22 β-stranded channel that traverses the OM, albeit, the central pores of TBDTs have much larger interiors, which are gated by a globular domain positioned in the pore (termed the 'cork' or 'hatch region'; reviewed by Krewulak and Vogel, 2008). To allow passage of iron-siderophores into the periplasm the 'plug domain' needs to be moved in order to open the pore, however, this process requires energy (Krewulak and Vogel, 2008). As consequence of the impossibility of a transmembrane potential across the OM and the absence of an energy-generating system in the periplasm, the energy required for the transport is derived from the proton motive force (pmf) across the inner membrane (IM; Rosen, 1987; Hancock and Braun, 1976). The system responsible for the energy-transduction from the IM to the OM receptors is embedded in the IM and called the TonB-system (Hancock and Braun, 1976; Holroyd and Bradbeer, 1984). This energy-transduction system is composed of the TonB, ExbB, and ExbD proteins. TonB and ExbD are embedded in the IM by their N-terminal hydrophobic domain, while the bulk of the proteins extends into the periplasm (Postle, 1990; Kampfenkel and Braun, 1992). ExbB, on the other hand, traverses the IM three times and has two short stretches localized in the periplasm, while two large portions of the protein reach into the cytoplasm (Kampfenkel and Braun, 1993). It is proposed that

TonB and ExbD bind to ExbB, as ExbB was shown to stabilize both proteins from degradation by proteases (Fischer et al., 1989; Skare and Postle, 1991). For TonB-dependent transport processes, however, all three proteins are essential (Ahmer et al., 1995). The mechanism by which the complex utilizes the pmf across the IM is still unknown. The transduction of the energy from the IM to the OM, nevertheless, is accomplished by the TonB protein. The TonB protein is proposed to span the entire periplasmic space, and the C-terminal domain of the protein has been shown to physically interact with TBDTs. Major experimental evidence for the interaction of TonB with TBDTs exists and comprises genetic suppressor analysis, cross-linking experiments with formaldehyde, cross-linking experiments between introduced cysteine residues and crystal structure determinations of TBDTs with bound C-terminal fragments of TonB (Heller et al., 1988; Schöffler and Braun, 1989; Bell et al., 1990; Günter and Braun, 1990; Skare et al., 1993; Ogierman and Braun, 2003; Pawelek et al., 2006; Shultis et al., 2006). The recognition and interaction site of the OM receptor has been identified as a hydrophobic stretch of seven amino acids at the N-terminus of TBDTs, termed the 'TonB box' (Postle, 1993). Even though the 'TonB box' is highly conserved among all TBDTs, it is suggested that the conformation rather than the sequence of the TonB box is important for TonB interaction (Larsen et al., 1997). Substrate binding to the OM receptor is assumed to trigger a conformational transition on the periplasmic face of the receptor, where a not well defined change in the conformation of the 'TonB box' would lead to interaction with the TonB protein (Merianos et al., 2000). This kind of mechanism is believed to ensure the substrate-specificity of the receptors and to 'direct' the TonB protein to substrate loaded transporters. Finally the interaction with the TonB protein alters the conformation of the TBDT in such a way that the pore is opened by moving of the plug domain, the substrate

is released from its binding site and transported through the channel into the periplasm (Braun and Hantke, 1991; Bradbeer, 1993).

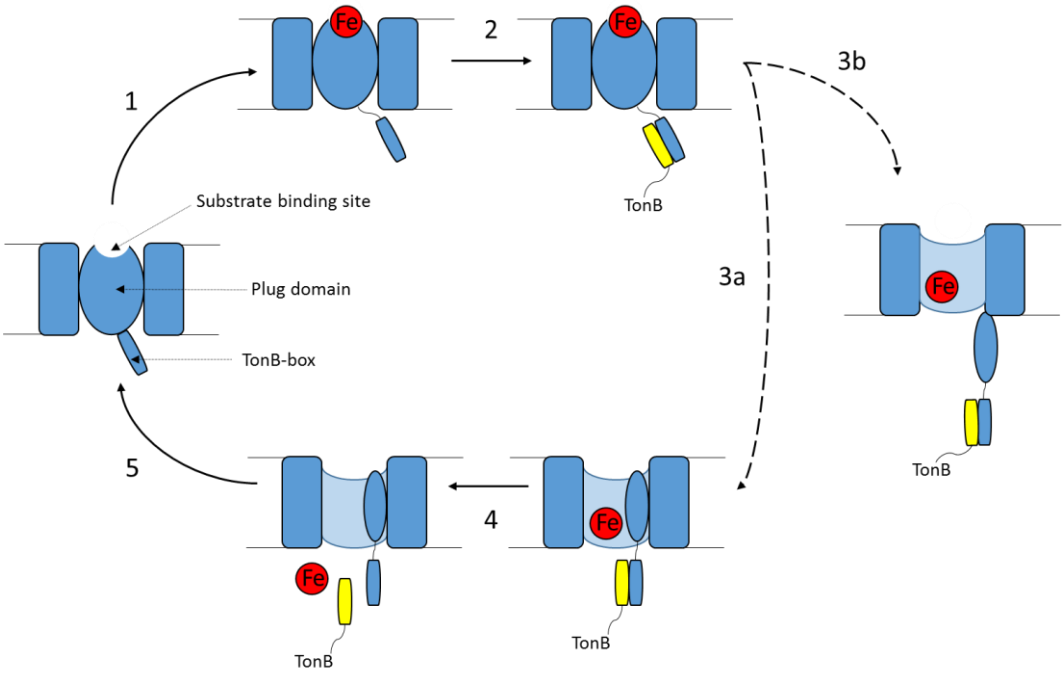

Figure I. Schematic diagram illustrating critical steps in the TBDT-dependent transport cycle. In the TBDT 'empty state' the plug domain is occluding the channel and the TonB-interaction site (TonB-box) resides close to the barrel. (1) Upon ferric-siderophore complex binding to the substrate binding site of the TBDT, the TonB-box undergoes a transition to a more periplasmic exposed state. (2) The C-terminus of the TonB-protein interacts with the TonB-box and initiates a conformational change of the receptors plug domain. Two theoratical ways to change the conformation of the plug domain exist: (3a) the plug domain is unfolded within the barrel or (3b) pulled into the periplasm to open the channel. (4) The ferric-siderophore complex is transported into the periplasm and the TonB interaction is detached. (5) The TBDT returns again to the 'empty state'.

Once the iron-siderophore complex has entered the periplasm it is further transported into the cytoplasm by a binding protein-dependent ATP-binding cassette (ABC)-type transporter (BP-dependent ABC transporter hereinafter). This kind of transporters commonly consists of three structural components: (1) a periplasmic substrate binding protein (BP), (2) one or two different polytopic integral membrane proteins (IMP) forming a channel, and (3) one or two nucleotide binding domains facing the cytosol, hydrolysing ATP and supplying the system with energy (reviewed by Davidson et al.,

2008). Bacterial ABC transporters are usually assembled from separate subunits encoded by different genes that are often organized in an operon, although some exceptions have been observed (Köster, 2001). Till today five ABC-transporter subtypes involved in ferric-siderophore uptake have been identified in Gram-negative bacteria (Braun and Hantke, 1997). Transport of siderophores across the IM is in general less specific than the translocation over the OM. In *E.coli* for instance three different OM receptors recognize siderophores of the catechol type, while only one ABC system is needed for the passage into the cytosol (Köster, 2001 and references therein). The best-characterized system is the ferric hydroxamate uptake (Fhu) system (Braun et al., 1982; Köster, 1991).

In the cytoplasm two mechanisms are possible to release iron from the siderophore: (1) hydrolysis of the siderophore or (2) reduction of ferric iron to ferrous iron by an iron reductase (Shalk and Guillon, 2013).

1.4 Iron homeostasis in cyanobacteria

Iron is not only an essential and not readily bioavailable element, but also potentially toxic, due to the interactions of iron with dioxygen and reactive oxygen species (ROS). Balancing sufficient iron supply and the danger of oxidative stress caused by iron is an acute problem that photosynthetic organisms face constantly (Shcolnick et al., 2009). The photosynthetic apparatus leads to an iron requirement that is 1 order of magnitude higher than that of non-photosynthetic organisms (Keren et al., 2004). Then, on the other hand, the photosynthetic electron transfer chain utilizes radicals and reduced metal species as part of its normal catalysis, all prone to cause oxidative damage if not balanced properly (Shcolnick and Keren, 2006; Shcolnick et al., 2009). Therefore, the cellular uptake and storage of iron in cyanobacteria needs to be tightly controlled.

Intracellular levels of free iron do not reach toxic levels, as iron is stored in a way that prevents its interaction with various redox reactive components. Responsible for the storage of iron are ferritins, which are widely distributed proteins among members of the different kingdoms (Theil, 1987). Ferritins catalyse the oxidation of ferrous iron to ferric iron via ferroxidase centres and the ferrous iron is subsequently stored in the form of ferric oxo products (Andrews, 2010). Ferritin-like proteins in cyanobacteria include the so called DNA-binding proteins from starved cells (Dps), bacterioferritins (Bfr) and in some cases also ferritins (Ftn; Andrews, 2010). In *Anabaena* four putative ferritin-like family genes with the highest similarity to Dps proteins were identified by genome search (Keren et al., 2004). While Ftns and Bfrs primarily use dioxygen to oxidize the ferrous iron, the Dps proteins exibit an over 100-fold higher rate with hydrogen peroxide (H_2O_2; Bou-Abdallah, 2010; Zhao et al., 2002). The preference of Dps proteins for H_2O_2 makes it an important factor in oxidative stress response, e.g. by catching and sequestering free Fe^{2+} while simultaneously reducing H_2O_2 to water and thereby preventing the Fenton reaction to occur (Ekman et al., 2014). For two of the four

identified putative Dps proteins in *Anabaena*, namely *all0458* and *all1173*, ferroxidase activity was experimentally shown (Wei et al., 2007; Sato et al., 2012).

To control iron uptake and storage the intracellular iron levels have to be sensed. This is monitored by the global iron-responsive transcriptional regulator Fur (ferric uptake regulator; reviewed by Andrews et al., 2003). In *Anabaena* three Fur homologues (FurA, FurB, FurC) have been identified (Hernández et al., 2004). The constitutive and essential protein FurA is the master regulator of iron homeostasis (Hernández et al., 2004 and 2006; González et al., 2012). In the presence of iron the FurA protein binds ferrous iron and acquires a conformation able to bind to cis-acting regulatory elements ('Fur boxes'), located in the promoter regions of target genes, and thereby inhibits transcription (Escola et al., 1999). On the contrary, upon iron-restricted conditions, the equilibrium is displaced to release Fe^{2+} bound by FurA, thereon Fur dissociates from the Fur box and the RNA polymerase is able to access cognate promoters. In this way FurA is controlling the expression of iron acquisition and storage systems, however, it can be considered as a global regulator, since also many genes and operons independent of a function in iron homeostasis are controlled by Fur (Lin et al., 2011; Gilbreath et al., 2012). Increased expression of FurA upon iron deprivation, oxidative stress and during heterocyst differentiation has been observed (Hernández et al., 2002; López-Gomollón et al., 2007 and 2009).

1.5 Homologs of the siderophore-dependent iron acquisition systems in *Anabaena*

Apart from the storage of iron and analysis of the global transcriptional regulator Fur family only little is known about the components involved in siderophore-dependent iron uptake in cyanobacteria. The only structurally characterized siderophore secreted by *Anabaena* is the citrate based hydroxamate type siderophore schizokinen (Goldman et al., 1983). A MFS type transporter (Major facilitator superfamily) putatively localized in the IM is involved in secretion of schizokinen into the periplasm (schizokinen exporter, SchE, encoded by *all4025*; Nicolaisen et al., 2010). The secretion of siderophores across the OM is like in *E.coli* a TolC-dependent process (Nicolaisen et al., 2010). The TolC homologue of *Anabaena* is encoded by *alr2887* and has, according to its function in heterocyst development, been named heterocyst glycolipid deposition protein D (hgdD; Moslavac et al., 2007).

In 2009 a database search for sequences with TonB-box signature led to the identification of 22 sequences coding for putative TBDTs in *Anabaena* (Mirus et al., 2009). Among the identified sequences five different transporter classes were identified, comprising FhuA (ferrichrome trasnporter), ViuA (ferric vibriobactin transporter), IutA (ferric aerobactin transporter), BtuB (cobalamin transporter) and HutA (heme trasnporter) type TBDTs. Out of the 22 putative TBDTs, so far only two TBDTs have been further characterized (Mirus et al., 2009). The TBDT encoded by *alr0397* was shown to be involved in schizokinen uptake and named accordingly schizokinen transporter (SchT; Nicolasien et al., 2008). The second characterized TBDT is encoded by *all4026* and is involved in copper and iron uptake (iron and copper transporter, IacT). The bioinformatic search for TonB proteins in *Anabaena* revealed four putative TonB candidates encoded by *alr0248* (annotated as *tonB1*), *all3585* (annotated as *tonB2*),

all5036 (annotated as *tonB3*) and *all5329* (annotated as *tonB4*; Stevanovic et al., 2012). Sequence similarity analysis of putative TonBs identified in *Anabaena* with the TonB protein from *E.coli,* showed differences in domain structure. Two of the putative TonBs, *tonB2* and *tonB4*, showed the *E.coli*-like structure. The putative *tonB3* showed prolonged sequences at the C- and N-terminal region localized in the periplasm. While *tonB1* showed an N-terminal extension in the cytoplasm and even more intriguing an incomplete C-terminal region; in particular missing the motif binding to the TonB-box of TBDTs (Stevanovic et al., 2012). Interestingly, the conserved SXXXH motif, which is believed to delimit the minimum required energy-transduction element of TonB proteins (Larsen & Postle, 2001; Pattanaik & Montgomery, 2010), is only found within the predicted transmembrane α-helix of TonB3 (Fig.IIA).

In order to complete the candidates of the putative TonB-system(s) in *Anabaena* the sequence search analysis was extended for putative *exbB* and *exbD* genes (Stevanovic et al., 2012). Two gene clusters coding for *exbB*s and *exbD*s have been identified and annotated as system 1 (encoded by *alr0643* and *alr0644*) and system 3 (encoded by *all5047* and *all5046*). A single standing gene with homology to *exbB* has also been identified and annotated as exbB2 (*alr4587*).

As potential components of BP-dependent ABC transporter a Fhu-like system (*all0387-all0389*), a Fut-like system (*alr1382-alr1384*) and three Fec-like systems (*all2586-alr2583*; annotated as system 1; *alr3240-alr3243*, annotated as system 2; *alr4031-alr4033*, annotated as system 3) were identified (Nicolaisen et al., 2008; Mirus et al., 2009; Stevanovic et al., 2012).

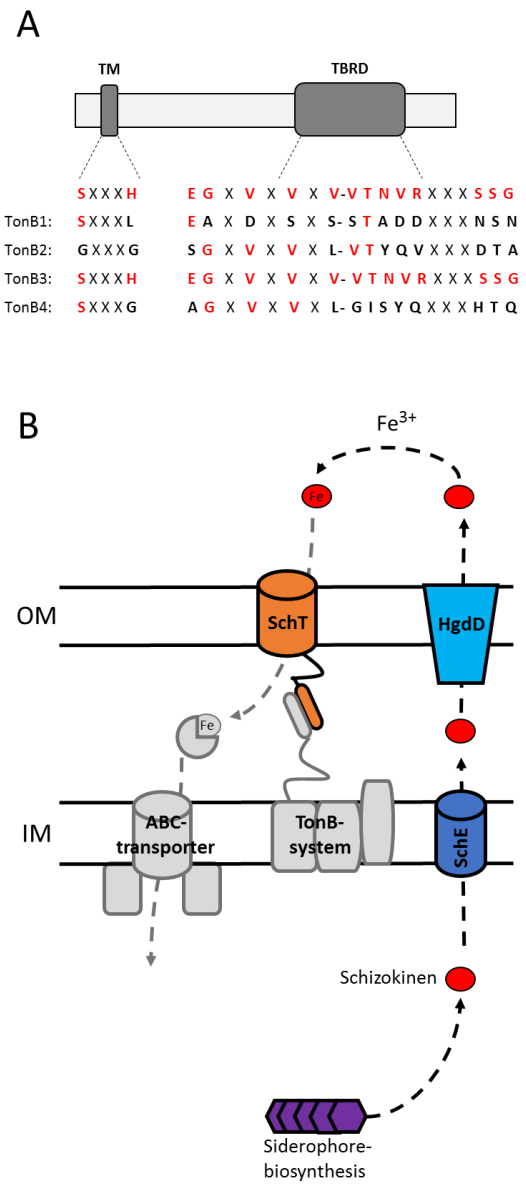

Figure II. (A) Sequence comperision of the four identified putative TonB proteins to the conserved TonB sequence. Marked in red are the identica sequences. (B) The proposed schizokinen cycle in *Anabaena*. Schizokinen (red) is synthesized and secreted by the schizokinen exporter (SchE) in a TolC (HgdD)-dependent manner. The ferric-schizokinen complex is transported by the schizokinen transporter (SchT; TBDT) over the outer membrane (OM). The TonB-system energizing SchT is unknown, as well as the ABC-transport system responsible for the transport over the inner membrane (IM).

An objective

A coordinated iron transport system in cyanobacteria is of great significance and can even be considered essential for survival of these organisms. As the iron requirement of cyanobacteria exceeds the requirement of non-photosynthetic prokaryotes and an interdependence of iron limitation and production of damaging radicals has been observed. Furthermore uptake needs to be tightly controlled, as iron is highly reactive and can result in the production of damaging radicals, which is particularly dangerous in photosynthetic bacteria. Iron homeostasis studies in cyanobacteria have so far most extensively been studied in unicellular non-siderophore producing cyanobacteria like *Synechocystis*. *Anabaena* sp. PCC 7120 however is a great model organism for the study of iron uptake, as it is a filamentous, diazotrophic and siderophore synthesizing cyanobacterium. Moreover *Anabaena* sp. PCC 7120 belongs to an evolutionary old cyanobacterial family. As depicted above the understanding of the molecular components involved in siderophore-dependent iron uptake in *Anabaena* is just beginning to emerge. Even though iron transport function for two TBDTs has been confirmed the energizing TonB-system has not been characterized. Since by bioinformatics means *Anabaena* encodes four putative TonB proteins, there arises the question whether indeed several TonB-systems do exist in *Anabaena*. Based on the high number of identified putative TBDTs it would be interesting to know if one TonB-system is specific for a certain TBDT class. As nowadays experimental evidence exists that the TonB-system is not only restricted to iron uptake, investigation of TonB substrates needs to be extended to other metals like copper, manganese, zinc and cobalt. Furthermore in order to complete the characterization of the schizokinen uptake cycle the BP-dependent ABC transporter(s) responsible for transport across the IM need to be identified.

2. Materials

2.1 Chemicals

Antibiotics used in this study: streptomycin (Carl Roth), spectinomycin (Sigma), Nystatin (Applichem) and chloramphenicol (Carl Roth).

2.2 Enzymes and kits

Enzymes and kits used for the cloning procedures were purchased as follows: Taq-polymerase and T4-DNA ligase from Eppendorf (Hamburg, Germany) and restriction enzymes from Fermentas (St. Leon-Rot, Germany). DNA extractions from agarose gels were performed with E.Z.N.A. Gel Extraction Kit (OMEGA bio-tek, USA).

For RNA isolation TRIzol reagent was used purchased from Invitrogen. gDNA digestion was performed with RNase-free DNase I (Roche). For reverse transcription Revert Aid Reverse Transcriptase from Thermo Scientific and random hexamer primer from Invitrogen were used. Quantitative real-time PCR (qRT-PCR) reaction was performed using the SYBR Green PCR Master Mix from Sigma.

2.3 Oligonucleotides

All oligonucleotides were purchased from Invitrogen (Karlsruhe, Geramany).

Table 1. List of oligonucleotides used in this study. The oligonucleotide names begin with the genenumber from the corresponding gene in *Anabaena* and end with the direction of amplification, whereby F stands for the forward and R for the reverse oligonucleotide.

