Microbiology (2007), **153**, 601–607 DOI 10.1099/mic.0.2006/002535-0

FraG is necessary for filament integrity and heterocyst maturation in the cyanobacterium *Anabaena* sp. strain PCC 7120

Asha S. Nayar, Hiroshi Yamaura, Ramya Rajagopalan, Douglas D. Risser and Sean M. Callahan

Department of Microbiology, University of Hawaii, Honolulu, HI 96822, USA

Correspondence
Sean M. Callahan
scallaha@hawaii.edu

Received 15 September 2006
Revised 18 October 2006
Accepted 20 October 2006

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium that differentiates nitrogen-fixing heterocysts when fixed nitrogen becomes growth limiting in the medium. The gene *alr2338* (designated *fraG* herein), located immediately upstream of the master regulator of differentiation *hetR*, was identified in a genetic screen for mutants unable to grow diazotrophically. Filaments with a mutation in *fraG* were unable to fix nitrogen or synthesize heterocyst-specific glycolipids, and they fragmented initially to approximately nine cells in length at 24 h after induction of heterocyst development and eventually became unicellular. The fragmentation phenotype could be duplicated in the presence of fixed nitrogen when differentiation of heterocysts was elicited by overexpression of *hetR*, suggesting that a defect in differentiation, and not a lack of fixed nitrogen in the medium, was the more direct cause of fragmentation. An intact *fraG* gene was necessary for differentiation of mature heterocysts, but was not required for proper pattern formation, as indicated by a normal pattern of expression of *hetR* in a *fraG* mutant. A transcriptional GFP reporter fusion indicated that the level of expression of *fraG* was low in vegetative cells in both nitrogen-replete and nitrogen-free media, and was induced in heterocysts. *fraG* appears to play a role in filament integrity and differentiation of proheterocysts into mature heterocysts.

INTRODUCTION

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium capable of fixing dinitrogen to ammonium. In the presence of a source of fixed nitrogen, such as nitrate, nitrite or ammonium, the cells in the filament are undifferentiated vegetative cells that carry out oxygenic photosynthesis. In the absence of fixed nitrogen, vegetative cells differentiate into nitrogen-fixing heterocysts at semi-regular intervals along the filament. The presence of two types of cells arranged in a 1D pattern makes *Anabaena* sp. strain PCC 7120 one of the simplest, as well as evolutionarily oldest, examples of multicellular pattern formation in developmental biology. The dedication of heterocysts to a single purpose, supplying the filament with fixed nitrogen, makes them an interesting system for the study of the 'ideal' cellular conditions that have evolved over billions of years for nitrogen fixation.

Heterocysts produced by *Anabaena* sp. strain PCC 7120 can be distinguished from vegetative cells microscopically by their larger size and thicker cell envelope. In order to create microaerophilic conditions for the activity of nitrogenase, they have two additional layers of envelope made of polysaccharides and glycolipids (Murry & Wolk, 1989). The glycolipid layer, which is the innermost of the two, is thought to act as a barrier against the entry of oxygen (Walsby, 1985; Winkenbach *et al.*, 1972). The exterior polysaccharide layer is thought to preserve the integrity of the glycolipid layer. Genes necessary for the production and localization of both layers have been found, and pathways for their synthesis have been proposed (Fan *et al.*, 2005; Huang *et al.*, 2005). Once a micro-oxic environment has been created inside the heterocysts, they fix atmospheric nitrogen, and transport it to vegetative cells, and in return they receive a source of reductant required for fixation from vegetative cells (Thomas *et al.*, 1977).

The pattern of heterocysts along a filament is determined by the interplay of positive- and negative-acting regulatory factors, and approximately 12 h after the removal of fixed nitrogen, selected cells have committed to terminal differentiation into heterocysts (Meeks & Elhai, 2002; Wolk, 2000; Zhang *et al.*, 2006). PatS and HetR appear to be the two central factors that control differentiation and pattern formation. HetR is the master regulator and has both DNA-binding and protease activity (Buikema & Haselkorn, 1991b; Huang *et al.*, 2004; Zhou *et al.*, 1998). It displays positive autoregulation, and expression of *hetR* is induced in proheterocysts prior to commitment to differentiation (Black *et al.*, 1993).