Purpose	Name	Sequence
cloning	0387-F	AGATCTGCATCCGTTGCGGTGAG
	0387-R	AGATCTTCCTGCTAATAATGC
	0388-F	AGATCTGGAATCCAACCTGTGGG
	0388-R	AGATCTTATAGATGAAGTTGGC
	0389-F	AGATCTCTTGCCAGACTTATGC
	0389-R	AGATCTCGCTATAGGCTGCGGC
	5036-F	ATTAATAGATCTCTGACACGAGTTCCTCAA GTTG

	5036-R	ATTAATAGATCTCTTCGTCTAGTTCTCGATTACCGC
	5047-F	GCTTGAACGAGTTTACGGCG
	5047-R	GCTGTAGATACTAGAGCTTCGC
	0248-F	GAATTCGTGTTAACCGTGATG
	0248-R	GATATCGGAGAGGGACTAGG
	3585-F	ATTAATAGATCTGTTGCAGTACCTCAGGGTTG
	3585-R	ATTAATAGATCTGATTTACCTTGCAGCGATC
Screen-ing of segre-gation	0387-F	CCAGTGGACACGTTTATCTAGC
	0387-R	GCCACTGATGGCTAAGGC
	0388-F	CGCCCTGGAATGGGTGTATGC
	0388-R	GCCGCCGAATAGCCAGGTGTC
	0389-F	CTCTGGGAGAAATCACCGC
	0389-R	GTTGGAGCATGATCAGGC
	5036-F	GTCGAGCAACGTTCCAAGG
	5036-R	CTCTAGCTGTGCCTGTTCTTG
	5047-F	GCTTGAACGAGTTTACGGCG
	5047-R	GCTGTAGATACTAGAGCTTCGC
	0248-F	GACTAATCCTAACGAGCG
	0248-R	CGCTTTGTGACTGGTCTC
	3585-F	CCATGGAATGTAAACTCTTCC
	3585-R	AAGCTTAACCGACTAGTTC
	C.SR3-F	CACGACGACATCATTCCGTGG
	C.SR3-R	CCACGGAATGATGTCGTCGTG
Operon-structure	0387-F	CCATCAGCAGCGATTTGGG
	0387-R	GGTACTAGTTGGCGGTGTCG
	0388-F	CTGCTTCCGCCTTAACTGTG
	0388-R	GTAGAACTTGGGCTGATAGGG
	0389-F	GCCTTAGTACGACGACTCAAC
	0389-R	GTAAGGCGGTGATTTCTCCC
	5047-F	CAATCGTCAACAGCGCGTAG
	5047-R	CTAGAGCTTCGCTGATCCC
	5046-F	GATGCTGTGGCTGGACAGTTG
	5046-R	GGCAAACCTTCTGAACGAG
	0643-F	GGATATTGCTAGACAGGC
	0643-R	GTGGTTGCGGAAACTTACCC
	0644-F	CACGCACAGCTACCTATAAC
	0644-R	GTGCTAACTCGTCTCTTC
PCR	isiA-F	GCCCGCTTCGCCAATCTCTC
	isiA-R	CCTGAGTTGTTGCGTCGTAT
	petE-F	GCATCGGCTGAAACATACACA
	petE-R	CGGCGACAGTGATTTTACCA
	furA-F	GCTGACGGTGAAGGAATC
	furA-R	GCACGTTGGCACTTG
	0834-F	AGCGATGTCTTATCAGG
	0834-R	GTCATTTGCTGTACCAC
	2231-F	TCTGCATTGGCTAGTGT
	2231-R	TCTCGATGGGACAACGT

	4499-F	AGATCTATCCCAACGGTACATACCGTGG
	4499-R	AGATCTGGATAGCATCGCTGAAGTTGAATGC
	4550-F	AGATCTGGTTGAGCGCTACGGTTGTATTGC
	4550-R	AGATCTCTGCTGCATAATGGCTATGTCG
	4741-F	AGATCTGTGATTAGTGAAGATGCTC
	4741-R	AGATCTGATATTAAGCCGATACG
	5191-F	AGATCTGGCACTACAAGCATTAAC
	5191-R	AGATCTCCAAAGCGTACAAAGTGACG
	7614-F	AGATCTGTTACATCAGTTTCC
	7614-R	AGATCTGAGAGTTGCCAACAGGGAAG
	sodA-F	CTCTGTGGCAACGGTTTATTG
	sodA-R	CTTTGCCGTGAAGCTTGG
	sodB-F	CCACTACCCTACGACTTTAATGC
	sodB-R	AAGCTGGGCGAGCATTTC
	rnpB-F	AGGGAGAGAGTAGGCGTTG
	rnpB-R	GGTTTACCGAGCCAGTACC
	0390-F	GGTTGTCTATGGCGCGAGAG
	0390-R	GTCCAGAAGGTCTGTTCTGGG
	0393-F	GCAAGGTTTGATGCTGC
	0393-R	CATGCTTTCCACCTCTG
	0396-F	CCATCGCAGAGTAGTG
	0396-R	CTGGCATTAACCCTGTC
	2635-F	GACAGGTGCGAGTTGTG
	2635-R	GCCGCATCTAAATGACC
	2649-F	CTCGCACCAAATCAAGC
	2649-R	GTGCGTATGCGTTCGAG
	2658-F	GCCGAACCAAATCAACC
	2658-R	CCTGCTGCTATCGGTTG
qRT-PCR	0643-F	GGATATTGCTAGACAGGC
	0643-R	GGACTCCAGTGCTAATCG
	0644-F	GGAGGTAGCCAAATTGTTAG
	0644-R	GTGCTAACTCGTCTCTTC
	4587-F	CGCCCTTATTAGGGTTAC
	4587-R	CTTTGTTCCTCCCACATC
	5047-F	GCTTGCTGGGTACAGTGTTAG
	5047-R	CTAGAGCTTCGCTGATCCC
	5046-F	GATGCTGTGGCTGGACAGTTG
	5046-R	GCCACTACCTTACCGTGTCC
	0248-F	GCACCTGCTATTACTCCTCAGCC
	0248-R	CCCGCTTTGTGACTGGTCTC
	3585-F	GAACGGGTTGCAGTACCTC
	3585-R	CAGCGTTTGGCGTAACAGG
	5036-F	GCTCAAACCTCCAGGGAAAC
	5036-R	CTCTAGCTGTGCCTGTTCTTG
	5329-F	CCAACGCCTGTCACTATTAC
	5329-R	CTAACGAGACTGAAAGCACC
	1383-F	GCGCCCATTGGCTTTAATAC
	1383-R	CTGATGCTTGGCTGTAGGAG
	2585-F	CCTTGTCGCTTGCATGGTAG

	2585-R	CGGGATCAGCTAGTGGGTTG
	2586-F	CGCCATTAGTGGGTCTGATTC
	2586-R	GTATTTCTGCCGCACCCAATG
	3240-F	GGCGATACAGTCTGTGTTGC
	3240-R	GCACCAGCCGTAGTCACTTC
	4032-F	GTTGTGCTGACAGCAAGTG
	4032-R	CCGAGACTTGCTGCTATAAC
	0387-F	GCTGCTGCTGCTGTCTCTAC
	0387-R	GGTACTAGTTGGCGGTGTCG
	0388-F	CTGCTTCCGCCTTAACTGTG
	0388-R	CCGCATACACCCATTCCAG
	0389-F	GCCTTAGTACGACGACTCAAC
	0389-R	CCTTGTTGGAGCATGATCAGG
	sodA-F	GATTTCTTGGGCGATGTCTC
	sodA-R	GATATTCGCACAACGGTACG
	sodB-F	CGCGCCTACAGGTGAATTCG
	sodB-R	TAGCCAAGCCCATCCGCTAC
	isiA-F	CGAGTTGTTTCGGGTCGTTC
	isiA-R	GCAGGTGCGCTCTTTCATAC
	furA-F	TTCCTCAAGGCGAACACCTG
	furA-R	AAATCCCCATCCGAGCCATC
	furB-F	ACTAGAAGGCTTGGTGCAGGTA
	furB-R	ACCGCATTGCAGGCACGTAA
	0834-F	CGCGATTAGAACTTGTCTCC
	0834-R	GCTAGCGAAGAACAGGTTAC
	2231-F	TACTAAACTGCGGGGAGAAG
	2231-R	CCTCAAGATTCGGTCTGCTA
	4499-F	CAGGATTAGCCGCACTATTC
	4499-R	TATCTACCGCCAAGCCTATG
	4550-F	CGCGTATCTGACAACATC
	4550-R	GAAGGTGGTTCTCAGAGT
	4741-F	TGCTGATATCCAGGCTCAAC
	4741-R	CTCCATCACCTGGACGATTC
	5191-F	ATCGCGCCATAGGGACTAGC
	5191-R	GGTGCAGGTATCGGGATTTC
	7614-F	GCTGCATAGTTACCATCG
	7614-R	GTCCCATTTGGTAGTGGTAG
	manS-F	ATGACTCACACGCAGATATCCC
	manS-R	TCCAGGAACGCAAAAGCTTC
	manR-F	GCCAACAACTCTCGCAGTTC
	manR-R	TCCTCACCGCCAAAGATACC
	mntB-F	CGCCTAAAGGATCGAAGGTG
	mntB-R	CCTTGGCGTGACTGTTGATG
	rnpB-F	GTAGGCGTTGGCGGTTG
	rnpB-R	CACTGGACGTTATCCAGC

2.4 Vectors

The vectors used for cloning or for conjugations into wild type *Anabaena* are a gift from the laboratory of Dr. Enrique Flores (CSIC, Seville, Spain).

Strain	Resistance	Genotyp	Relevant properties
Anabaena sp. PCC 7120	/	Wild type	Wild type
AFS-I-*fhuB*	Sp^RSm^R	*all0387*::pCSV3	Gene interrupted by pCSV3
AFS-I-*fhuD*	Sp^RSm^R	*all0388*::pCSV3	Gene interrupted by pCSV3
AFS-I-*fhuC*	Sp^RSm^R	*all0389*::pCSV3	Gene interrupted by pCSV3
AFS-I-*exbB3*	Sp^RSm^R	*all5047*::pCSV3	Gene interrupted by pCSV3
AFS-I-*tonB3*	Sp^RSm^R	*all5036*::pCSV3	Gene interrupted by pCSV3
AFS-I-*tonB1*	Sp^RSm^R	*alr0248*::pCSL24	Gene interrupted by pCSV3
AFS-I-*tonB2*	Sp^RSm^R	*all3585*::pCSL24	Gene interrupted by pCSV3
AFS-I-*iacT*	Sp^RSm^R	*all4026*::pSCV3	Gene interrupted by pCSV3
AFS-I-*schT*	Sp^RSm^R	*alr4167*::pSCV3	Gene interruption by gene-cassette C.S3
AFS-I-*schE*	Sp^RSm^R	*all4025*::pCSCW11	Gene interrupted by pCSV3
AFS-I-*hgdD*	Sp^RSm^R	*alr2887*::pSCV3	Gene interrupted by pCSV3

Table 2. List of vectors used in this study.

Vector	Resistance	Purpose	Reference
pCSEL24	Sp^RSm^R	cargo vector	Olmedo-Verd et al., 2006
pCSV3	Sp^RSm^R	cargo vector	Elhai & Wolk, 1988
pRL623	Cm^R	helper vector for conjugation	Elhai & Wolk, 1988
pRL443	Ap^R	conjugal vector	Elhai & Wolk, 1988

2.5 Bacterial strains

Table 3a. E.coli strains used in this study.

Strain	Resistance	Purpose
DH5α	/	Cloning
HB101	Cm^R	Conjugation
RP-4	/	Conjugation

Table 3b. Anabaena strains used in this study.

Sp, Spectinomycin; Sm, Streptomycin; TS, this study; CP, from C.P. Wolk; 1, Nicolaisen and colleagues (2010); 2, Nicolaisen and colleagues (2008); 3, M. Moslavac and colleagues (2007).

3. Methods

3.1 Molecular biological Methods

3.1.1 General molecular biological Methods

The growth conditions of the E. coli cultures, phenol/chlorophorm extraction or isopropanol/ethanol precipitation of DNA, agarose electrophoresis and transformation were performed as described in Sambrook et al. (1989). Competent *E.coli* cells were prepared according to Hanahan et al. (1985). Restriction, ligation, purification and extraction from agarose gels of PCR products, plasmid DNA and DNA fragments were performed according to the recommendation of the manufacturer of the corresponding kit (see “Materials”).

3.1.2 Polymerase chain reaction (PCR)

PCR was performed with the Triple master PCR System (Eppendorf) using the PCR cycle as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of incubation each consisting of 30 sec denaturation at 94°C, 30 sec annealing at 56°C, 2 min extension at 72°C and a final extension of 10 min at 72°C.

3.1.3 RT-PCR

For reverse transcriptase PCR (RT-PCR) experiments, 1 µg of *Anabaena* total RNA was mixed with 0.5 µg of random hexamer oligonucleotides in the presence of 9 µl bidest and heated for 5 min at 70°C. After short cooling on ice 10 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA (Revert Aid Reverse Transcriptase-buffer, 1x), 0.10 mM dNTP and 200 U of Revert Aid Reverse Transcriptase (Thermo Scientific) wre added. The mixture was incubated for 1 h at 42°C and the reaction stopped by heating to 70°C for 10 min. To control for the presence of contaminating DNA, samples containing 1 µg of RNA were processed as described above but with reverse transcriptase omitted (-RT

control). PCR was carried out with 2 to 5 µl of a retrotranscription mixture (or -RT control mixture) as the template.

3.1.4 Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) reaction was performed as described (Bohnsack et al., 2008) using the SYBR Green PCR Master Mix (Sigma) on an Mx3000P Real-Time PCR System (Stratagene). Amplification efficiency of each oligonucleotide pair was calculated using standard curve dilutions. The specificity of fragment amplification was verified through melting curve analysis. For each oligonucleotide set a no-template reaction was used as negative control. The qRT-PCR results were analysed using the Mx3000P computer software. The values were normalized to the expression of the control gene *rnpB*. Results represent the average of technical and biological triplicates. The oligonucleotides used are listed in Table 1.

3.1.5 Cloning strategy for single-recombinant insertion mutants

To generate the insertion mutants, an internal fragment of the coding region was amplified by PCR on wild type genomic DNA (oligonucleotides are listed in Table 1) introducing *Bgl*II restriction sites for cloning the products into pCSV3 or *Eco*RV/*Eco*RI restriction sites for cloning the products into pCSEL24. The produced plasmids were used for generation of single-recombinant insertion mutants (Table 3b).

3.2 *Anabaena* related methods

3.2.1 Media and growth conditions

Anabaena sp. wild type and mutants strains were grown photoautotrophically at 30°C in liquid BG11 medium (Rippka et al., 1979) supplied with 17.6 mM of $NaNO_3$ as a source of bound nitrogen and under constant illumination from incandescent lamps at 70 mol photons $m^{-2}s^{-1}$. The same medium without source of bound nitrogen was $BG11_0$. For qRT-PCR analysis *Anabaena* strains were grown in different media, which are based on BG11 without the source of iron (no ferric ammonium citrate added; -Fe), copper (no CuSO4 x 5H2O added; -Cu) or both (-Fe/-Cu). For media with elevated metal content BG11 with elevated levels of iron (1 mM ferric ammonium citrate added; ++Fe) or copper (1 µM CuSO4 x 5H2O added; ++Cu) or combinations thereof were used. Further BG11 without citric acid (-citrate) was used. Agar plates were prepared with 1.5 % Bacto™ Agar (BD, USA).

Mutant strains were grown in the presence of 3 µg ml^{-1} streptomycin, 3 µg ml^{-1} of spectinomycin and 100 µg ml^{-1} nystatin.

3.2.2 Determination of Chl concentration and growth rates

To determine chlorophyll a (Chl) concentration, 100 µl of a 50-ml shaking culture of *Anabaena* without additional air/CO2 bubbling was mixed with 1 ml of methanol and vortexed vigorously for 1 min. 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 growth rates, 50-ml shaking cultures (100 rpm), without additional air/CO_2 bubbling, of the strains 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 of 0,05 OD_{750}, where OD_{750} is the optical density at 750 nm. Samples of 1 ml were taken immediately after the reinoculation and afterwards regularly every 24 hours for 10-14 days. To determine the growth on plates, a concentration of 1, 0.1 and 0.01 OD_{750} was used for 5-µl spots.

3.2.3 DNA isolation

Total DNA of *Anabaena* was isolated according to Cai and Wolk (1990) from 50 ml of 1-2 week old cultures. Cells were collected by centrifugation at 4 000 rpm, 5 min, room temperature, resuspended in 10 mM Tris/HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0 up to 400 µl final volume. Three small spoons of (~150 µl) of glass beads and 400 µl of phenol: chlorophorm = 1:1 (v/v) was added. Mixture was vigorously vortexed four times for 1 min, while keeping the cells 1 min on ice between every vortexing period, and subsequently centrifuged 15 min, 14 000 rpm, 4°C. The clean upper phase was extracted twice with phenol/chlorophorm and twice with chlorophorm. DNA was precipitated out of the water phase with 2,5 V of ethanol absolute and 0.1 V of 3 M sodium acetate, pH 5.2, overnight at -20°C. Then the samples were centrifuged 15 min, 14 000 rpm, 4°C and the DNA pellet was washed with 70% ethanol, air dried and dissolved in sterile water.

3.2.4 RNA isolation and DNase disgestion

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. gDNA contaminations were digested by treatment with RNase-free DNase I (Roche) according to the manufacturer's instructions. DNA contaminations were excluded by PCR in the absence of reverse transcriptase.

3.2.5 Conjugation

Anabaena sp. wild type strain was transformed with plasmids according to the method of three-parental mating (Elhai and Wolk, 1988b). The cargo strain HB101 was created by pre-transformation with helper plasmid pRL623 and subsequent transformation with the cargo plasmids bearing cloned constructs (pCSV3 or pCSM1). The cargo strain was mixed gently with the conjugal strain ED8654, carrying conjugal plasmid pRL443, and with wild type *Anabaena* sp cells (three parents). The mixture was spread onto nitrocellulose filter (Immobilon® Transfer Membranes, Millipore) placed on BG11 agar medium supplemented with 5% LB medium. After 24 hours of incubation in the growth chamber the filter was first transferred to fresh BG11 plates without antibiotics for the next 24 hours and then every second day on the fresh BG11 plates with antibiotics for at least two weeks. After the thick green background of *Anabaena* sp. cells had died out, the single colonies of exconjugants appeared on the filters.

3.2.6 Chromazurol S (CAS) agar plates preparation

Chromazurol S (CAS)-containing agar plates were prepared according to Schwyn and Neilands (1987). 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 $FeCl_3$x6 H2O; 10 mM HCl), and while this solution was stirred, 72.9 mg HDTMA (hexadecyltrimethylammonium) dissolved in 40 ml water was added. CAS agar plates were prepared by the addition of a 1/10 V of the CAS stock solution to liquid BG11 and BG11 without iron and copper ($BG11_{-Fe/-Cu}$).

3.3 Light microscopy

Anabaena filaments were visualized with a use of the standard reverse light microscope (DM1000, Leica, Germany). Autofluorescence was monitored by collection through the window of 630-700 nm.

4. Results

4.1. Identification of iron-regulated acquisition systems in *Anabaena*

4.1.1 Expression analysis of putative *tonB* candidates

The role of the TonB-system in iron acquisition is well characterized, based on this role the synthesis of the TonB-energy-transducing components is regulated by intracellular iron availability (see section 1.3). In *E.coli* iron-deplete conditions lead to a 2.5-3-fold increase of TonB-system proteins in consequence of transcriptional regulation (Higgs et al., 2002; Zimbler et al., 2013). After identification of four putative TonB proteins in *Anabaena* (Fig.1A) their gene expression was analysed in response to variable iron concentrations in the culture medium (Fig.1B). Different copper concentrations in combination with iron were also tested, as a connection between iron and copper homeostasis exists in cyanobacteria. Under iron limitation the iron-containing ferredoxin is replaced by the copper-containing flavodoxin (LaRoche et al., 1995; Doucette et al., 1996). Furthermore the existence of a schizokinen-independent, but copper-dependent iron uptake system is proposed for *Anabaena* (Nicolaisen et al., 2010). Another tested condition was BG11 medium without any combined nitrogen source (termed $BG11_0$). In $BG11_0$ medium *Anabaena* differentiates heterocysts in order to fix elemental nitrogen (reviewed by Buikema, W. J., and R. Haselkorn, 1993). Even though, nitrogen-fixation results in a higher iron demand, *Anabaena* is not able to simultaneously fix-nitrogen and synthesize siderophores (Schindelin et al., 1997; Shi et al., 2007; Hutchins et al., 1991). Therefore $BG11_0$ serves as a control condition under which the classical TonB-system is not needed.

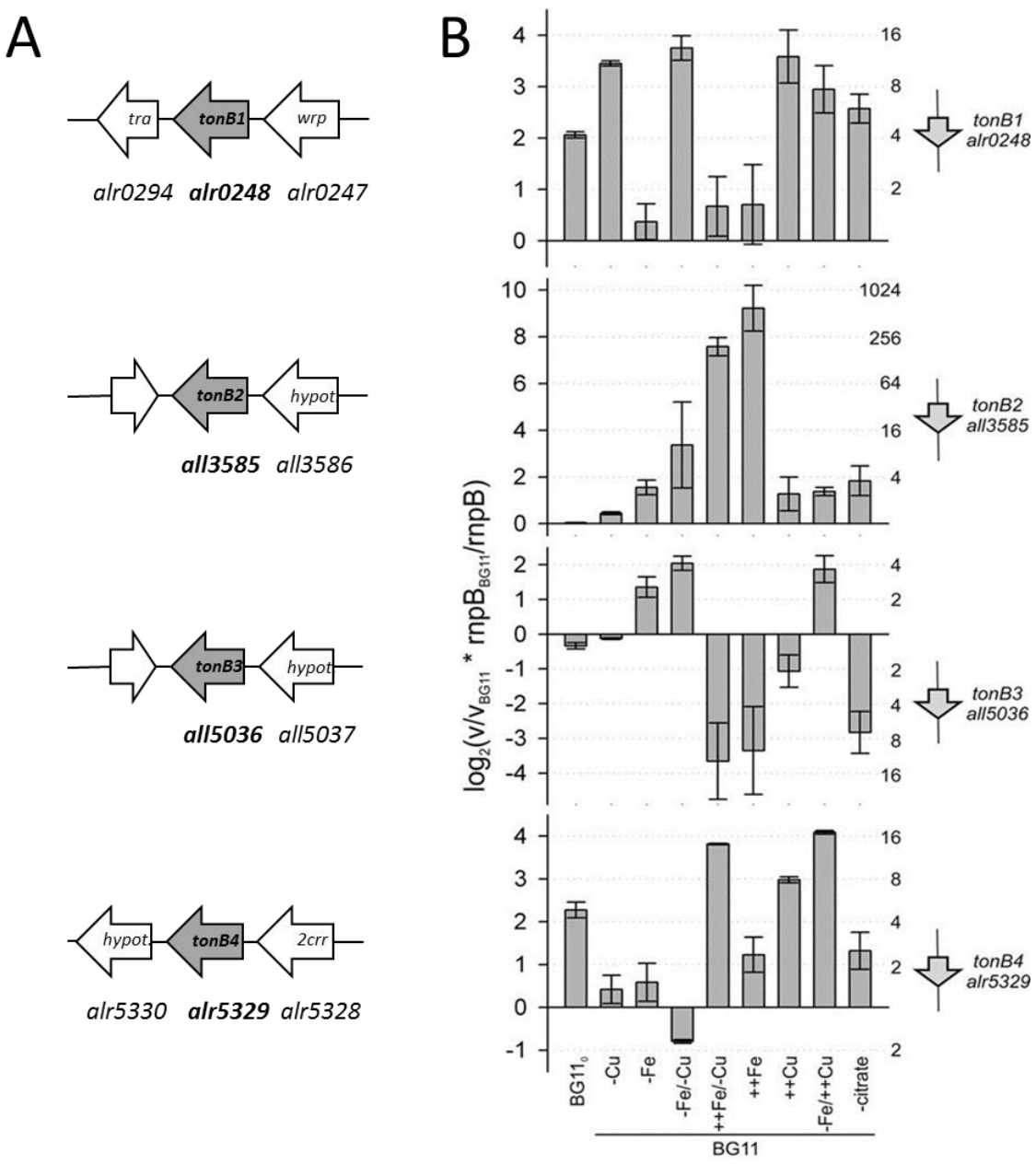

Figure 1. (A) Genomic organization of putative *tonB* genes according to annotations deposited in cyanobase (Nakamura et al., 1998; Kaneko et al., 2001). Abbreviations: tra, transposase; wrp, WD40-repeat protein; hypot., hypothetical protein; 2crr, two-component response regulator. (B) qRT-PCR analyses of putative *tonB* genes in wild type *Anabaena* in response to growth in different media. The different media are based on BG11 either without nitrogen (BG110), copper (-Cu), iron (-Fe), with elevated levels of iron (1 mM Fe; ++Fe), copper (1 mM Cu; ++Cu) or combinations thereof. The data are expressed on a log scale relative to expression levels in BG11 medium and relative to the changes of the internal standard *rnpB*. The scale on the right side indicates the x-fold changes. The data are means of three independent experiments. Figures are reproduced from Stevanovic et al., 2012.

The expression analysis of the putative *tonB* candidates was performed by qRT-PCR analysis (see Methods, section 3.1.4). For this purpose RNA from wild type *Anabaena* grown for 7 days under the indicated condition was extracted (see Methods, section 3.2.4). The transcript levels of *rnpB* (RNaseP subunit B) served as an internal standard, as its transcript levels are not significantly affected by the various nutrient conditions (e.g. Vioque, 1992). Furthermore, the transcript level of each gene under each growth conditions was also normalized to those under control condition in standard BG11 medium.

The expression analysis revealed a 10-fold up-regulation of *tonB1* under copper-limiting (-Cu) and copper and iron-limiting conditions (-Fe/-Cu), but no change of expression under iron limitation (-Fe) could be detected (Fig.1B). The results suggest that copper-starvation is the actual transcription trigger for *tonB1* expressio. However, *tonB1* also responds to high copper concentrations (++Cu; -Fe/++Cu).

For *tonB2* the most significant increase in transcript abundance was observed in the presence of enhanced iron concentrations, irrespective of the copper concentration (++Fe; ++Fe/-Cu; Fig.1B).

In contrast, *tonB3* is 2-fold up-regulated under iron limitation (-Fe) and further enhanced (4-fold up-regulated) if also copper is omitted from the medium (-Fe/-Cu). Growth of *Anabaena* in iron supplemented, but copper-limiting medium (-Cu), showed no change of *tonB3* expression in comparison to BG11. In agreement with an iron-regulated system the expression levels of *tonB3* were down-regulated under elevated iron concentrations independent of the copper concentration (++Fe; ++Fe/-Cu). Interestingly, the expression of *tonB3* was slightly down-regulated under high copper concentrations (++Cu), but again 4-fold up-regulated if iron was missing (++Cu/-Fe). The extent of the up-regulation corresponds more to the condition with no iron and copper (4-fold change) as to the condition with only iron missing in the medium (2-fold change) indicating an

effected iron uptake under enhanced copper concentration. Under the control condition $BG11_0$ *tonB3* showed no alteration compared to BG11, which is strengthening the function in siderophore-dependent iron uptake.

The expression pattern of *tonB4* is clearly not related to iron limitation, but is rather reacting to metal stress, since an up-regulation of expression was observed for elevated copper and minus iron (-Fe/++Cu) as well as for elevated iron concentration and minus copper (++Fe/-Cu) concentrations. Based on the expression analysis a prediction of the *tonB4* function cannot be made and further experimental evidence is needed.

Summarizing the qRT-PCR analysis strongly indicates, that *tonB3* is expressed in response to iron limitation. Moreover the regulation of *tonB3* under control conditions, as $BG11_0$ and high metal concentrations (++Fe; ++Cu) confirms a 'classic' *tonB* gene involved in siderophore-dependent iron uptake.

4.1.2 Expression analysis of the putative *exb*-system

The cytoplasmic membrane proteins ExbB and ExbD are required for TonB-dependent transport and encoded in an operon in *E.coli* (see section 1.3; Higgs et al., 1998). Expression of *exbB* and *exbD* is iron-dependent and regulated by Fur (Higgs et al., 2002; see section 1.4). The sequence similarity search for *exb*-homologous in *Anabaena* revealed two gene clusters encoding putative ExbB and ExbD proteins, respectively (Fig.3A; Stevanovic et al., 2012). To define if the flanking exb-genes are contained on the same transcriptional unit (operon) overlapping RT-PCR was performed. For that purpose oligonucleotides flanking the intergenic region were used (Fig.2, scheme; see Material, Tabel.1). The observed RT-PCR products for *exbB1-exbD1* and *exbB3-exbD3*, respectively, imply that the exb-clusters share a common transcript (Fig.2).

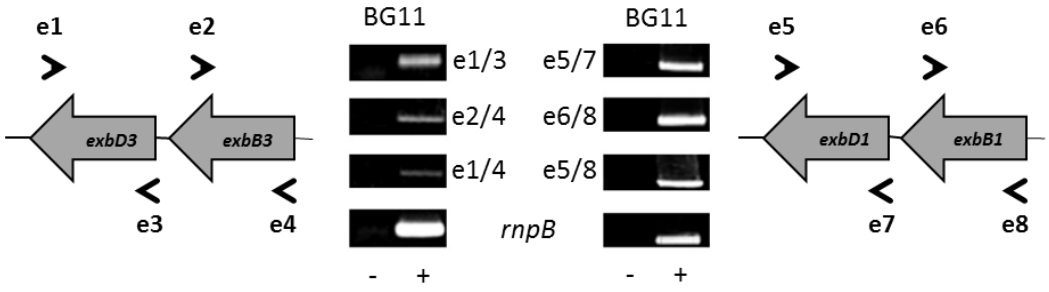

Figure 2. Analysis of the *exbB3*/*exbD3* and *exbB1*/*exbD1* operon structure was performed by RT-PCR. The gene models of the corresponsing genes are given sidewise to the PCR results. Oligonucleotides used for PCR are indicated in the gene model (e1-e8). PCR reaction was performed on RNA isolated from wild type *Anabaena*. Presence or absence of reverse transcriptase (RT) is denoted by (+) and (-) respectively. *rnpB* expression served as a PCR control.

To test if one of the putative *exb*-clusters is regulated by iron-availability, the expression of the predicted *exbB* and *exbD* genes was tested under the same conditions applied for the *tonB* candidates (see section 4.1). The transcript levels of the *exb*-system 1 (encoded by *alr0643* and *alr0644*) were consistently up-regulated at elevated copper levels,

irrespective of the iron concentration (++Cu; -Fe/++Cu; Fig.3B). In medium with elevated iron concentration and without copper added the expression of the *exb*-system 1 was also up-regulated (++Fe/-Cu; Fig.3B). The system also responds to copper or iron limitation with an approximately 2-fold up-regulation, respectively (-Fe; -Cu). Nevertheless, a combination of iron and copper deprivation (-Fe/-Cu) leads to a down-regulation of the system (Fig.3B). Therefore the expression levels of system 1 are difficult to interpret and a clear functional assignment cannot be made.

The expression of the single *exbB2* gene encoded by *all4587* was most significantly influenced by nitrogen starvation, as the expression was 12-fold increased ($BG11_0$; Fig.3B). Since *Anabaena* cells which fix nitrogen do not synthesize siderophores it is unlikely that *exbB2* is involved in siderophore-dependent iron-uptake (Hutchins et al., 1991). However, the expression levels of *exbB2* under all other tested conditions were unchanged in comparison to BG11.

On the contrary the *exb*-system 3 (encoded by *all5047* and *all5046*) shows a clear iron-dependent regulation of expression. The expression of system 3 is 2-fold up-regulated under iron deprivation (-Fe) and further enhanced under iron and copper deprivation (4-fold; -Fe/-Cu), whereas the transcript levels were reduced under high iron concentrations irrespective of the copper concentration (++Fe; ++Fe/-Cu). Furthermore, enhanced copper concentration influenced expression of system 3 only if iron was missing in the medium (++Cu; -Fe/++Cu; 6-fold up-regulation; Fig.3B). Interestingly, the general expression profile of *exb*-system 3 resembles the profile of *tonB3* under both iron limiting and control conditions. For this reason it is tempting to speculate that TonB3 and ExbB3-D3 act in concert as the iron acquiring TonB-system in *Anabaena*. *TonB3* and the exb3-system genes are encoded in close genomic neighbourhood, but an overall operon structure covering *exbB3* (*all5047*) to *tonB3* (*all5036*) is unlikely, because of the reversed genomic orientation of *alr5045*.

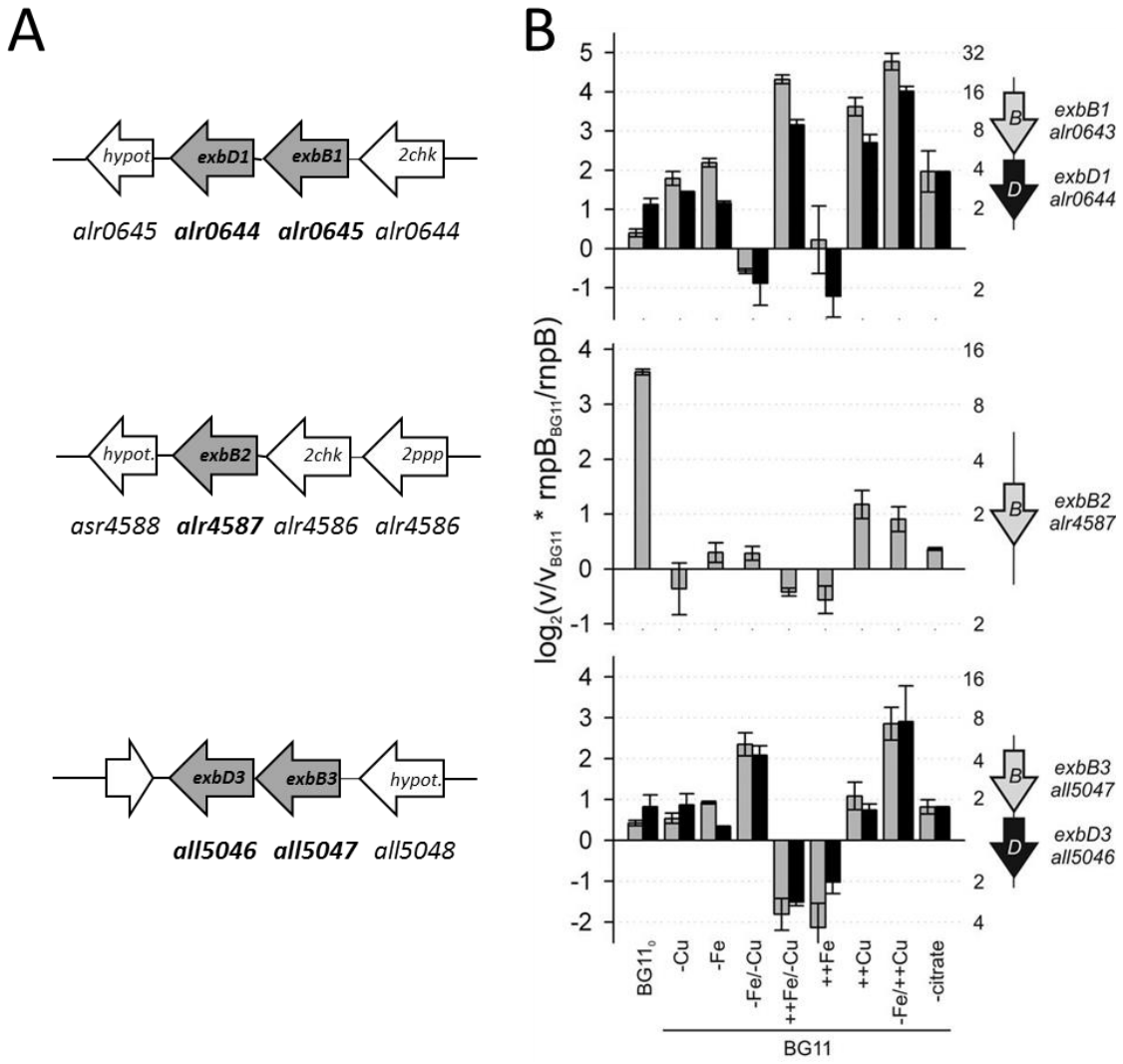

Figure 3. (A) Genomic organization of putative *exb* genes according to annotations deposited in cyanobase (Nakamura et al., 1998; Kaneko et al., 2001). Abbreviations: hypot., hypothetical protein;. 2chk, two-component sensor histidine-kinase; 2ppp, periplasmic phosphate-binding protein of a phosphate ABC transporter. (B) qRT-PCR analyses of putative *exb* genes in wild type *Anabaena* in response to growth in different media. The different media are based on BG11 either without nitrogen (BG110), copper (-Cu), iron (-Fe), with elevated levels of iron (1 mM Fe; ++Fe), copper (1 mM Cu; ++Cu) or combinations thereof. The data are expressed on a log scale relative to expression levels in BG11 medium and relative to the changes of the internal standard *rnpB*. The scale on the right side indicates the x-fold changes. The data are means of three independent experiments. Figures are reproduced from Stevanovic et al., 2012.

4.1.3 Expression analysis of putative permeases in *Anabaena*

In *Synechocystis sp.* strain PCC 6803 the BP-dependent ABC-transporter class that plays a major role in iron acquisition is the constitutively expressed Fut-system (Katoh et al., 2001). The genome of *Anabaena* encodes several putative BP-dependent ABC-transporter, namely the Fhu-, Fec- and Fut-like system (Fig.4A; Stevanovic et al., 2012). To test if one of these ABC-transporter is involved in siderophore uptake under iron limitation, expression of the permeases of the predicted ABC-transporters was analysed (Fig.4B).

The expression of *fhuB* shows a clear iron-dependent regulation, since an up-regulation under iron (-Fe; 2-fold) and iron and copper limitation (-Fe/-Cu; 4-fold) was observed. Whereas under elevated iron concentration with (++Fe) or without copper (++Fe/-Cu) the expression of *fhuB* was down-regulated. Also under the control condition $BG11_0$ or in BG11 medium with elevated copper concentration (++Cu) *fhuB* shows down-regulation of expression. Accordingly, *fhuB* expression was only enhanced if iron was missing in the medium, as it was shown in media with elevated copper and without iron added (-Fe/++Cu). Therefore *fhuB* is resembling the expression pattern of *tonB3* and exb3 with one exception and that is the enhanced expression under copper starvation (-Cu).

Transcriptional regulation of the other predicted permeases is not as clear as for *fhuB*. *FutB* expression for instance is enhanced under the absence of iron and copper (-Fe/-Cu), yet, the expression is decreased if iron (-Fe) or copper (-Cu) each are absent. *FecC1* expression was not affected by most of the tested conditions and the strongest change of expression was observed for elevated iron concentrations without copper added (++Fe/-Cu) showing a 4-fold up-regulation. The expression of *fecD1* was strongly enhanced under nitrogen starvation ($BG11_0$; 16-fold) and elevated copper concentration (++Cu; 16-fold). *FecD2* and *fecD3* showed similar expression patterns for many of the

tested conditions, however, under other conditions the two genes showed opposite behaviour. For example while *fecD2* expression is slightly up-regulated under copper starvation (-Cu), *fecD3* expression is 2-fold down-regulated. Altogether for *fecD2* and *fecD3* it was difficult to observe a consistent response to a given environment parameter. Taken together the expression analysis revealed that the only BP-dependent ABC-system clearly responding to iron-limitation in *Anabaena* is the *fhu*-system.

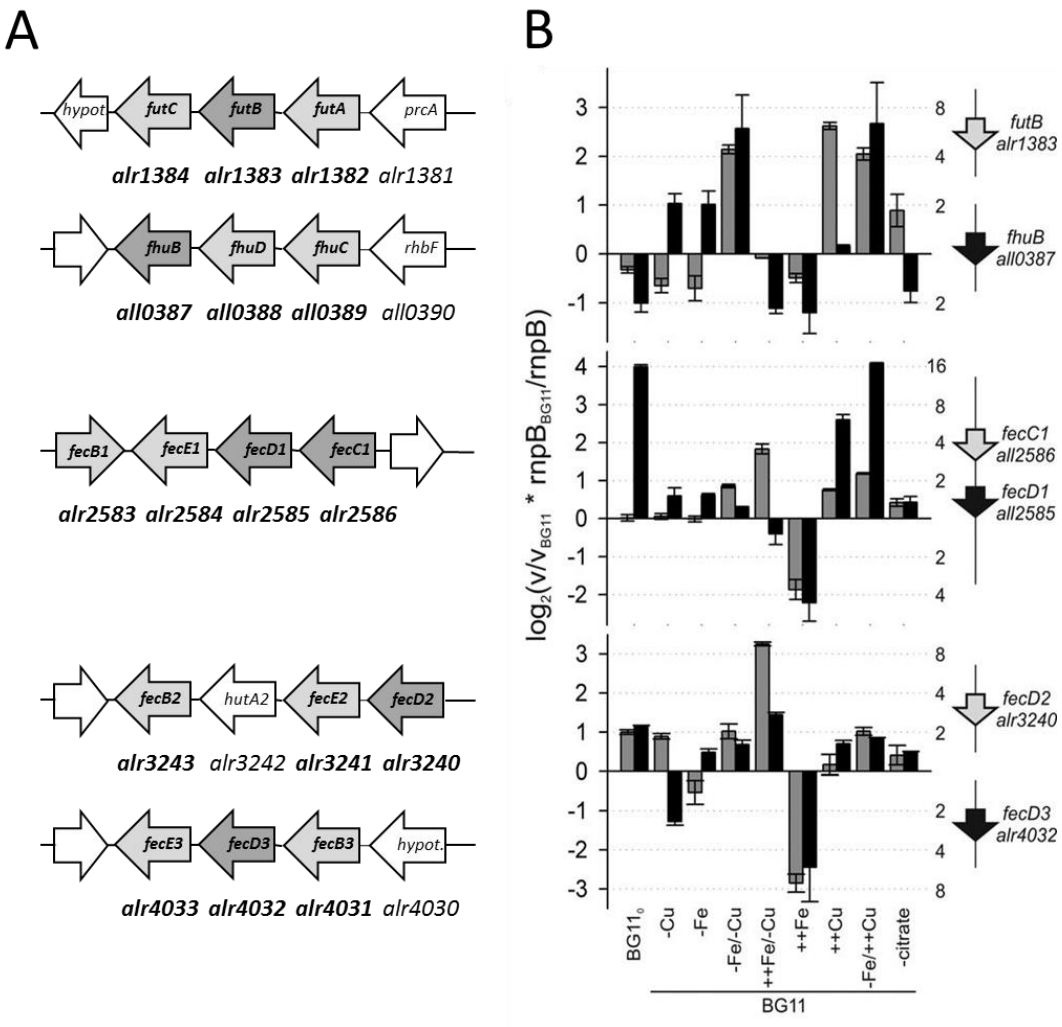

Figure 4. (A) Genomic organization of putative fhu/fut/fec genes (represented in grey) according to annotations deposited in cyanobase (Nakamura et al., 1998; Kaneko et al., 2001). The genes analysed by qRT-PCR are highlighted in dark grey. Abbreviations: hypot., hypothetical protein;. prcA, trypsin; rhbF, rhizobactin siderophore biosynthesis protein F; hutA, heme transporter protein. (B) qRT-PCR analysis of genes encoding permeases of the putative fhu/fut/fec systems in wild type *Anabaena* in response to growth in different media. The different media are based on BG11 either without nitrogen (BG110), copper (-Cu), iron (-Fe), with elevated levels of iron (1 mM Fe; ++Fe), copper (1 mM Cu; ++Cu) or combinations thereof. The data are expressed on a log scale relative to expression levels in BG11 medium and relative to the changes of the internal standard *rnpB*. The scale on the right side indicates the x-fold changes. The data are means of three independent experiments. Figures are reproduced from Stevanovic et al., 2012.

As the *fhu*-system appears to be the main BP-dependent ABC transporter under iron limitation in *Anabaena*, the remaining genes *fhuC* and *fhuD* were also analysed by qRT-PCR (Fig.5A). While *fhuB (*encoding the permease) and *fhuD* (encoding the periplasmic-binding protein) showed a similar expression pattern, *fhuC* the gene

encoding the ATP-binding domain was expressed differently under certain conditions, in particular under elevated iron concentration (++Fe) and elevated iron concentration without copper (++Fe/-Cu). Under these conditions the expression of *fhuB*/*fhuD* was down-regulated, while *fhuC* expression levels were unaffected (++Fe) or even up-regulated (++Fe/-Cu).

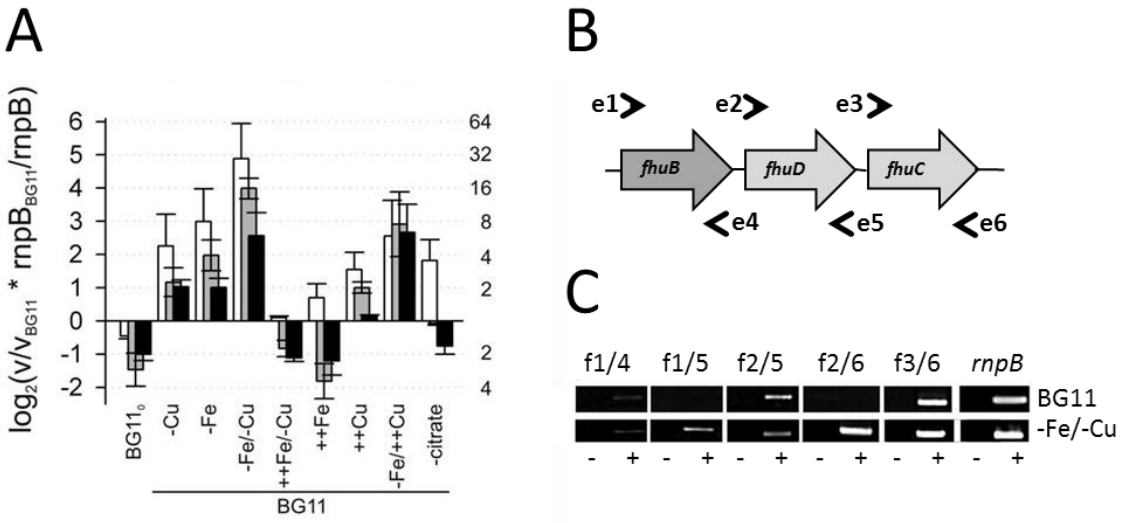

Figure 5. (A) Expression analysis of *fhuC* (white bar), *fhuD* (grey bar) and *fhuB* (black bar) by qRT-PCR on isolated RNA after growth in various media. The media are based on BG11 either without nitrogen (BG110), copper (-Cu), iron (-Fe), with elevated levels of iron (1 mM Fe; ++Fe), copper (1 mM Cu; ++Cu) or combinations thereof. The data are expressed on a log scale relative to expression levels in BG11 medium and relative to the changes of the internal standard *rnpB*. The scale on the right side indicates the x-fold changes. The data are means of three independent experiments. (B) Model of the genomic region coding for the three fhu genes. Oligonucleotides used for operon structure analysis of the *fhu* genes by RT-PCR are indicated in the gene model. (C) RT-PCR reaction was performed on RNA isolated from wild type *Anabaena* grown in BG11 or BG11 lacking both iron and copper (-Fe/-Cu). Presence or absence of reverse transcriptase (RT) is denoted by (+) and (-) respectively. *rnpB* expression served as a PCR control. Figure 5A&B are reproduced from Stevanovic et al., 2012.

In consequence of the unusual expression pattern of the *fhu*-system the operon structure of the system was analysed with oligonucleotides flanking the intergenic region of the *fhu* genes (overlapping RT-PCR; Fig.5C). Remarkably, a transcript for the intergenic regions could only be observed if RNA was extracted from wild type grown in BG11 without iron and copper added (-Fe/-Cu), while under control condition (BG11) three individual transcripts existed. Taken together both qRT-PCR and the overlapping RT-PCR results strongly indicate the existence of internal promoters in the fhu-operon.

4.1.4 Growth phase-dependent expression of iron uptake systems

The secretion of schizokinen has been examined with respect to the growth-phases in iron-limited cells of *Anabaena*. Schizokinen accumulation in the growth medium was proportional to cell density throughout growth phases and continued into stationary phase (Clarke et al., 1987). Quantification of ferric-siderophore uptake rates in *Anabaena* revealed that the uptake systems saturate at high concentrations of iron irrespective of the growth phase (Clarke et al., 1987). However, repression of the iron uptake systems on transcriptional level, with respect to the growth phases, has not been examined. Furthermore, it remains unclear which component of the siderophore-dependent iron uptake system is the first one to respond to iron limitation. As growth phase-dependent response to iron starvation in bacteria is known (Shcolnick and Keren, 2006; Merell et al., 2003), expression of the several predicted *tonB* and *exB*-system encoding genes in *Anabaena* needs to be analysed with respect to different growth stages.

In order to examine the expression of the predicted iron uptake systems in *Anabaena* under different growth phases, RNA from the ‘early growth stage’ (assigned as stage 0,1), logarithmic phase (assigned as stage 2,0) and stationary phase (assigned as stage 4,0) was isolated and analysed by qRT-PCR. Furthermore wild type was grown in BG11, under iron and copper limitation, as well as under enhanced concentrations of both metals. In addition to the *tonB*, *exbB*, *exbD* and *fhuB* encoding genes, expression of the schizokinen exporter (*schE*) and schizokinen transporter (*schT*) encoding gene was investigated.

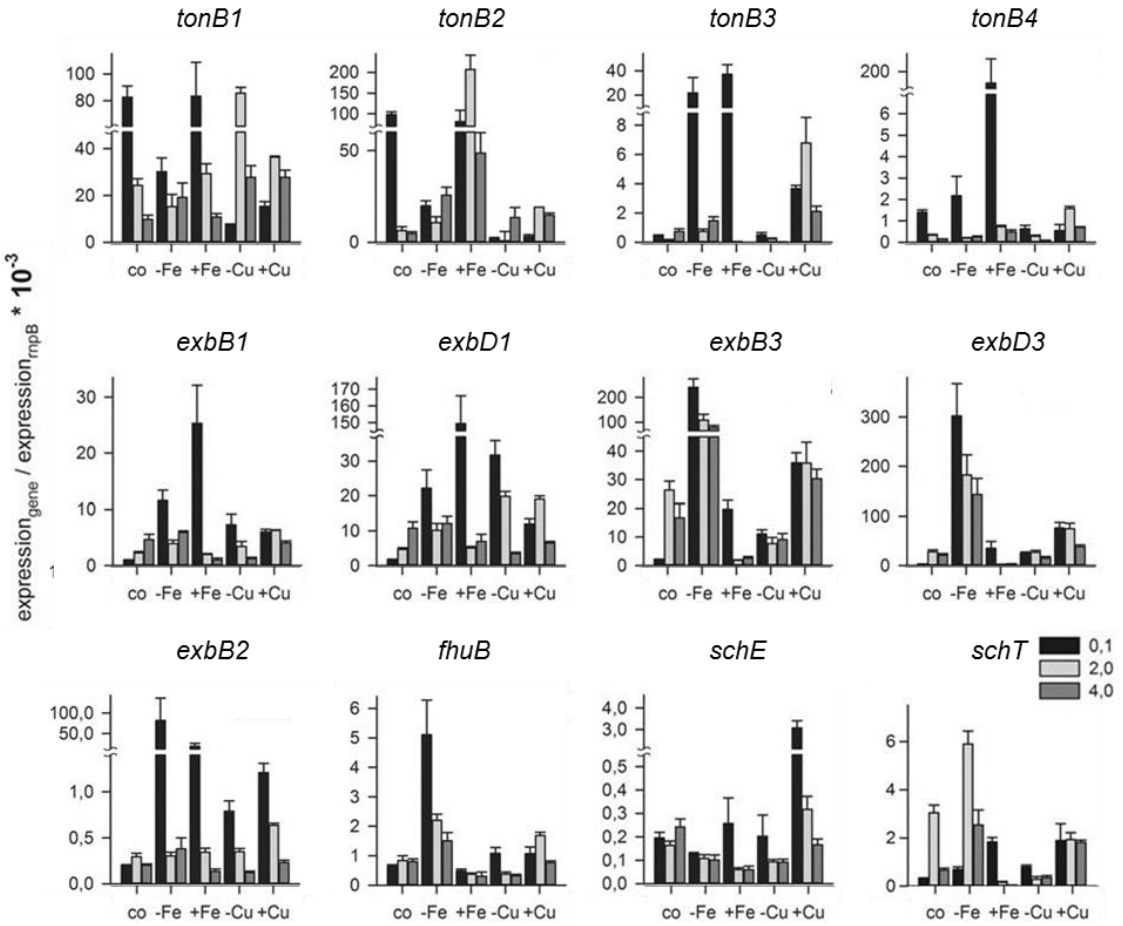

Figure 6. Expression analysis of putative iron-uptake systems in *Anabaena* with respect to different growth stages and medium conditions are presented as bar diagrams. The tested media are BG11 (co), BG11 without copper (-Cu) or iron (-Fe), or with elevated levels of iron (1 mM Fe; +Fe) or copper (1 mM Cu; +Cu). Black bars represent the results for the early growth phase, grey bar for the logarithmic growth stage and the dark grey bar for the stationary phase.The data are expressed relative to the changes of the internal standard *rnpB* and are means of four independent experiments. Figure reproduced from Stevanovic et al., 2013.

Expression of *tonB1* is not growth phase-dependant, as an increased expression can be observed under all growth stages, but under different conditions (Fig.6). The most prominent expression change is seen in the early growth stage in BG11 (co) and with enhanced iron concentration (+Fe). In the medium without copper added (-Cu) an up-regulation of expression was observed in the logarithmic growth stage (grey bar). Transcription of *tonB2* was increased in the medium with elevated iron concentration (Fig. 6, *tonB2*, +Fe) throughout all growth stages, but most strongly in the logarithmic growth stage (grey bar). An elevated expression was also observed in the early growth

stage in BG11 (*tonB2*, co, black bar). The expression of *tonB3* was enhanced in BG11 media without iron added in the early growth stage (*tonB3*, -Fe, black bar), but decreased in the logarithmic and stationary phase (grey and dark grey bar). A comparable increase is also seen with elevated iron concentration in the early growth stage (tonB3, +Fe, black bar). However, expression of *tonB3* is strongly decreased in the logarithmic and stationary growth stage with elevated iron (+Fe, grey and dark grey bars). Interestingly, *tonB3* shows also an enhanced expression with elevated copper concentration particularly in the logarithmic growth stage (+Cu, grey bar), this observation was not seen in the previous expression analysis (section 4.1.1; Fig.1). The transcription profile of *tonB4* reveals a growth stage-dependence, as the gene expression is enhanced in BG11 (co), BG11 without iron (-Fe) and BG11 with elevated iron concentrations (+Fe) but only in the early growth stage (black bar).

The expression profile of *exbB1* and *exbD1* was compareable, as the most prominent change in gene expression was observed in the early growth stage (black bar) when wild type was grown without iron (-Fe), copper (-Cu) or with elevated iron (+Fe). A compareable transcription profile is also observed for the *exbB3* and *exbD3* genes, since the expression of the genes is enhanced in BG11 without iron (-Fe) and BG11 with enhanced copper concentration (+Cu) throughout all growth stages. In BG11 with elevated iron concentration the expression is only enhances in the early growth stage (+Fe, black bar). This expression profile resembles the expression of *tonB3* under the same growth conditions. The only exception is the enhanced expression of *exbB3* and *exbD3* in the logarithmic and stationary growth phase in BG11 (co, grey and dark grey bars). Expression of *exbB2* on the other hand is mostly enhanced in the early growth stage in BG11 without without iron (-Fe), copper (-Cu) and with enhanced concentrations of both metals (+Fe and +Cu), respectively.

A noticeable enhanced expression of *fhuB* is observed under iron-limiting conditions, whereby the expression is mostly enhanced in the early growth stage (Fig.6, *fhuB*, -Fe, black bar) and decreases in the logarithmic (grey bar) and stationary stage (dark grey bar).

Expression of the schizokinen exporter (*schE*) is surprisingly not noticeably enhanced under iron-limiting conditions (-Fe), but rather in the early growth stage without copper (-Cu) and with elevated concentrations of iron (+Fe) and copper (+Cu). The enhanced expression of *schE* under elevated copper concentration indicates that copper affects iron uptake under this condition. The same impact was seen for *tonB3, exbB3* and *exbD3* expression under elevated copper concentration.

Transcription of the schizokinen transporter (*schT*) on the other hand is mostly up-regulated in the logarithmic growth stage (grey bar) in BG11 (co) and under iron limiting condition (-Fe).

4.2. Characterization of the iron starvation induced acquisition systems in *Anabaena*

4.2.1 Inactivation of the *fhuB/C/D*, *exbB3* and *tonB3* genes

In order to investigate the cellular function of the systems shown to be regulated by iron-availability (*tonB3*, *exbB3*, *exbD3*, *fhuB*/*C/D*), deletion mutants of the corresponding genes were generated. For this purpose a plasmid containing a C.S3 cassette and a fragment of the gene of interest was inserted by homologous recombination into the corresponding gene, thereby interrupting the orf (see Methods, section 3.2.5). Integration of the plasmid was tested by PCR using one oligonucleotide flanking the fragment used for inactivation and one oligonucleotide binding in the C.S3 cassette. Chromosomal DNA from the mutant strains was used as template whereas wild type DNA served as control (Fig.7A). The generated mutants are denoted as AFS-I-*fhuB* (insertion in *all0387*), AFS-I-*fhuD* (insertion in *all0388*), AFS-I-*fhuC* (insertion in *all0389*), AFS-I-*exbB3* (insertion in *all5047*) and AFS-I-*tonB3* (insertion in *all5036*).

Anabaena has multiple copies of the same chromosome (Herdman et al., 1979) and mutation of a gene with essential function will never result in a fully segregated mutant, i.e. wild type copies of the gene will remain under any selective pressure. Segregation of mutant chromosomes was analysed with oligonucleotide pairs flanking the fragment used for inactivation (Fig.7A, cartoon). Failure to amplify a fragment of wild type size from a mutant strain's DNA confirms the absence of the intact gene in mutant cells, therefore these mutants are termed fully segregated. Screening of the mutant strains grown in BG11 revealed that only AFS-I-*exbB3* was fully segregated, while for all other mutants wild type copies of the corresponding gene could still be detected (Fig.7A). A successful depletion of the *tonB* gene in *E.coli* was shown to require excess of iron in the growth medium (Wang and Newton, 1969). To promote segregation of AFS-I-*tonB3*

the mutant was as well grown in BG11 medium enriched with iron. Consistent with the observation made by Newton, a complete segregation of the *tonB3* mutant grown in BG11 enriched with iron could be observed. However, a subsequent shift of the mutant back to BG11 resulted in a fast (10-12 h) reappearance of wild type copies of the gene, as a result of re-recombination (Fig.7B). The full segregation of the mutant confirms, in addition to the observed qRT-PCR results (see section 4.1.1; Fig.1), that *tonB3* is not needed in the presence of elevated iron concentrations and by this negatively regulated by iron. The fast re-recombination of the mutant in turn indicates, that *tonB3* is essential in BG11 and moreover that its function cannot be complemented by any of the other predicted TonB proteins.

The fact that mutants of the *fhu*-system could not be fully segregated, on the other hand, indicates that the fhu-system is essential and cannot be complemented by the other predicted BP-dependent ABC-transporter. The importance of the fhu-system is in agreement with the obtained qRT-PCR results, showing that the fhu-system is the only BP-dependent ABC-system clearly regulated by iron availability (see section 4.1.3).

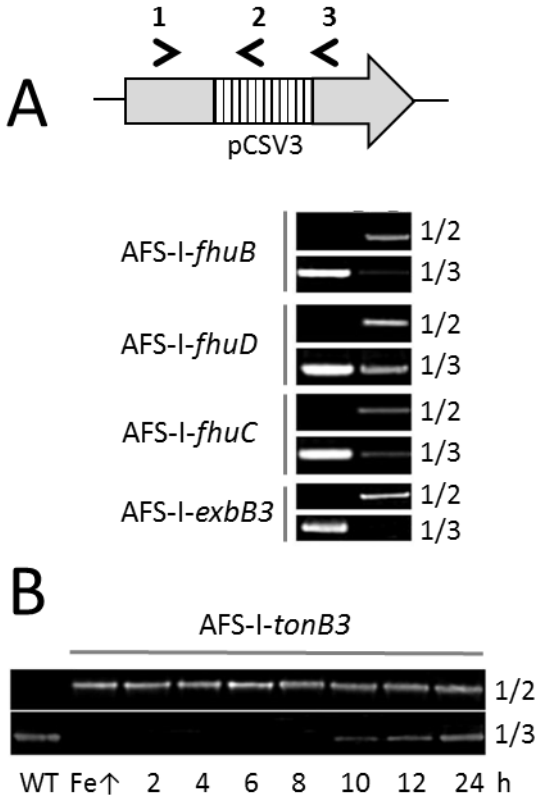

Figure 7. (A) Mutants were generated by homologous recombination via insertion of pCSV3 as shown in the cartoon on top. Segregation efficiency was analysed by PCR on gDNA using oligonucleotide combinations indicated in the cartoon on top of the figure. gDNA of wild type *Anabaena* served as PCR control. (B) AFS-I-*tonB3* was grown for four weeks with elevated iron concentrations (Fe↑) before being shifted to control conditions (BG11). Segregation efficiency was analysed by PCR using the indicated olgonucleotides combinations on genomic DNA isolated from wild type and mutant strain. Figures 6A&B are reproduced from Stevanovic et al., 2012.

4.2.2 Interdependence of components involved in siderophore-dependent iron-uptake

To assess the impact of the putative iron-acquisition systems on general iron homeostasis, the expression of *tonB3*, *exbB3* and the *fhu*-system was analysed under iron limitation in the generated mutant lines. Furthermore, the expression of *hgdD*, the outer membrane component involved in siderophore secretion (Nicolaisen et al., 2010), was analysed. Expression of *hgdD* was up-regulated in all mutant lines, indicating enhanced siderophore secretion as a result of iron starvation. The only exception was the unchanged transcription of *hgdD* in AFS-I-*exbB3*, again hinting towards a complementation of ExbB3. The transcript of the *fhu*-system could not be detected in the AFS-I-*exbB3* and -*tonB3* mutants, but rather in the mutants of the other *fhu* genes (Fig.8A), whereas the transcript of the *exb3*-system was increased in all three *fhu*-mutants and the *tonB3*-mutant. The same holds true for the expression of *tonB3*, which showed enhanced transcripts in all *fhu*-mutants and the *exbB3*-mutant. Summarizing, the expression analysis in the mutant lines revealed, that *tonB3* and the *exb3*-system respond to iron starvation by up-regulation of expression. Whereas expression of the *fhu*-system seems to be substrate-dependent, since transcripts of the *fhu*-system components could not be detected in mutants affected in siderophore-dependent iron uptake (AFS-I-*exbB3* & AFS-I-*tonB3*).

To follow up the idea of a substrate-dependent feedback-regulation of the fhu-system expression analysis was extended to mutants already characterized to be affected in siderophore-dependent iron-uptake (Nicolaisen et al., 2008 and 2010). Siderophore secretion has been shown to involve beside the already mentioned HgdD protein also the MFS-type schizokinen exporter (SchE) localized in the cytoplasm membrane. Insertion mutants of these two genes showed a drastic reduction of siderophore secretion

and consequently an iron starvation phenotype (Nicolaisen et al., 2010). In addition, expression of the iron-responsive system was also analysed in mutants of *schT* (AFS-I-*schT*) and *iacT* (AFS-I-*iacT*), both coding for TBDTs involved in iron uptake (Nicolaisen *et al*., 2008; Nicolaisen *et al*.,2010). The transcript level of the *fhu* genes was decreased in AFS-I-*schE* and AFS-I-*schT* and not detectable in the *hgdD* mutant (Fig.8B), again supporting the notion that the fhu-system expression is dependent on iron-loaded siderophores in the periplasm. Expression of the *fhu*-system in the AFS-I-*iacT* mutant on the other hand is detectable (Fig.8B). In contrast, the expression of *exbD3* and *tonB3* was up-regulated in the same mutants suggesting transcriptional regulation in respond to iron-limitation (Fig.8B). All0075 encodes an Omp85-like protein (Nicolaisen et al., 2009). Omp85 like proteins are considered to be generally involved in outer membrane protein biogenesis. Therefore the enhanced expression of *exbD3* and *tonB3* might indicate an altered TBDT biogenesis and therefore enhanced iron starvation. Slightly enhanced expression of hgdD could be also interpretated as a result of altered TBDT biogenesis, alternatively HgdD biogenesis could be influenced.

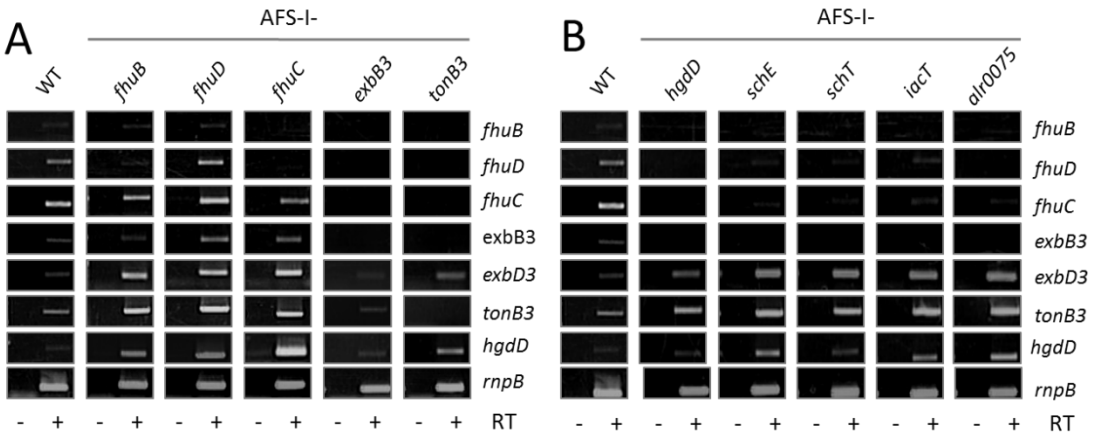

Figure 8. (A) Expression analysis of genes involved in iron uptake under iron limited conditions in the generated mutant strains. (B) Expression analysis of genes involved in iron uptake under iron limited conditions in mutants of genes involved in siderophore secretion and uptake by the TBDT system. RT-PCR was performed on RNA isolated from *Anabaena* sp. wild type (WT) or mutant cells grown under control conditions (BG11). The presence or absence of reverse transcriptase (RT) is denoted by (+) and (-) respectively. RT-PCR of *rnpB* was performed as a control. Figures are reproduced from Stevanovic et al., 2012.

4.2.3 Iron starvation phenotype of the systems involved in siderophore-dependent iron uptake

To substantiate the conclusion that the *tonB3, exbB3 and fhu*-systems have an impact on iron uptake under iron-limiting conditions, iron starvation of the mutants was investigated using several methods. Primary the expression of genes known as indicator of iron starvation were analysed in wild type and mutants' background grown in BG11 and in BG11 without iron and copper (-Fe/-Cu). One of the iron starvation indicator genes is *furA* (*all1691*), the master regulator of iron homeostasis (see section 1.4), and the iron stress inducible gene *isiA* (*all4001*; Escolar et al., 1999; Latifi et al., 2005). The *isiA* gene is expressed as a respond to iron deficiency in cyanobacteria, hereby large IsiA aggregates are produced and some of them associate with PSI as a protective mechanism (Van der Weij-de Wit et al., 2007).

While transcripts of *furA* and *isiA* could only be observed in wild type grown in -Fe/-Cu, the expression of both genes was detectable in mutants grown in BG11 (Fig. 9A). These results indicate iron starvation already in BG11. In order to proof that the starvation phenotype of the mutants is iron limitation specific and not caused by copper limitation the expression of the gene coding for plastocyanin (*petE*) was analysed. Plastocyanin functions under copper-replete conditions as an alternative electron carrier between the cytochrome b_6-f complex and PSI. Therefore the expression of *petE* is copper-dependent and down-regulated in the absence of copper (Ghassemian et al., 1994). Since *petE* transcript was detected in all mutant lines copper limitation can be excluded (Fig.9A).

Next to investigate iron starvation the expression of gene clusters involved in siderophore biosynthesis were analysed (Fig.9B), as siderophores are only synthesized under iron starvation. One of the analysed gene cluster comprises *all0390,* a gene which

shows similarity to the rhizobactin biosynthesis gene *rhbF*, and *all0393*/*all0396* genes with similarity to *iuc* genes involved in aerobactin biosynthesis (Nicolasien et al., 2008). The second gene cluster comprises *all2658-2635* and encodes several non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) described to be involved in siderophore synthesis in *Anabaena* (Jeanjean et al., 2008). Analysis of wild type grown in BG11 showed, that only a transcript for *all0390* and *all0396* could be observed (Fig.9B), while expression of the other genes could not be observed even after 45 PCR cycles (data not shown). Analysis of siderophore bisosynthesis genes expression in the *tonB3*-, *exbB3*- and *fhu*-system mutants grown in BG11, revealed transcript for *all0396* in all mutant strains. In AFS-I-*fhuB* the expression, however, could be shown only after extension of the PCR cycles (data not shown). Transcript of *all2635* was observed in all strains, but in lower abundance in AFS-I-*tonB3* and -*exbB3*. These results confirm iron starvation of the mutants already observed by expression of iron starvation indicator genes (Fig.9A).

The results above showed that expression of siderophore biosynthesis genes is increased in some mutant strains. To determine if increase in transcription correlates with actual changes in production of siderophores, levels of siderophore secretion were analysed using the chrome azurole S (CAS) plate assay (Fig.8C; Schwyn and Neilands, 1987). This assay uses a blue colored CAS-Fe(III) complex containing the detergent hexadecyltrimethylammonium bromide (HDTMA). Secreted siderophores remove iron from the CAS/HDTMA complex, resulting in a color change to yellow. The assay was first performed on BG11 agar plates and consistent with the RT-PCR results, AFS-I-*tonB3*, I-*exbB3*, I-*fhuD* and I-*fhuC* showed enhanced siderophore secretion (Fig.8C, left part). However, siderophore secretion is even more pronounced for AFS-I-*tonB3* and I-*fhuC*, even though both strains are growing slower than the wild type as judged from the size of the colonies (Fig.9C, top, left part). On CAS agar plates without iron and

copper the assay showed that the siderophore secretion of AFS-I-*tonB3* and AFS-I-*fhuC* was even stronger than on BG11 CAS agar plates. Again comparison of growth of colonies shows the obvious alteration of the mutant lines with only AFS-I-exbB3 growing wild type-like (Fig.9C, right part). At last all three experiments demonstrated iron starvation of the mutants, with an especially drastic effect of the AFS-I-*tonB3* and AFS-I-*fhuC* mutants.

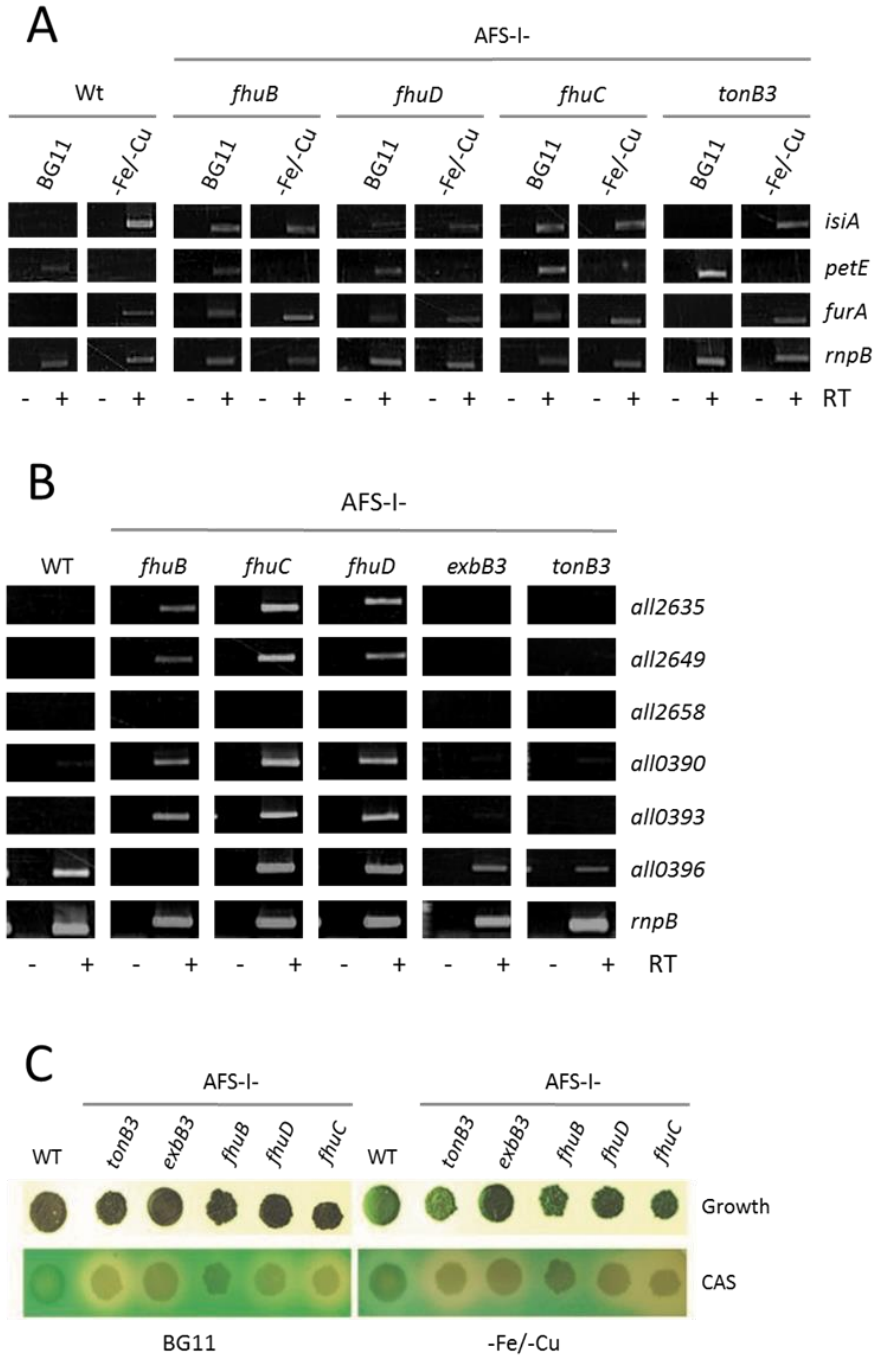

Figure 9. (A) Transcript levels of iron starvation indicator genes *isiA*, *petE*, *furA* and, *rnpB* were determined by RT-PCR. RNA was isolated from wild type or mutant strains listed on top of the figure. The cells were either grown in control medium (BG11) or under limiting iron and copper conditions (-Fe/-Cu). The presence or absence of reverse transcriptase (RT) is denoted by (+) and (-) respectively. (B) Transcript levels of *all0396*, *all2635* and *rnpB* genes were determined by RT-PCR using RNA isolated from the various mutants listed on top of the figure grown in BG11. The presence or absence of reverse transcriptase (RT) is denoted by (+) and (-) respectively. (C) Wild type and mutants were grown on CAS plates prepared with BG11 or BG11 without iron and copper added (-Fe/-Cu). Growth density of the colonies (top) and siderophore secretion (bottom) are shown after 14 days of growth. Figures are reproduced from Stevanovic et al., 2012.

4.3 Characterization of the non-iron related TonB proteins

4.3.1 Mutant characterization

Four putative TonB proteins were predicted by bioinformatic analysis (Stevanovic et al., 2012), but only the *tonB3* gene showed iron-dependent expression by qRT-PCR analysis (see section 4.1.1; Fig:1B). Moreover the *tonB3* single recombinant mutant exhibited an iron starvation phenotype, as judged from the expression of iron starvation indicator genes and the enhanced siderophore secretion of the mutant on CAS plates (see section 4.2.3; Fig.9C). In contrast iron-limitation dependent expression of the remaining three *tonB*-genes could not be observed by expression analysis with qRT-PCR (see section 4.1.1; Fig.1B). To define the cellular function of the remaining putative TonB proteins, single recombinant insertion mutants thereof were generated and named accordingly AFS-I-*tonB1* and AFS-I-*tonB2* (see Methods, section 3.2.5). Integration of the plasmid used for homologous recombination of the mutants was tested by PCR on gDNA of mutant strains (oligonucleotides used are listed in Table 1, see Material section 2.3). For this purpose one oligonucleotide flanking the fragment used for inactivation and one oligonucleotide binding to the C.S3 cassette was used (Fig.10A, top). Investigation of the segregation status of the mutants with oligonucleotides flanking the fragment used for inactivation revealed only AFS-I-*tonB2* as a fully segregated mutant, which implies that TonB2 may not be essential under the tested condition or that TonB2 can be complemented by another putative TonB protein (Fig. 10A). To test whether TonB2 can be complemented by one of the other putative TonB proteins expression of *tonB1*, *tonB3* and *tonB4* was analysed by RT-PCR in the *tonB2* mutant background (Fig.10B). Since no up-regulation of any other *tonB* gene could be observed by RT-PCR a complementation of TonB2 is unlikely.

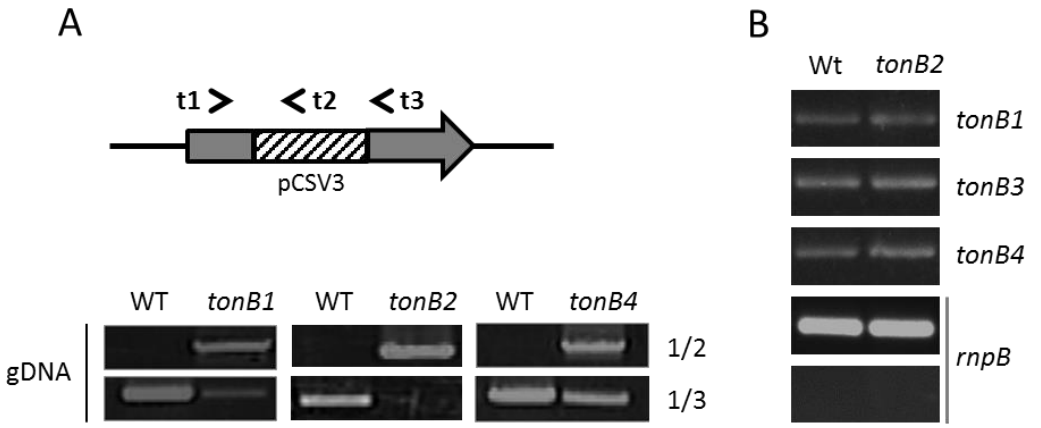

Figure 10. (A) Cartoon indicating the oligonucleotide combinations used for screening of the single recombinant mutant strains is shown on top of the figure. Segregation efficiency was analysed by PCR using the indicated oligonucleotide combinations (on the right) on genomic DNA isolated from wild type or mutant strains. (B) RT-PCR on reverse transcribed RNA isolated from the *tonB2* mutant with oligonucleotides for the remaining putative *tonB* genes. PCR with *rnpB* oligonucleotides on the bottom of the figure is performed on RNA without reverse transcriptase.

To further investigate a possible inveolvment of the predicted TonB proteins in siderophore-dependent iron uptake, characterization of *tonB*-mutants started by testing the growth of the mutants under iron limitation. The conditions tested were liquid BG11, BG11 without iron ($BG11_{-Fe}$) and BG11 without iron and citric acid ($BG11_{-Fe/-Cit}$). Citric acid is a component of the BG11 concentrate (Rippka et al., 1979), however, citric acid is also known to chelate metals and in particular iron (Silva et al., 2009 and references therein). Therefore in order to avoid chelation of iron traces, citric acid was in addition to iron left out in the medium (-Fe/-Cit). To be able to apply the same conditions for control and mutant strains, with respect to the use of antibiotics, instead of the wild type the strain AFS-I-*nucnui* was used. The mutant is carrying the C.S3 cassette in a neutral site (*nucA*-*nuiA* region) located in the α-megaplasmid of *Anabaena* (Olmedo-Verd et al., 2006).

The growth curves of the mutants in the first week revealed, that growth of all mutants under all tested conditions was wild-type like, with only AFS-I-*tonB3* showing slightly

decreased growth in BG11$_{-Fe}$ (data not shown). However, it has been observed that cyanobacteria need to be transferred to iron-limiting conditions several times to ensure depletion of intracellular stored iron (Jiang et al., 2012). Thus, all strains were inoculated a second time to the corresponding medium, meaning that the cultures grown in BG11 were transferred a second time to BG11 et cetera. Following the second transfer the difference in growth of AFS-I-*tonB3* to all other strains grown in BG11$_{-Fe}$ became obvious (Fig.11B), supporting the assumed involvement of *tonB3* in iron uptake under iron-limiting conditions. In Bg11$_{-Fe/-Cit}$ the *nucnui* and *tonB1* strains showed identical growth behavior like in BG11$_{-Fe}$, while AFS-I-*tonB3* did not show a drastic growth alteration. Interestingly, when grown in BG11$_{-Fe/-Cit}$ AFS-I-*tonB2* revealed strongly altered growth (Fig.11C). As no growth alteration for the *tonB2* mutant was observed in BG11$_{-Fe}$ the growth in BG11$_{-Fe/-Cit}$ implies a citrate based phenotype. Another interesting growth behavior was observed, as AFS-I-*tonB1* showed altered growth in BG11 following the second transfer (Fig.11A). Since growth of the *tonB1* mutant was not thus strong affected in the media without iron a phenotype not specifically related to iron can be assumed.

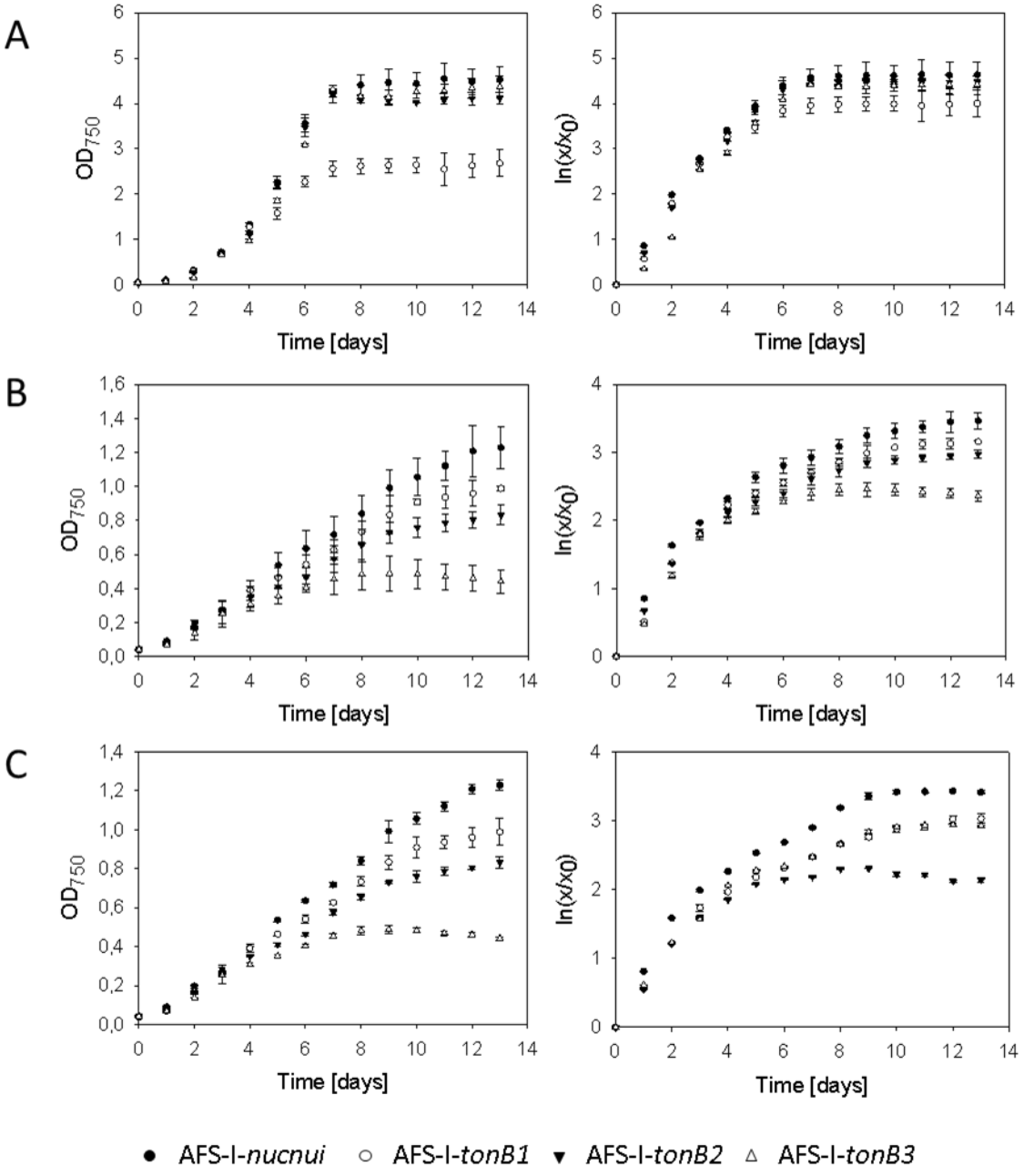

Figure 11. Growth curves after second transfer of wild type substitution (AFS-I-*nucnui*) and mutant strains cultured in liquid BG11 medium (A), BG11 without iron (B) and BG11 without iron and citrate (C). Growth was recorded as absorbance at 750 nm. The data are presented as means of four independent measurements.

To examine if the growth of the mutant strains is indeed influenced by iron the strains AFS-I-*nucnui*, AFS-I-*tonB2* and AFS-I-*tonB3* previously grown in $BG11_{-Fe}$ were again inoculated in $BG11_{-Fe}$ (Fig.12A). This time every culture was twice inoculated and one of the cultures was resupplied with 0.22 µM iron(III) citrate while the second culture

remained in the respective medium (FeCit; Fig.12B). Indeed, growth of AFS-I-*tonB3* could be rescued by iron, demonstrating that growth alteration of AFS-I-*tonB3* was caused by iron limitation and also that a TonB3-independent iron uptake mechanism must exist. The strain AFS-I-*tonB2* which experienced only a minor growth alteration when grown in $BG11_{-Fe}$ showed wild type-like growth when resupplied with iron(III) citrate.

The same procedure as described above was also applied for the cultures AFS-I-*nucnui*, AFS-I-*tonB3* and, AFS-I-*tonB2* previously grown in $BG11_{-Fe/-Cit}$, with the exception of the iron source used to resupply the cultures, since in this case 0.22 µM $FeCl_3$ (Fig.11D) was used. Surprisingly, growth of AFS-I-*tonB2* could be restored to wild type-level by this iron source. The rescue of AFS-I-*tonB2* indicated that the phenotype observed for the mutant cannot be exclusively explained by citrate, but has to be connected to iron.

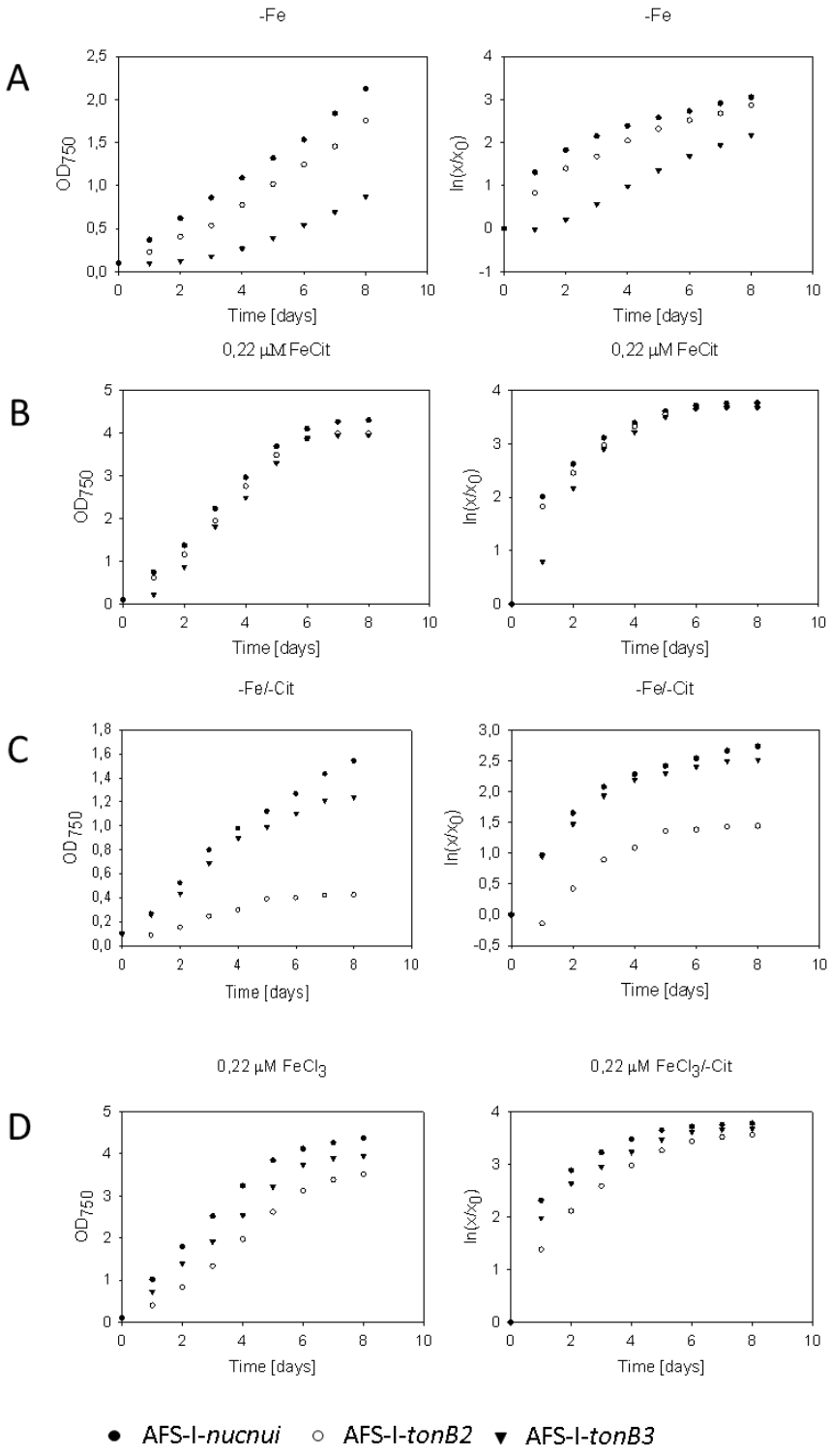

Figure 12. Growth curves after third transfer of wild type substitution (AFS-I-*nucnui*) and mutant strains cultured in liquid BG11 without iron (A), resuplied with 0,22 µM ferric citrate (B), BG11 without iron and citrate (C) and, BG11 without citrate but resupplied with 0,22 µM ferric chloride (D). Growth was recorded as absorbance at 750 nm. The data are presented as means of three independent measurements.

The *tonB1* mutant which showed a growth alteration in BG11 was again transferred to BG11 (Fig.13A) and to BG11 resupplied with iron (Fig.13B). As expected the *tonB1* mutant could not be rescued by iron (Fig.13B), which supports the hypothesis that the observed AFS-I-*tonB1* growth alteration after second transfer to BG11 (Fig.11) was not caused by iron.

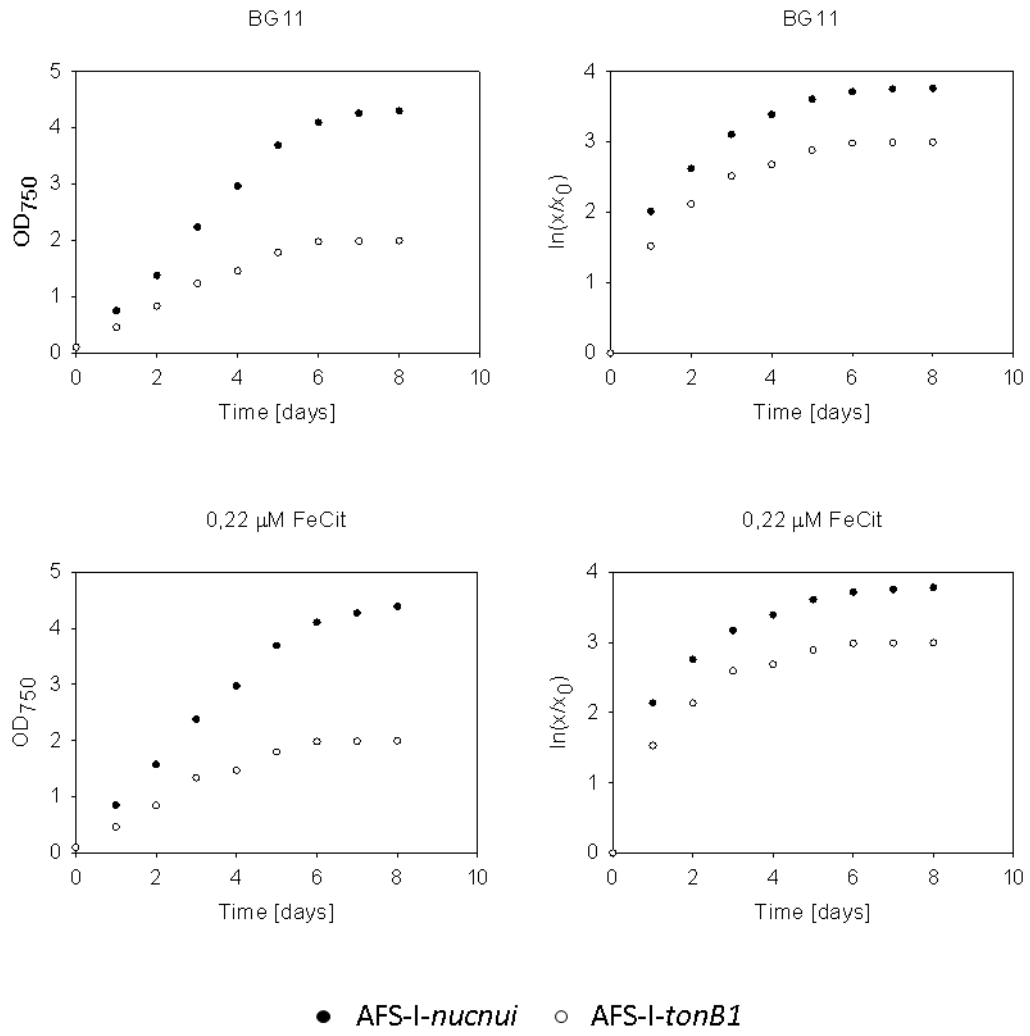

Figure 13. Growth curves after third transfer of wild type substitution (AFS-I-*nucnui*) and *tonB1* mutant strains cultured in liquid BG11 and resuplied with 0,22 µM ferric citrate (FeCit). Growth was recorded as absorbance at 750 nm. The data are presented as means of four independent measurements.

The TonB-system is not restricted to the transport of iron complexes, considering that TonB dependence of the transport of nickel and different carbohydrates has been established (Schauer et al., 2008). Furthermore, two TBDTs of *Anabaena* (*alr3242* and *alr4028-29*) are postulated to be under the control of the zinc transcriptional regulator Zur (*all2473*; Napolitano et al., 2012), indicating that they might be involved in zinc uptake. In this light, the growth of the *tonB*-mutants on agar plates with different metal concentrations was tested (Fig.14). On the one hand, growth under metal limiting conditions was tested and complementary growth of the mutants under high metal concentrations was analyzed, assuming that a decreased metal uptake would allow the mutants to grow under usually toxic concentrations. The growth analysis was performed with altered concentration of zinc ($ZnSO_4$), cobalt ($CoCl_2$), ferric citrate (FeCit), and manganese ($MnCl_2$). The analysed conditions are plates with no addition of the corresponding metal (0), plates with 10-fold the original concentration in comperision to BG11 (x10) and plates with 100-fold the original concentration in comperision to BG11 (x100).

The growth of AFS-I-*tonB1* on plates with different concentration of zinc, cobalt and iron showed no difference compared to AFS-I-*nucnui* (Fig.13). In contrast, a clear difference could be observed on plates with different manganese concentrations. AFS-I-*tonB1* was growing slower in comparison to AFS-I-*nucnui* under manganese limiting conditions (Fig.14;0). The growth of the mutant got facilitated with higher manganese concentrations (Fig.2; x10), and the mutant was even able to withstand a concentration of manganese obviously toxic for AFS-I-*nucnui* (Fig.2; x100). Thus function of TonB1 is not related to iron concentrations, but rather shows a phenotype connected to manganese.

Neither AFS-I-*tonB2* nor AFS-I-*tonB3* showed a growth alteration on agar plates without iron (FeCit, 0). Therefore it can be reasoned that growth on plates was not

sufficient to deplete the intracellular stored iron. However both mutant strains were able to withstand the 10-fold concentration of iron(III) citrate, whereas AFS-I-*tonB1* and AFS-I-*nucnui* could not survive (FeCit, x10). This phenotype again suggests an iron related function of the proteins. Since no difference in growth compared to the control strain was observed for the other tested metals, it can be further assumed that TonB2 and TonB3 are not involved in the uptake of zinc, cobalt or manganese.

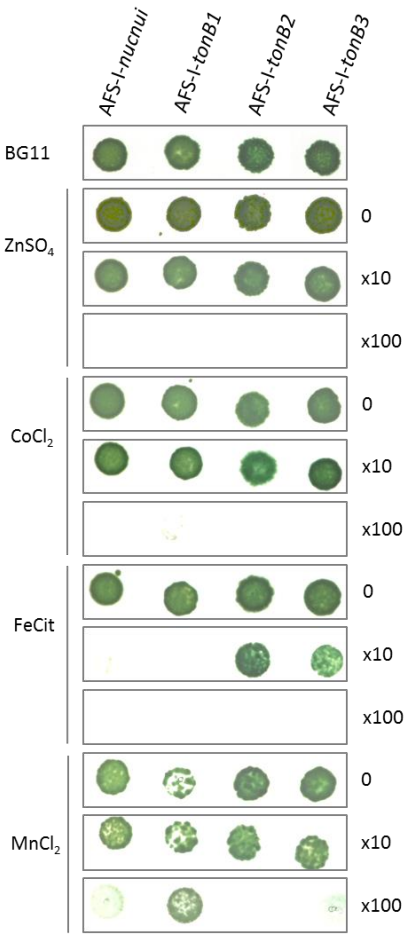

Figure 14. AFS-I-*nucnui* (wild type substitution) and mutant strains were spotted at a concentration of 0.1 OD_{750} on agar plates composed of BG11 supplemented with different metal variations. The metal concentration is indicated at the right part of the figure, whereby 0 means that the corresponding metal is not added to the agar, x10 is the tenfold concentration and x100 the hundredfold concentration of the metal compared to BG11. Cells were grown for 7 days.

4.3.2 The role of TonB1 in manganese uptake

The TonB1 protein is among all putative TonB candidates the most exceptional one, especially because of the missing TBDT-interaction domain motif (Stevanovic et al., 2012). Blast search with the amino acid sequence (http://www.ncbi.nlm.nih.gov/BLAST) relates TonB1 to the Z interaction protein A (ZipA), for which no other obvious homolog is found in *Anabaena* (Rothfield et al., 1999). ZipA belongs to the group of proteins involved in cell division; it is recruited to the septal ring at a very early stage, where it interacts directly with FtsZ (Hale and de Boer, 1997; Liu et al., 1999). Overexpression or depletion of ZipA in *E.coli* leads to formation of non-septal filaments (Hale and de Boer, 1997, 1999). However, microscopic examination of AFS-I-*tonB1* cultures in the exponential growth phase revealed no morphological changes or a stronger fragmentation in the strain compared with its parental strain (Fig. 15). Therefore, it seems unlikely that TonB1 is involved in cell division in *Anabaena*.

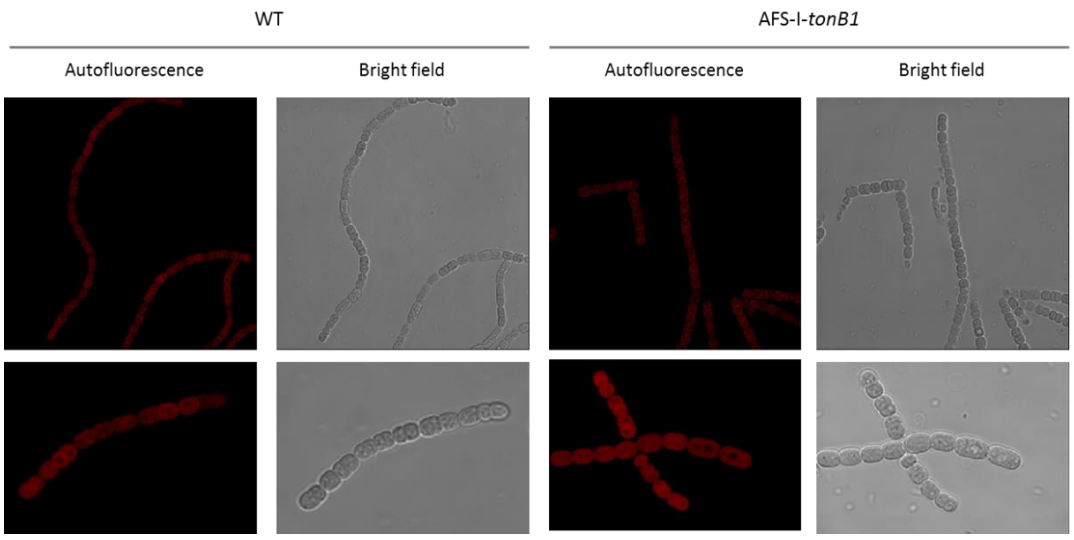

Figure 15. Leica confocal microscopy of wild type *Anabaena* and *tonB1* (AFS-I-*tonB1*) mutant in the exponential growth phase in BG11 medium. Shown are auto-fluorescence and bright field images of the strains.

The inactivation of *tonB1* did not lead to an iron starvation phenotype; whereas a phenotype connected to manganese could be observed on agar plates with low and high manganese concentrations (see section 4.3.1). To confirm the manganese related phenotype AFS-I-*tonB1* and AFS-I-*nucnui* were grown in BG11$_{-Mn}$. The growth analysis revealed a prominent growth alteration of the *tonB1* mutant compared to AFS-I-*nucnui* (Fig.16A). However, manganese acquisition by gram negative bacteria has never been described to be TBDT-dependent, whereas the transport is believed to be carried out by porins. Recently a porin involved in high-affinity manganese uptake in the gram negative bacterium *Bradyrhizobium japonicum* has been identified (Hohle et al., 2011). Guided by the finding in *B. japonicum* the expression of putative porins in the AFS-I-*tonB1* strain and wild type *Anabaena* grown in BG11 was compered. According to CyanoBase (http://genome.kazusa.or.jp/*cyanobase*) the genome of *Anabaena* contains six putative porins, encoded by *alr0834*, *alr2231*, *all4499*, *alr4550*, *alr4741*, *all5191* and *all7614*. HMMPfam analysis (CyanoBase: http://genome.kazusa.or.jp/ *cyanobase*) shows, that sequences of all seven putative porins share an N-terminal motif with significant homology to 'S-layer homology' domains (PF00395) and the OprB domain (PF04966), indicating them as similar to members of the carbohydrate-selective porin OprB family. Certainly it has to be considered, that the high similarity among porins, driven by the 14 to 16 stranded β-barrel regions, makes distinguishing general porins from carbohydrate-selective porin problematic. Thus without direct experimental characterization it is difficult to predict the substrate specificity of a porin. Nevertheless, the expression of all seven putative porins was analyzed in the *tonB1* mutant grown in BG11 and BG11 without manganese added. Remarkably mRNA levels of all seven genes were strongly down-regulated in *tonB1* mutant background grown in BG11 (Fig.16B, dark grey bars). However, growth of the *tonB1* mutant in BG11 minus manganese (Fig.16B, light grey bars) led to a

differentiated expression pattern. The transcript abundance of *all4499* and *alr4550* remained comparable in BG11 and BG11-Mn. Also the transcript abundance of *alr0834* and *all7614* was still reduced but not as drastic as in BG11. Whereas, *alr2231*, *alr4741* and *all5191* showed transcript abundance almost comparable to that found in wild type when grown in the absence of manganese.

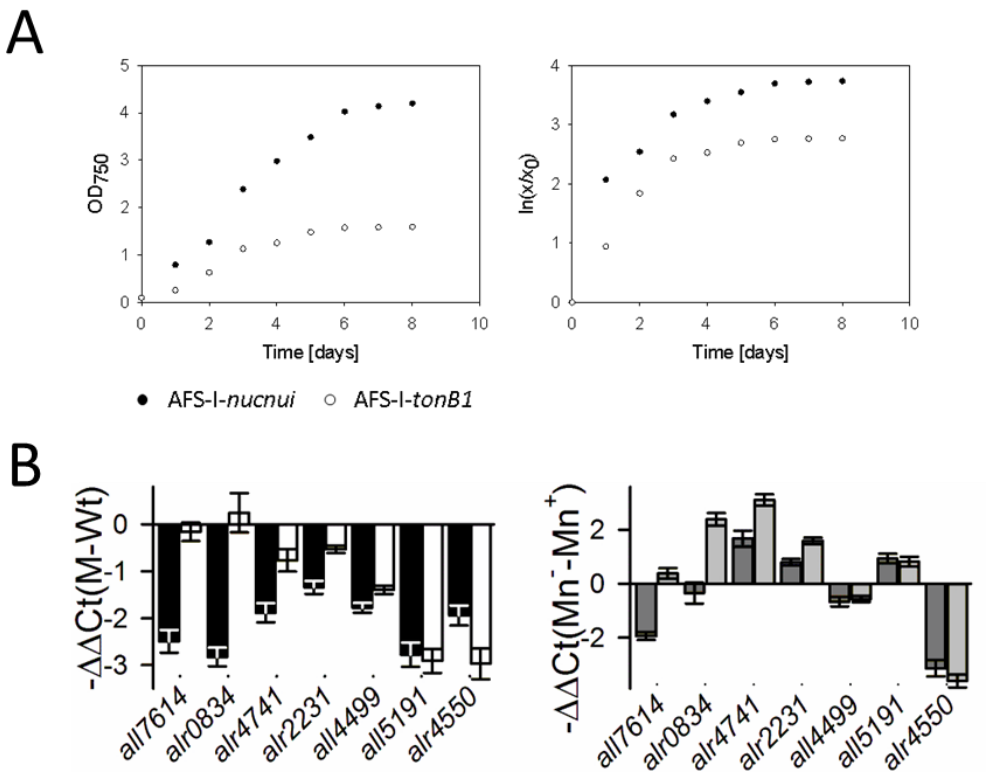

Figure 16. (A) Growth curves of AFS-I-*nucnui* (wild type substitution) and AFS-I-*tonB1* in liquid BG11 without manganese added. Growth was recorded as absorbance at 750 nm. The data are presented as means of three independent measurements. (B, left) Expression of putative porin encoding genes in the *tonB1* mutant background grown in BG11 (black bars) and BG11 medium without manganese added (white bars). Data are expressed relative to wild type and *rnpB* expression. (B, right) Transcript abundance of putative porin encoding genes in wild type (dark grey bars) and *tonB1* mutant (light grey bars) under manganese limitation (Mn^-) relative to expression levels in BG11 (Mn^+) and changes of the internal standard *rnpB*.

These results are consistent with the manganese related phenotype of AFS-I-*tonB1* and might suggest that porins of *Anabaena* are indeed involved in manganese uptake. To examine, which impact a mutation of *tonB1* has on manganese homeostasis the expression of genes known to be involved in manganese uptake under manganese starvation were analyzed. Interesting for the analysis were the components described to

be part of a manganese-dependent two-component signal transduction pathway (Huang and Wu, 2004a and 2004b). This pathway includes the membrane-bound histidine kinase *manS* (*all3587*), the transcriptional response regulator *manR* (*all1964*) and the permease (*mntB*, encoded by *all3574*) as a component of the plasma membrane localized ABC-transporter *mntABC* (*all3575*, *all3574*, *alr3576*). Surprisingly, the expression of the manganese-dependent components manS and mntB was down-regulated in the *tonB1* mutant grown in BG11, the transcript abundance of the manganese-regulator manR is slightly higher than in wild type (Fig.16C; dark grey bars). However, growth of the mutant in BG11 without manganese added led to a wild type-like expression (Fig.16C, light grey bars). Summerizing the observed expression of the components involved in manganese uptake suggests no manganese starvation phenotype of the *tonB1* mutant in BG11. To further investigate the putative manganese limitation phenotype of the *tonB1* mutant grown in BG11 the expression of the superoxide dismutases *sodA* (*all0070*) and *sodB* (*alr2938*) was also tested, hence manganese limitation is described to cause oxidative stress in cyanobacteria (Krieger and Rutherford, 1998).

Table 4. Effects of *tonb1* mutation on the manganese-dependent two-component signal transduction (manR-manS; mntB) and sod expression. The amount of transcript of each gene was determined, corrected for the signal observed without reverse transcriptase in the reaction, set in relation to *rnpB* abundance and normalized to the expression in wild type. Represented is the average of three biological replicas.

-ΔΔCt(M-Wt)	**BG11**
manR	1,63 ± 0,34
manS	-2,43 ± 0,68
mntB	-2,76 ± 0,9
SodA	-3,58 ± 0,68
sodB	0,88 ± 0,37

While the expression of *sodB* was wild-type like, the expression of *sodA* at the same time was significantly reduced (-3-fold; (Fig.16D). Transcription of *sodA* is regulated by manganese-availability and is significantly down-regulated (3.8-fold) under manganese limitation in *Streptococcus pneumonia* (Eijkelkamp et al., 2014). With regard to this obersation the down-regulation of *sodA* in AFS-I-*tonB1* can be interpreted as a consequence of manganese limitation. Taken together the observed overall reduction of gene expression in the *tonB1* mutant indicates a relation of the phenotype to porins.

It is intriguing to assume, that the down-regulation of porin expression is a direct consequence of TonB1 depletion. Following up the porin related phenotype of AFS-I-*tonB1*, growth of the mutant was tested on agar plates with varying copper concentrations, as it is believed that the small and hydrophilic copper ions utilize porins to enter bacterial cells (Lutkenhaus, 1977; Niederweis, 2008; Nies and Herzberg, 2013). Furthermore down-regulation of porin expression in respond to copper stress has been previously reported in *E.coli* and two strains of marine *Synechococcus* (Stuart et al., 2009; Lutkenhaus, 1977). Mutation of *tonB1* indeed enhanced resistance towards toxic copper concentrations (Fig.17A), supporting the observed porin connected phenotype of AFS-I-*tonB1* (see Fig. 16). To further substantiate this notion, growth of the mutant on plates with macrolide antibiotics was tested, as it is assumed that porins are most likely involved in the macrolide transport in *Anabaena* (Hahn et al. 2012, 2013). AFS-I-*nucnui* and AFS-I-*tonB1* were spotted on plates with 30 ng/ml Tylosin (Tyl) and 30 ng/ml Roxitromycin (Rox, Fig.17A). Again, this concentration of macrolides altered the growth of AFS-I-*nucnui*, while AFS-I-*tonB1* was able to grow in the presence of these compounds (Fig.17A).

To learn more about the link between *tonB1* and porin expression, RT-PCR was performed with wild type *Anabaena* grown for 16 h under conditions influencing outer

membrane permeability (salinity : 0,1 M NaCl; 0,1 M KCl; chemoheterotrophic growth: 5 mM glucose in the dark). Initially expression of *tonB1* was tested under the described conditions. In BG11 and with sodium chloride the transcript levels of *tonB1* were weak, but upon glucose and potassium chloride treatment the expression was enhanced. Thereafter expression of the putative porins was analyzed with RT-PCR. Out of the seven putative porins in *Anabaena* only three, namely *alr0834*, *alr4550* and *all4499*, showed in BG11 a detectable expression. Chemoheterotrophic growth of *Anabaena* with glucose did not change the expression of *alr4550* and *all4499*, but significantly decreased the expression of *alr0834*. Upon sodium chloride treatment porin expression was unchanged in comparison to BG11, with one exception and that is the up-regulation of *alr0834* expression. In contrast potassium chloride treatment induced the expression of two additional porins (*all5191*; *all7614*) and enhanced the expression of *alr0834*, *alr4550* and *all4499*. Finally the expression of both of them *tonB1* and the porins is mostly affected by addition of potassium chloride.

Salinity is known to cause osmotic stress and oxidative stress as a secondary effect (Zhu, 2001). In order to assess if *tonB1* expression is rather controlled by oxidative stress than by outer membrane permeability changes, expression of *sodA* and *sodB* was tested (Fig.17B). The analysis showed that either of the salt stress conditions led to oxidative stress. In BG11 supplemented with sodium chloride *sodB* expression was strongly increased, while *sodA* expression was unchanged. Salt stress caused by potassium chloride resulted in enhanced transcript levels of both superoxid dismutases (Fig.17B). Since *tonB1* expression is unchanged upon sodium chloride treatment, whereby oxidative stress response of the cells could be detected, it seems unlikely that oxidative stress is controlling *tonB1* transcription.

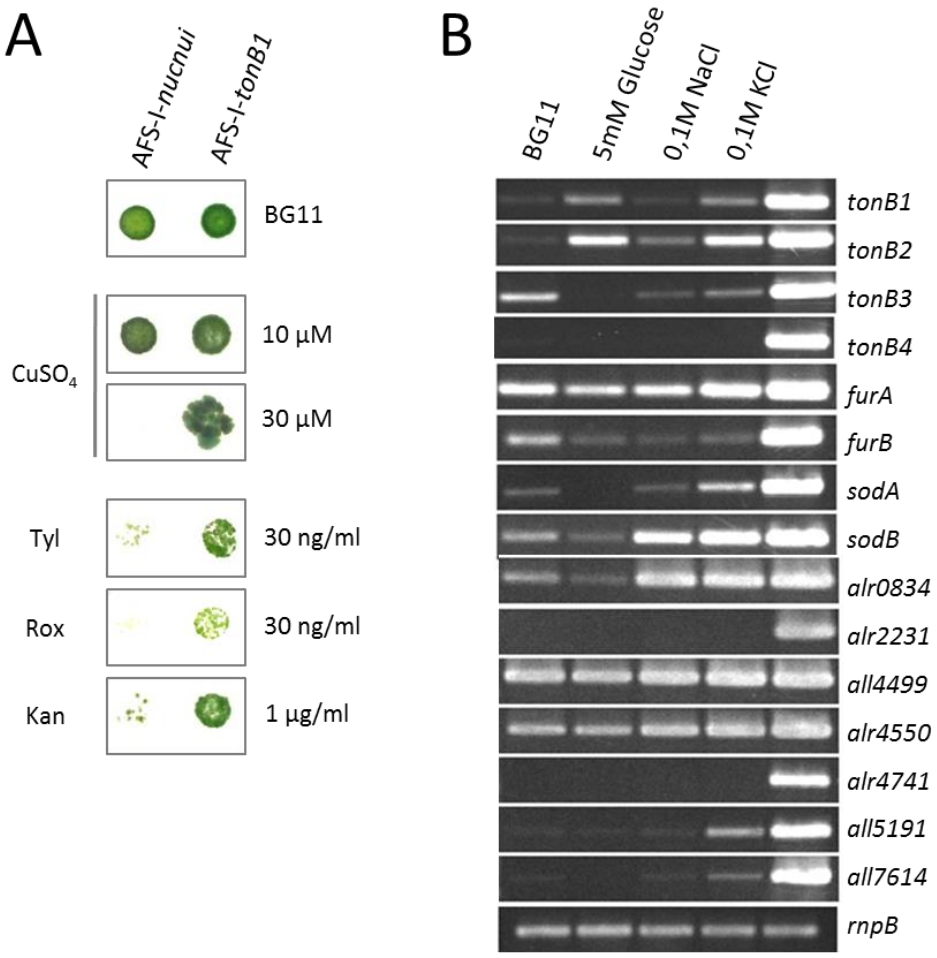

Figure 17. (A) Growth analysis of AFS-I-*nucnui* and AFS-I-*tonB1* under control condition (BG11), different copper concentrations ($CuSO_4$) and antibiotic treatment (Tyl; Rox; Kan). Concentrations are given on the right sight of the figure. Abbreviations: Tyl, tylosin; Rox, roxithromycin; Kan, kanamycin. (B) Expression analysis of the *tonB1* candidates (*tonB1-4*), transcriptional regulators (*furA*; *furB*), superoxide dismutases (*sodA*; *sodB*) and putative porins.

4.3.3 The role of TonB2 in citric acid uptake

Expression of *tonB2* in wild type *Anabaena* was noticeably up-regulated upon high concentrations of iron(III) citrate (see section 4.1.1; Fig.1). Investigation of the *tonB2* mutant showed an interesting phenotype related not only to iron, but also to citric acid (see section 4.3.1; Fig.11). Intriguingly this observation suggestes citrate as the possible reason for the increased expression of *tonB2* in BG11 medium with high iron(III) citrate concentration. This hypothesis is based on the fact that beside the high number of identified putative TBDTs in *Anabaena* no FecA homolog could be identified (Mirus et al., 2009). FecA is a class of TBDTs responsible for the transport of iron(III) dicitrate, therefore it is possible that *Anabaena* grown in the classical BG11 medium needs to secrete schizokinen to take up iron, which in turn would mean that citrate remains in the medium. To test if citrate has any effect on the expression of *tonB2* wild type was grown in altered BG11 medium, without citrate in the medium and supplied with different sources of ferric iron (Fe(III)Cl_3, Fe(III)-EDTA, Fe(III)citrate). Indeed, the expression analysis showed the highest expression in the medium supplied with iron(III) citrate (Fig.18A). To further investigate effects of the *tonB2* mutation, qRT-PCR was performed testing the expression of the iron starvation indicator gene *isiA* and expression of genes related to oxidative stress (*furB, sodA* and *sodB*; Fig.18B). Beside wild type and AFS-I-*tonB2* the mutant strain AFS-I-*tonB3* was used as a control, because this mutant was shown to be affected in siderophore-dependent iron uptake. All strains were grown in BG11 and $BG11_{-Fe.}$ Growth of the *tonB2* mutant in BG11 results in oxidative stress, as reflected by the 2-fold upregulated expression of *sodB*. In the case of the *tonB3* mutant an up-regulation of mRNA was observed for *furA* (2.3-fold), *furB* (2.5-fold) and *sodA* (2.6-fold). These data are suggesting oxidative stress in both mutant strains already in BG11, as judged from the expression of the superoxide dismutases *sodA* and *sodB* (Fig.B).

Growth in BG11$_{-Fe}$ further enhanced the oxidative stress of AFS-I-*tonB2*, as seen by further up-regulation of *sodB* expression (5-fold, respectively) and up-regulation of *furB* and *isiA* expression. TonB3 than again showed no significant changes, except for a slight up-regulation of *isiA* expression (Fig.18B).

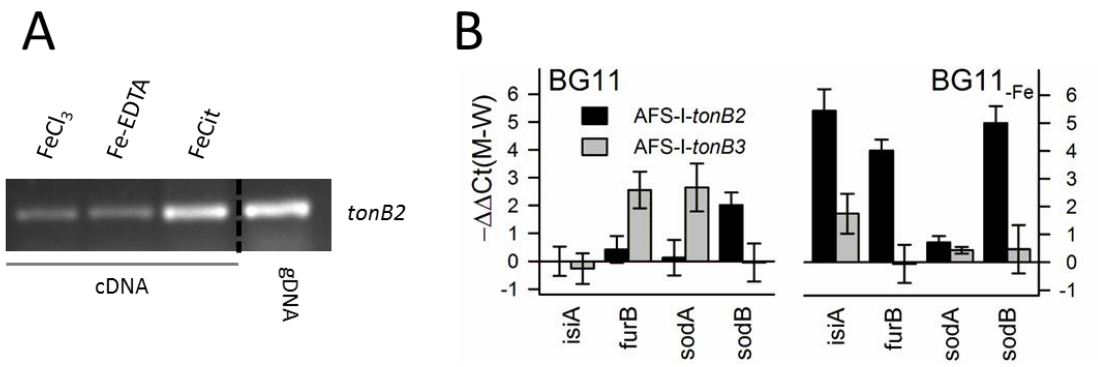

Figure 18. (A) Expression analysis of *tonB2* in wild type *Anabaena* grown with different iron sources (FeCl3, iron(III)chlorid; Fe-EDTA, iron(III)-EDTA; FeCit, iron (III)citrate). (B) qRT-PCR analysis of iron-limitation (*isiA*) and oxidative stress indicator genes (*furB*, *sodA*, *sodB*) in the *tonB2* and *tonB3* mutant strains. Data are shown relative to wild type mRNA levels and represent the average of three biological replicas.

5. Discussion

5.1 The TonB family proteins

The TonB protein is generally considered to contain three domains: (i) an N-terminal domain containing a single transmembrane helix with a conserved SXXXH motif, found to be essential for the function of TonB in *E.coli*, (ii) a periplasmic linker domain containing a proline-rich region, and (iii) a C-terminal domain which is the most well studied and best conserved portion of the TonB protein (Larsen et al., 2007; Swayne and Postle, 2011). The bioinformatic search for TonB proteins in *Anabaena* revealed four putative candidates (Stevanovic et al., 2012). However, out of this four predicted proteins only TonB3 fulfills all criteria expected from a 'classical' TonB protein. TonB3 contains the conserved specific SXXXH motif within its predicted transmembrane domain (see section 1.5; Fig.II A). Like in many other TonB-family proteins TonB3 contains a proline-enriched domain between the transmembrane domain and the C-terminal domain. Sequence comparison between the TonB protein from *E.coli* and TonB3 from *Anabaena*, showed prolonged sequences at regions of the protein that are localized in the periplasm (Stevanovic et al., 2012). This observation is further supporting the proposed TonB3 function, since TonB proteins physically interact with TBDTs in the outer membrane, and consequently need to span the periplasmic space (Kadner, 1990). Despite their overall Gram-negative structure, cyanobacteria contain a peptidoglycan layer considerably thicker than that of most Gram-negative bacteria. Unicellular strains such as *Synechococcus*, have a peptidoglycan layer of about 10 nm thickness, whereas in filamentous species the peptidoglycan layer thickness is reaching 15 to 35 nm (Gromov et al., 1986; Hoiczyk and Baumeister, 1995; Hoiczyk and Hansel, 2000). In *Anabaena* sp. PCC7120 the estimated peptidoglycan layer thickness is 14 ± 2 nm and the periplasmic space has a width of 21 ± 3 nm (Wilk et al., 2011). Therefore

compared to the periplasmic space of *E.coli*, which is approximately 15 nm thick (Leduc et al., 1989), the TonB protein in *Anabaena* needs to be larger in order to span the periplasmic space and this request can be fulfilled by TonB3. A longer periplasmic domain is not rare, as it was already observed that this region is highly variable, and can range from 22 to 283 residues (Byron et al., 2007). Sequence comparison of TonB3 with the TonB protein from *E.coli* reveals the presence of conserved TonB motifs, including the RY and the SSG motif, at the C-terminus of the protein (Stevanovic et al., 2012). Furthermore, sequence comparison of TonB3 with four other cyanobacterial TonB proteins (FdTonB from *Fremyell diplosiphon*; TonB3 from *Anabaena sp*. PCC 7120; Ava29413 from *Anabaena variabilis*; Np73102 from *Nostoc punctiforme*; Nsp9414 from *Nodularia spumigena*) shows significant sequence similarity beyond the conserved motifs and with a high number of identical residues (Pattanaik and Montgomery, 2010). All five strains belong to the same order of Nostocales, though *Fremyell diplosiphon* belongs to a different family (*Microchaetaceae*) than the other strains. Unfortunately, besides TonB3 only FdTonB has been further characterized and shown to fulfill a novel iron utilization independent function. FdTonB is proposed to participate in the photoregulation of cellular morphology during complementary chromatic adaptation (CCA; Pattanaik and Montgomery, 2010).

The remaining three putative TonB proteins identified in *Anabaena*, differ in their sequences, and all of them are missing the SXXXH motif in their transmembrane domain (see section 1.5; Fig.II A). The reason for the importance of the histidine (H20) residue, included in the SXXXH motif, remains unresolved. It was hypothesized that the protonable histidine residue is involved in shuttling protons through the TonB-complex channel (Ollis et al., 2009). Though, this hypothesis was disproved by Swayne and Postle in 2011, which showed that the replacement of histidine by asparagine (H20N) retained the TonB protein functional in *E.coli*. This observation is surprising,

as the asparagine residue is similar in bulk to the histidine side-chain, but is non-protonable. The universal importance of the histidine residue is further questioned by the observation that in *Pseudomonas aeruginosa* for instance the histidine residue-aligning to the SXXXH motif has by site-directed mutagenesis been altered and shown to be non-essential for TonB1 function (Zhao and Poole, 2002). TonB proteins without the SXXXH motif have also been characterized (annotated as TonB2) in *Vibrio cholerae* and *Vibrio anguillarum*. Interestingly the TonB2 complex in both organisms requires beside the classical ExbB and ExbD proteins an additional protein. The new complex partner is named TtpC, standing for TonB complex Transport associated Protein C (Stork et al., 2004 and 2007). The transmembrane domains of TtpC show high peptide sequence similarity to the ExbB, TolQ and MotA proteins, and based on this similarity a function of TtpC in proton channel arrangement is proposed (Germon et al., 2001; Cascales et al., 2001). In this context the presence of single standing *exbB* gen in the genome of *Anabaena* (*exbB2*, encoded by *all4587*) is noteworthy. *ExbB2* showed a constitutive expression and could theoretically be a TtpC candidate. However, *exbB2* expression was most significantly enhanced in $BG11_0$ and that speaks against a classical TonB-system component, as it was shown that *Anabaena* does not synthesize siderophores simultanesouly to nitrogen fixation (Hutchins and Rueter, 1991). Certainly the hypothesis of a TtpC-like function of ExbB2 needs further investigation.

The C-terminal domains of TonB1, TonB2 and TonB4 show only slight homology to the conserved C-terminal domain of other TonB proteins (Stevanovic et al., 2012). Nevertheless, despite the conservation of certain residues especially in the C-terminal domain of TonB proteins, Postle and colleagues (2010) reported only partial correlation between the conserved residues and the functional important residues in *E.coli*. Based on this observation a possible TonB-like function of TonB1, TonB2 and TonB4 can not be excluded solely by the missing motifs.

Possession of more than one TonB-system has been described in several species and examples are known where individual TonBs are assigned to specific functions. The TonB-systems in *Vibrio cholera* show both specific and redundant functions, as uptake of schizokinen is TonB1-dependent, while TonB2 is required for enterobactin utilization. Furthermore, both TonB-complexes are needed for heme transport, but TonB2 shows strict low osmolarity dependence for heme uptake, while TonB1 allows the use of heme at a wide range of medium osmolarities (Seliger et al., 2001). Another example are the two TonB-systems in *Actinobacillus pleuropneumoniae,* where both systems are required for iron-loaded transferrin uptake, but only *tonB2* was found to be essential for growth when the sole source of iron was hemin (Beddek et al., 2004). Some species even possess one particular TonB dedicated to a specific TBDT. Such a system is described for *Serratia marcescens,* where the TonB-like protein HasB specifically interacts with its dedicated receptor HasR for heme internalization (Létoffé et al., 2004; Izadi-Pruneyre et al., 2006). Another example is the TonB1 protein from *Caulobacter crescentus,* which is needed for the interaction with the receptor MalA for maltodextrin transport (Lohmiller et al., 2008). On the basis of the above-mentioned examples, existence of several specific TonB-systems in *Anabaena* seems thinkable.

Furthermore, it has been observed, that the extreme carboxy terminus of TonB crystallizes as a dimer (Chang et al., 2001; Sauter et al., 2003), which led to the assumption of a functional TonB dimer *in vivo*. Owing to the dimer hypothesis a heterodimeric TonB-complex in *Anabaena*, consisting of TonB3 with non-His containing TonB proteins, would be another possibility. However, a study proposing that neither of the two dimeric crystal structures represent the functional conformation of the TonB carboxy terminus *in vivo* also exists (Postle et al., 2010). Moreover, the observation that a truncated TonB C-terminal domain that forms dimers in solution was unable to interact with FhuA further supports the doubts concerning the existence of *in*

vivo dimer conformations of the TonB protein (Koedding et al., 2004). Even if TonB dimers do exist *in vivo*, it is obvious that this conformation(s) cannot be observed by crystallization, since the components known to influence TonB conformation *in vivo* are missing. These components are the proton motive force and the transmembrane domains of ExbB and ExbD (Laresen et al., 1996 and 1999; Ghosh and Postle, 2005; Ollis et al., 2009).

5.2 TonB1-a component involved in OM stability

Protein sequence comparison of TonB1 with the TonB protein from *E.coli* did not only reveal a missing SXXXH motif in the transmembrane domain (Fig.IIA), more intriguing the C-terminus of the protein did not show the conserved 'TonB-box interacting domain' (Stevanovic et al., 2012). In general TonB1 is the shortest of all four proteins, as it is only 218 aa and 23,5 kDa large. The PFAM analysis of TonB1 revealed three insignificant hits, two for domains of unknown function (PF09972, PF07129) and one for a flagella basal body-associated protein (FliL, PF03748; Stevanovic et al., 2012). In the flagellar system of *Salmonella* and *E.coli* FilL is one of the least understood proteins, as no apparent mutant phenotype associated with FilL was observed (Raha et al., 1994). The protein is localized in the cytoplasmic membrane, most likely in the vicinity of the MotA-MotB proteins (Schoenhals and Macnab, 1999). MotA and MotB form a complex in the cytoplasmic membrane and are direct components of the flagellar motor. The MotA-MotB complex is involved in proton conductance and functions as a force generating unit (reviewed by Schuster and Khan, 1994). Sequence similarity, the same transmembrane topologies and function homologies of MotA towards ExbB/TolQ and MotB towards ExbD/TolR, led to the assumption that the three systems are distantly related (Braun and Herrmann, 2004). In the case of TonB1 it seems much more plausible that the protein is a TonB- or a TolA-like protein than that it belongs to the flagellar protein family, as even motile cyanobacteria lack flagella and *Anabaena* belongs to the non-motile strains.

A Blast search with the protein (http://www.*ncbi*.nlm.nih.gov/*BLAST*), on the other hand, revealed a conserved domain with homology to the ZipA protein. In *E.coli* ZipA (Z interaction protein A) belongs to the group of proteins involved in cell division, it is recruited to the septal ring at a very early stage, where it interacts directly with FtsZ

(Hale and de Boer, 1997; Liu et al., 1999). Involvement of ZipA in cell division was demonstrated in *E.coli* by overexpression or depletion of ZipA, which in both cases led to the formation of non-septal filaments (Hale and de Boer, 1997 and 1999). Nevertheless, mutation of *tonB1* in *Anabaena* did not result in altered cell division phenotypes (Fig.15), as it was shown for *zipA* mutants in *E.coli* which elongate to form filaments (Hale and de Boer, 1997 and 1999) or for $FTN2_A$ and $FTN6_A$ mutants in *Anabaena*, which show up to 60-fold longer vegetative cells than those of the wild type strain (Koksharova and Wolk, 2002).

Based on the expression pattern of *tonB1* (Fig.1 & 6) a clear metal-dependent expression cannot be made, nevertheless, an iron-dependent expression was not observed. However, the *tonB1* mutant showed a manganese related growth phenotype (Fig.16) and insensibility towards toxic copper and manganese concentrations (Fig.17). In bacteria manganese is best understood in its role in oxidative stress responses, as it is required as a cofactor of the superoxide dismutase A (SodA) or can form non-proteinaceous complexes (Aguirre and Culotta, 2012). In cyanobacteria, moreover, it is required in the reaction centre complex of PSII, which contains a tetra-Mn cluster (Yocum and Pecoraro, 1999). It is known that high manganese concentrations inhibit growth of cyanobacteria more drastic than growth of green algae or diatoms; however, the reason for this is still unclear (Guseva, 1958). A recent study in *Bradyrhizobium japoniucum* revealed that Mn^{2+} mainly occupies Mg^{2+} binding sites in cells (Hohle and O'Brian, 2014). Copper is another essential element that is required as a cofactor for many enzymes involved in oxygen chemistry or redox reactions (Mosely et al., 2000). Cyanobacteria have various levels of copper tolerance, but have in general been shown to be particularly sensitive to copper (Brand et al., 1986). Copper toxicity is mediated by two key aspects: (i) copper can catalyse the production of ROS through the Fenton reaction and (ii) copper competes with other metals for their binding sites in proteins

(Giner-Lamia et al., 2014). Manganese and copper are both proposed to be transported by porins in bacteria, considering that in *Mycobacterium tuberculosis* the porin MspA is required for copper uptake, while in *Bradyrhizobium japonicum* a porin responsible for manganese uptake was identified (Speer et al., 2013; Hohle et al., 2011). Porins are the most abundant proteins of the bacterial outer membrane, they are essentially trimeric β-barrels forming channels mainly responsible for the outer membrane permeability to hydrophilic solutes. General or non-specific porins allow the diffusion of hydrophilic molecules and show no particular substrate specificity (Galdiero et al., 2012). Albeit, also substrate specific porins have been described, like the sucrose-specific ScrY from *Salmonella typhimurium* (Forst et al., 1998; Charbit, 2003) and the maltooligosaccharide-specific maltoporin LamB from *E.coli* (Meyer et al., 1997). Evidence that porins may function in metal uptake is nowadays available not only for manganese and copper, but also for zinc, as expression of several porins in *Pseudomonas protegens* and OprD in *Pseudomonas aeruginosa* is zinc-dependent (Lim et al., 2013; Conejo et al., 2003). Interestingly enough TolA, the TonB homolog of the Tol-system, was shown to interact with trimeric porins of *E.coli* and might be involved in the assembly of porins into the OM (Derouiche et al., 1996; Rigal et al.; 1997). The Tol-system (TolA-TolQ-TolR) shares some important features with the TonB-system, as both systems have one protein that spans the periplasm (TonB; TolA) and couples the proton motive force to the OM (Davies and Reeves; 1975; Fognini-Lefebvre et al.; 1987). While TonB and TolA have limited sequence similarity, interestingly their carboxy-terminal domains are structurally analogous, implying an evolutionary relationship of the proteins (Witty et al., 2002). Although the physiological function of the Tol-system is not well understood, it is assumed that the system is involved in maintaining the OM integrity, a notion which is derived from the hypersensitivity of the *tol* mutants to drugs and detergents (Davies and Reeves; 1975; Fognini-Lefebvre et al.;

1987). The manganese and copper related phenotypes of the *tonB1* mutant indicate an altered OM integrity, particularly pointing towards porins. In line expression of all seven putative porins was down-regulated in the *tonB1* mutant when grown in BG11 (Fig.16). Growth of AFS-I-*tonB1* in BG11 without manganese added led to a still decreased expression of four porins, while three showed a wild type-like expression (*alr2231*, *alr4741* and *all5191;* Fig.16B). Two hypotheses may be proposed: (1) TonB1 regulates expression of porins under metal sufficient conditions (BG11), and (2) signals overruling *tonB1* expression control do exist as in $BG11_{-Mn}$ three porins show wild type-like expression.

As porins control the permeability of polar solutes across the outer membrane it is reasonable that their expression needs to be regulated and adjusted to specific conditions. The best-understood model of porin expression control exists for *E.coli*. Expression of the two major porins, OmpC and OmpF, is regulated on transcriptional level by the two-component-regulatory system EnvZ-OmpR (Nara et al., 1986). EnvZ is the sensor kinase that monitors external osmolarity and modifies the activity of the response regulator OmpR by phosphorylation (Forst et al., 1989). High osmolarity leads to more phosphorylated OmpR molecules and low osmolarity results in fewer phosphorylated OmpR molecules (Slauch and Silhavy, 1989). High level of phosphorylated OmpR (high osmolarity) activates transcription of *ompC* and represses *ompF* expression (Forst et al., 1988; Mizuno and Mizushima, 1990). The divergent expression of the two porins is reasonable since OmpF forms a larger pore than OmpC (Nikaido and Rosenberg, 1983). Therefore, the smaller pore-forming porin OmpC is expressed under high osmolarity conditions, whereas under low osmolarity expression of OmpF diminishes the level of OmpC (Pratt et al., 1996). Other known factors regulating porin levels in *E.coli* include the sigma factor RpoS and non-coding antisense

RNAs (Pratt et al., 1996). In general characteristics and functions of cyanoabcterial porins are poorly understood.

The best characterized porins in cyanobacteria are SomA and SomB from the marine cyanobacterium *Synechoccocus* PCC 6301. Investigation of the two porins revealed that porins in cyanobacteria are larger (50-70 kDa) than their eubacteria counterparts (30-40 kDa). The higher molecular weight arises from a 120 aa long N-terminal domain with similarities to the surface layer homology (SLH) domain described for S-layer proteins (Hansel et al., 1998). The SLH-domain is known to connect the cell wall or external layer proteins to the peptidoglycan layer and thereby stabilize the cell wall (Lupas et al., 1994; Engelhardt and Peters, 1998). Furthermore a lower conductance of cyanobacterial porins was measured (0,5 nS SomA) in comparison to other typical Gram-negative bacterial porins (2-3,5 nS; Hansel et al., 1998). The low conductance led to the hypothesis that cyanobacteria require porins only large enough to facilitate uptake of small solutes, since organic compounds are synthesized by the bacteria themselves (Hoiczyk and Hansel, 2000). However, this hypothesis is under debate, since in *Anabaena* for instance the uptake of erythromycin (734 kDa) and ethidium bromid (394 kDa) is porin-dependent and the established permeability coefficient is comparable with the one found in proteobacteria (Hahn et al., 2012). The understanding of the regulation of porin expression is even more obscure, and only a few porins in cyanobacteria have been shown to be altered in their expression dependent on certain nutrient conditions. Recently for instance a porin (SYNW222) up-regulated during growth in zinc-depleted conditions has been identified in *Synechococcus sp*. WH810 (Barnett et al., 2014). Certainly the exact mechanism controlling the expression of the porin in *Synechococcus sp*. is not characterized. Cyanobacteria can survive in both oligotrophic and eutrophic ecosystems; moreover a number of freshwater species are able to withstand relatively high concentrations of sodium chloride (Watson et al., 1997; Salmaso, 2000; Issa et al.,

2014). In view of this kind of lifestyle a well-controlled porin expression system is needed, but remains to be characterized. Based on the decreased porin expression in the *tonB1* mutant background grown in BG11 an involvement of TonB1 in porin expression regulation seems possible. Admittedly TonB1 does not show any conserved domain of a two-component response system, like the HisKA (dimerization and phosphoacceptor) or HATPase (histidine kinase ATPase) domains. Despite everything the long cytoplasmic domain (65 aa) of TonB1 is conspicuous and for instance not seen in the TonB3 protein where the cytoplasmic part of the protein is only 10 aa long. Finally, further investigation with a focus on the cytoplasmic domain of TonB1, which could play a role in signal transduction, is needed.

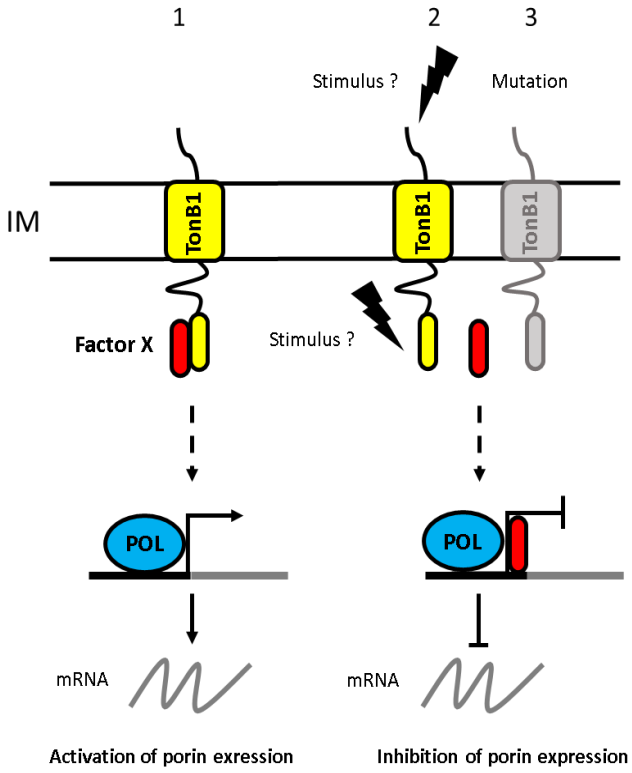

Figure III. The hypothetical TonB1 function in regulation of porin expression. (1) Under certain conditions TonB1 forms a complex with a transcriptional regulator (Factor X) and as a result the expression of porins is activated. (2) As a result of an unknown stimulus (from the periplasm or the cytosol) the Factor X is released. Subsequently the Factor X is able to bind to promoter regions of porin encoding genes and thereby to inhibit their expression. (3) The same effect as described for case 2 occurs if TonB1 is mutated (3). Abbrevation: POL, polymerase.

Growth of the *tonB1* mutant in BG11$_{-Mn}$ revealed that the repressed expression of porins observed in BG11 is overcome for *alr2231, alr4741* and *all5191* which show a wild type-like expression (Fig.16B). Under manganese starvation the two component signal

transduction system ManR-ManS is activated and controls in turn the expression of the ABC-transporter *mntBCA* (Huang and Wu, 2004). Expression analysis of these components in the *tonB1* mutant background grown in BG11 showed a slightly decreased transcript for *manS* and *mntB* (Fig.16C; dark grey bars), suggesting that the mutant is not experiencing manganese starvation. Expression of the components in the *tonB1* mutant grown in $BG11_{-Mn}$ is wild type-like (Fig.16C; light grey bars). So it may well be that under manganese starvation the expression of *alr2231, alr4741* and *all5191* is controlled by the manganese-response regulator ManR and therefore the possible regulation by tonB1 is 'overruled'.

Alternatively to a connection of the *tonB1* mutant phenotype to regulation of porin expression, the phenotype could be interpreted as a 'general stress response', since one of the consequences of a general acclimation response is the overall reduction of protein synthesis (Aldehni et al., 2003; Sauer et al., 2001). Such a general reduction of protein synthesis would explain why the ABC transporter MntABC is down-regulated in its expression, even though AFS-I-*tonB1* shows a manganese limitation related phenotype (Fig.16A & 17). Otherwise certain proteins are strongly enhanced upon a general stress response like proteins involved in oxidative stress protection (superoxide dismutases, Sod's; catalases, Cat's) and in protection of the genomic material (DNA-binding proteins from starved cells Dps; Pena et al., 1995; Aldehni et al., 2003). In *tonB1* mutant background, however, the expression of superoxide dismutases is not enhanced (Fig.16D). To the contrary transcript of the manganese containing *sodA* is decreased (Fig.16D, dark grey bar). This observation speaks on the one hand clearly against a 'general stress response' and on the other hand in favour of a manganese limitation in the mutant.

5.3 TonB2-and the citrate dilemma

The expression of *tonB2* in wild type *Anabaena* was drastically enhanced in BG11 supplemented with enhanced concentration of ammonium ferric citrate throughout all growth stages (Fig.1 & 6), indicating that *tonB2* is not a 'classical TonB' protein, which shows under this condition decreased expression, as it is the case for *tonB3* (Fig.1 & 6). In line the *tonB2* mutant showed initially no growth arrest in media without iron (Fig.11B), indicating a function not related to iron uptake. However, if additionally to iron also citric acid (citrate) was omitted from the medium a distinct impact on growth was observed (Fig.11C). Citrate is a weak iron chelator and was left out of the medium to ensure that iron is not complexed by any other chelator than by the siderophores secreted by *Anabaena*. The phenotype observed without iron and citrate was quite surprising and initially indicating towards a function strictly related to citrate. Yet, the growth of the mutant could be rescued with iron(III) chloride (Fig.12D), clearly suggesting that the phenotype is also somehow connected to iron uptake. To test for a starvation phenotype qRT-PCR of the mutant strain was performed and revealed enhanced expression of *isiA*, *sodB* and *furB* already in BG11 and strengthen in medium without iron (Fig.18B). Undoubtable a close interrelationship between iron homeostasis and oxidative stress exists, considering that PSI and PSII are greatly affected under iron limitation, due to their high demand for iron, and lead to a decreased decomposition of ROS (Lundringen et al., 1997; Michel and Pistorius, 2004). The observed strong increase of *isiA* transcript in the iron-starved *tonB2* mutant indeed indicates not only iron limitation, but also oxidative stress (Fig.18B), since it is believed that oxidative stress is the superior signal for expression of *isiA* (Michel and Pistorius, 2004). IsiA is a general photoprotector under stress conditions, as it shows an impressive capacity to dissipate excited-state energy (Berera et al., 2009). Especially the PSI-IsiA

supercomplex assembled under iron-starvation has been accurately described (Andrizhiyevskaya et al., 2002; Melkozernov et al., 2003). The light-harvesting antenna function of IsiA under iron-limitation is of particular importance, due to the fact that superoxide anion can be produced via the interaction of O_2 with the acceptor side of PSI (Asada, 2006). The third candidate showing enhanced expression in the mutant was the soluble iron-containing superoxide dismutase *sodB* (Fig.18B), which is catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (McCord and Fridovich, 1969). Owing to the suggested iron-limitation phenotype of the mutant and the need for iron as a cofactor for SodB function, the observed up-regulation of *sodB* is unexpected. Surely a post-translational regulation cannot be excluded, as in *E.coli* for instance, the small regulatory RNA *ryhB*, a master regulator of iron homeostasis, has been described to mediate *sodB* silencing under iron-limiting conditions. RyhB binds to *sodB* mRNA and inhibits the initiation of translation. The translational repression is followed by the rapid concomitant degradation of the sRNA-mRNA duplex by RNaseE (reviewed by Salvail and Massé, 2012). Since no potential equivalent of *ryhB* has been discovered in cyanobacteria, yet this kind of posttranscriptional regulation seems unlikely. Moreover, it can be assumed that if such a regulation would exist the mRNA would be degraded and not show such high mRNA levels of *sodB*. Page and colleagues (2012), on the other hand, have nicely shown that under iron-limiting conditions in the chloroplasts of *Chlamydomonas reinhardtii* the plastid FeSOD is by *de novo* synthesis preferentially retained over other abundant Fe proteins. Based on the findings that the closest bacterial homologs of plastids are cyanobacteria, a similar hierarchy of Fe distribution is conceivable, but needs further investigation (Douglas and Raven, 2003). Nonetheless, SodB expression is reasonable, since it has been shown that iron starved cells of *Anabaena* exhibit a 100-fold increase in the amount of ROS compared to non-starved cells (Latifi et al., 2005). As expression of *furB* is particularly up-regulated under

oxidative stress in *Anabaena* (López-Gomollón et al., 2009), the enhanced expression in the tonB2 mutant further substantiates oxidative stress in the mutant (Fig.18B). Other observations also point towards an involvement of FurB in oxidative stress response, since overexpression of FurB in *E.coli* prior to oxidative challenge increased cell survival (López-Gomollón et al., 2009). Furthermore, an in vitro assay with saturating concentrations of FurB showed sequence independent binding of FurB to DNA and protection thereof from cleavage caused by hydroxyl radicals and DNaseI (López-Gomollón et al., 2009). Napolitano and colleagues (2012) proposed FurB as the Zur (zinc uptake regulator) in *Anabaena*, since they showed that many of the proteins controlled by FurB are related to adaptation to zinc deficiency. However, in consideration of FurB's role in oxidative stress response, it seems as FurB is a so called 'moonlighting protein', with so far two identified functions. Taken together the enhanced expression of *sodB, isiA* and *furB* in AFS-I-*tonB2* is in line with an oxidative stress response.

5.4 The systems activated under iron-limitation

Expression analysis of wild type *Anabaena* clearly showed, that the expression of *tonB3* and the exb-system 3 (*exbB3*; *exbD3*), hereinafter referred as the *tonB3*-system, is regulated by iron availability (Fig.1), like it is proposed for the *tonB*-sytem in *E.coli*. Transcript abundance of the *tonB3*-system was up-regulated under iron-limiting conditions and even further enhanced if also copper was omitted from the medium (Fig.1). The iron containing enzyme ferredoxin can be replaced by the functionally equivalent copper-containing protein flavodoxin under iron limitation (LaRoche et al., 1995; Doucette et al., 1996), therefore copper can compensate to a certain extent iron limitation. Furthermore, the existence of a schizokinen-independent but copper-dependent iron uptake system was proposed in *Anabaena* (Nicolaisen et al., 2010). Beside the expression under iron-limitation the system showed also the expected expression pattern under control conditions, as for instance high iron and high copper concentrations (Fig.1). Schizokinen the siderophore secreted by *Anabaena* is moderating copper-toxicity, since it is able to complex copper but the resulting complex cannot be taken up by the cells (Clarke et al., 1987). However, this advantageous effect of schizokinen is considered to be coincidental, as cultures supplemented with sufficient iron but high copper concentrations are sensitive to copper, implying that repressed siderophore synthesis by iron cannot be overcome by high copper concentrations (Clarke et al., 1987). Therefore the expression profile of the *tonB3*-system under high copper concentrations fits perfectly to the expected pattern. The strong up-regulation of the system under high copper and limiting iron conditions (-Fe/++Cu; Fig.1), is a consequence of schizokinen molecules complexed with copper which are therefore not anymore available for iron uptake. Even though the *tonB3*-system shows clear expression controlled by iron-availability, the nature of the regulator(s) remains unknown in *Anabaena*. Three Fur homologs have been identified in *Anabaena* and the

protein FurA is the master regulator of iron homeostasis (Hernández et al., 2004; González et al., 2012). FurA regulated genes involved in iron metabolism comprise many TBDTs (González et al., 2014), but neither *tonB3* nor the *exb3*-system are among them. Nonetheless, the down-regulation of the *tonB3*-system expression under elevated iron concentrations and the activation of expression under iron limitation perfectly fit the criteria for Fur-regulated systems, since FurA typically acts as a repressor, which is sensing intracellular free iron and modulating transcription in response to iron availability (Bagg and Neilands, 1987; De Lorenzo et al., 1987). In conclusion expression of the *tonB3*-system in *Anabaena* is modulated by iron availability, albeit the transcriptional regulator(s) of the *tonB3*-system remain to be elucidated.

Segregation analysis of the *tonB3* single recombinant mutant (AFS-I-*tonB3*) demonstrated the essential nature of the gen, since the mutant could not be fully segregated in BG11 (Fig.7B). The mutant of *exbB3,* on the other hand, could be fully segregated (Fig.7A). Held and Postle discovered in 2002 two important facts about ExbB and ExbD function: (i) both ExbB and ExbD are required for TonB to conformationally respond to proton motive force, and (ii) both ExbB and ExbD are also needed for association of TonB with the cytoplasmic membrane. The full segregation of the *exbB3* mutant in *Anabaena* can, according to the findings by Held and Postle that ExbB and ExbD are equally essential as it is the case for the TonB protein, only be explained by complementation of ExbB3 by another protein. To take this observation as an indirect proof for the existence of a second ExbB protein in *Anabaena* has to be considered with caution, since cross-complementation of ExbB-ExbD with TolQ-TolR, partner proteins of the Tol-system, is possible. ExbB and ExbD have ~60 % sequence similarity with TolQ and TolR, respectively (Braun and Herrmann, 2004). This sequence similarity and the most likely similar function are the reasons for observed cross-complementation of the systems, as it was demonstrated, that *exbB*-*exbD* mutants

can be partially complemented by *tolQ-tolR* and vice versa (Braun and Herrmann, 1993). Thus ExbB3 in *Anabaena* could theoretically be complemented by a TolQ protein and due to the high sequence similarity it is hard to distinguish ExbB-ExbD proteins from TolQ-TolR proteins.

The CAS-plate assay in standard BG11 and BG11 without iron and copper added showed that both mutants, AFS-I-tonB3 and –I-exbB3, secrete more siderophores than the wild type (Fig.9). However the siderophore secretion by the *exbB3* mutant is not as by the AFS-I-*tonB3* mutant (Fig.9C). Growth of AFS-I-*tonB3* was altered in BG11 not supplemented with iron (Fig.11B), but the growth could be restored by iron(III) citrate resupply (Fig. 12B), indicating a TonB3-independent iron uptake. However survival of the AFS-I-tonB3 mutant on elevated iron(III) citrate concentrations (10x; Fig.14), which proofed to be toxic for the wild type substitution AFS-I-*nucnui*, imply altered iron uptake ability.

Out of the many predicted binding protein-dependent ABC-transporter in *Anabaena*, only the *fhu*-system revealed to be clearly regulated by iron availability (Fig.4 & 5A). Actually González and colleagues recently validated (2014), that the *fhu*-system belongs to the group of FurA-regulated genes in *Anabaena*. Despite the regulation by FurA, the expression of the *fhu*-system seems also to be substrate-dependent, since its expression could not be detected in mutants of *hgdD*, *schT*, *exbB3*, and *tonB3* (Fig.8). All of these mutants are affected in different steps of the siderophore-dependent iron uptake, but what they have in common is the consequence that the iron-siderophore complexes cannot cross the outer membrane anymore. A similar substrate-dependent induction is known for the FecABCD system in *E.coli*, where binding of ferric dicitrate to the TBDT (FecA) triggers a signal transduction cascade, regulated by alternative sigma factors, which leads to the transcription of the *fecABCD* operon (Van Hove et al., 1990). The Fec-system is also additionally Fur-regulated and repressed by intracellular iron, which

shows that the regulation by Fur does not exclude substrate-dependent regulation (Hussein et al., 1981; Zimmermann et al., 1984). Strikingly despite the high number of TBDT encoding genes in *Anabaena* no FecA homolog has been identified (Mirus et al., 2009). Though the FecBCD system has been identified and according to CyanoBase (http://bacteria.kazusa.or.jp/cyanobase) putative FecI (*alr3280*) and FecR (*all3916, alr4739, all2198*) homologues do exist, though the function needs experimental verification. Another possible regulation in addition to FurA includes two-component sensory transduction systems, like the PfeR-PfeS system of *P.aeruginosa* that induces the PfeA Fe-enterobactin receptor (TBDT) by sensing periplasmic Fe-enterobactin (Dean et al., 1996). A functional genome approach from 2002 (Wang et al.) showed that in *Anabaena* 211 genes encode two-component signalling elements, and 66 genes encode Ser/Thr kinases and phosphateses. Taken together these genes represent 4,2 % of the coding capacity of the whole genome, making *Anabaena* a leading member among prokaryotes in terms of its signalling potential. Since only few of the signalling proteins-most of them related to heterocyst differentiation-have been studied so far in *Anabaena*, it can be assumed that understanding of the signalling network is just starting to emerge (Liang et al., 1992; Zhang, 1993). Considering the importance of iron uptake especially for cyanobacteria, as well as the need for control of the uptake in terms of oxidative stress prevention, it can be assumed that some of these signalling proteins will be related to iron homeostasis.

Another interesting characteristic of the *fhu*-system is the operon organisation. By overlapping RT-PCR intergenic regions, indicating one polycistronic transcript, could only be observed under iron and copper limitation (Fig.5C). Whereas in BG11 three individual transcripts exist (Fig.5C), which strongly imply the existence of internal promoters of the individual genes in the *fhu*-system. Surprisingly, conditions exist under which the expression of these genes is differently regulated, for instance under high iron

concentrations where *fhuC* is transcribed while the expression of *fhuB-fhuD* is down-regulated (Fig.5A). This observation suggests activation of *fhuC* transcription by a stimulus which keeps *fhuB-fhuD* expression repressed.

A possible reason for independent expression of *fhuC* could be assembly with other ABC-transporter subunits than FhuBD. As the ATP-binding proteins are the components which are conserved the most among all ABC transporter components, it is likely that the structural features and the mechanisms of energetization is very similar in all these systems (Koester, 2001). Furthermore, it is assumed that ATP-binding subunits do not participate directly in substrate recognition, since binding protein dependent ABC transporter initially recognize their substrates by binding to the periplasmic binding protein, making an ATP-binding protein subunit interchange even more achievable (Schneider and Hunke, 1998). Consistent with the notions are the findings by Hekstra and Tommassen (1993), that MalK and UgpC from *E.coli*, both ATP-binding subunits involved in transport of chemically different substrates, are exchangeable. Beyond that it was shown that the LacK protein of *Agrobacterium radiobacter* can substitute for MalK in *S. typhimurium* and *E.coli* (Wilken et al., 1996). Finally Speziali and colleagues showed in 2006 that in *Staphylococcus aureus* the FhuC protein functions with FhuB, FhuG, and FhuD1/FhuD2 to transport iron(III)-hydroxamates and is the "genetically unlinked" ABC-ATPase that functions with SirA, SirB, and SirC to transport iron(III)-staphylobactin. Although this hypothesis in case of *Anabaena* needs further investigation, it would explain the more drastic siderophore secretion phenotype of the *fhuC* mutant in comparison to the fhuB- and fhuD-mutants (Fig.9C), because in this case a mutation of *fhuC* would not only affect the *fhu*-system, but also other systems.

The *fhuD* mutant shows a less pronounced siderophore secretion phenotype (Fig.9C), which can be explained by the observation made by Mirus and colleagues in 2009, that

in the genome of *Anabaena* many scattered periplasmic substrate binding proteins are annotated. Conspicuously, 13 of these genes are encoded downstream of an *fhuA*-like encoding gene, which is raising the chances that these proteins are actually functional, as all of the *fhuA* homologous in *Anabaena* are expressed. Many of the periplasmic substrate-binding proteins are annotated as dicitrate-binding proteins. Though the identification of a particular system solely based on genome sequence analysis should be taken cautiously, and the function of these proteins needs to be estimated based on experimental studies. In the *fhuB* mutant, on the other hand, siderophore secretion was decreased compared to wild type levels (Fig.9C). The simplest explanation would be that the mutant is not exposed to iron limitation. Albeit, *isiA* expression was increased in the *fhuB* mutant (Fig.9A), which indicats iron limitation. Alternatively, siderophore secretion could be influenced caused by the iron-loaded FhuD protein in the periplasmic space. This, however, is somewhat speculative and remains to be investigated in future studies.

Summerising out of several predicted *tonB*, *exb*-system and BP-dependent encoding genes, only the *tonB3*, *exbB3*, *exbD3* and the *fhu*-like genes showed a particularly enhanced expression under iron limitation. Their expression profile under control conditions, such as $BG11_0$ or enhanced iron concentrations, further strengthened their possible role in siderophore-dependent iron uptake. Mutant characterization revealed iron starvation phenotypes, even though not similarly strong for every mutant. Despite the high number of possible TBDTs it can be assumed that only one main TonB protein (TonB3) operates under iron-limiting conditions.

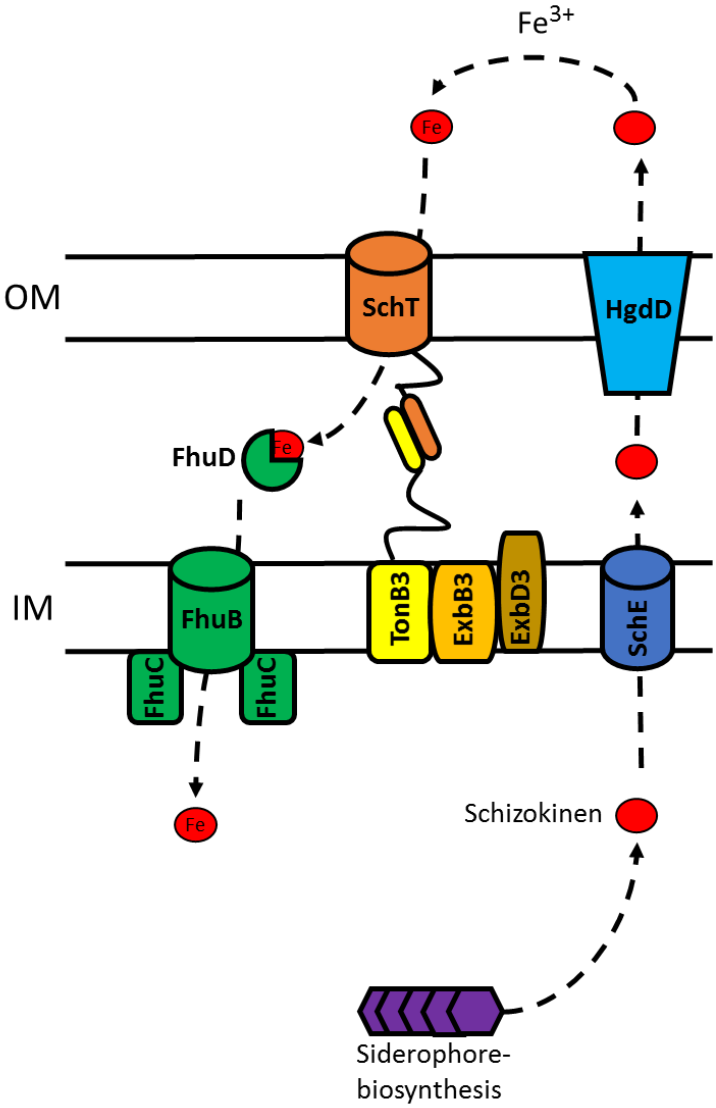

Figure IV. The schizokinen-dependent iron uptake system in *Anabaena*. Under iron-limitation schizokinen is synthesiszed and transported by the MFS-type schizokinen exporter (SchE) over the inner membrane (IM). Transport over the outer membrane (OM) is accomplished by the TolC (HgdD) protein. The ferric-schizokinen complex is transported by the schizokinen transporter (SchT; TBDT) over the outer membrane. Transport is energyzed by the TonB-system 3 (TonB3-ExbB3-ExbD3). Once the ferric-schizokinen complex has reached the periplasm it is bound by the periplasmic binding protein FhuD and transported to the permease FhuB. The ATPase FhuC couples ATP binding and the energy of ATP hydrolysis to the transport of ferric-schizokinen. It is suggested that the whole FhuD-ferric-siderophore complex is transported into the cytosol, where ferric-iron is reduced by a reductase and by this released from the complex.

Future directions

In terms of the ‘classical’ TonB-system the following study was restricted to the siderophore secreted by *Anabaena* (schizokinen) and revealed TonB3 as the main TonB protein functioning under iron starvation. Further analysis should be extended to different siderophore classes. In line a substrate-induced expression of the remaining TonB-system should be analysed. To confirm the function of TonB3 uptake experiments with radioactively labelled iron should be performed.

In the case of TonB1 the most likely regulatory function of the protein needs further investigation. Here the cytoplasmic domain of the protein is particularly interesting as a possible involvement in signal transduction needs to be examined. Generation of a truncated *tonB1* mutant, missing the cytoplasmic region of the protein could be generated and analysed with respect to porin expression.

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