In order to identify the genes involved in diazotrophic growth and differentiation of heterocysts by *Anabaena* sp. strain PCC 7120, a genetic screen was conducted to isolate mutants incapable of growth in the absence of fixed nitrogen. Interruption of the coding region of *fraG*, the gene upstream of *hetR*, by a transposon resulted in a fragmentation mutant that was unable to grow in the absence of a fixed source of nitrogen. The predicted protein is similar to permeases and is necessary for filament integrity and maturation of heterocysts to the point of glycolipid layer formation.

METHODS

Strains and culture conditions. Table 1 describes the *Anabaena* strains, plasmids and oligonucleotides used in this study. The wild-type strain *Anabaena* sp. strain PCC 7120, and its derivatives, were grown in BG-11 medium, and for induction of heterocysts they were transferred to BG-11_0 medium as described previously (Borthakur *et al.*, 2005). Transposon mutagenesis, screening for mutants unable to grow diazotrophically and recovery of the transposon insertion site were performed as described by Wolk *et al.* (1991), except that spectinomycin and streptomycin, each at a concentration of 2.5 μg ml^{-1}, were used to select for cells in which the transposon had transposed into the genome of *Anabaena* sp. strain PCC 7120.

Table 1. *Anabaena* strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Characteristic(s)	Source
***Anabaena* strains**		
PCC 7120	Wild-type	Pasteur Culture Collection
UHM103	Δ*hetR*	Borthakur *et al.* (2005)
UHM127	Δ*fraG*	This study
Plasmids		
pAN120	Suicide plasmid to delete *fraG*	This study
pAN116	Shuttle vector carrying P_{fraG}–*gfp* fusion	This study
pAN129	Shuttle vector carrying P_{fraG}–*gfp* fusion using larger region of DNA upstream of *fraG*	This study
pSMC127	Shuttle vector carrying P_{hetR}–*gfp* fusion	Callahan & Buikema (2001)
pDR138	Shuttle vector carrying *hetR* under the control of its native promoter	This study
pPetHetR	Shuttle vector carrying P_{petE}–*hetR* for copper-inducible expression of *hetR*	Buikema & Haselkorn (2001)
pAN130	Shuttle vector carrying *fraG* under the control of its native promoter	This study
pHY101	pBluescript SK(+) containing Ω cassette conferring resistance to Sm and Sp	This study
pRR106	Suicide plasmid with transposon, Sp^r Sm^r	This study
pRL278	Mobilizable suicide vector	Black *et al.* (1993)
pAM505	Mobilizable shuttle vector	Wei *et al.* (1994)
pAM1956	pAM505 with promoterless *gfp*	Yoon & Golden (2001)
pGEMT	Cloning vector	Promega
pBluescript SK(+)	Cloning vector	Stratagene
Oligonucleotide	**Sequence (5′–3′)***	
alr2338 UF	**CTCGAG**GAGAAGCGACCAGACAACGACCC	
alr2338 UR	**ATCGAT**GTTACTGGTGTTGATTTCTCAGGAG	
alr2338 DF	**CTGCAG**GGTGGTTGTACCACTAATGATAC	
alr2338 DR	**ACTAGT**GCCAATACCAAAAGCAATTGCCC	
alr2338promoterUp	**GAGCTC**GTTGCCAAGTATCCAATTGCAGAAC	
UpPalr2338SacIA	AATG**GAGCTC**GTTCTGAAATATGAGTTATGGCTGG	
alr2338promoterDown	**GGTACC**CACCTTGACGATACAGCTGCCGC	
Rev 2338	CAGT**GGTACC**CACCTTGACGATACAGCTGCCGC	
alr2338 end KpnI	TTAA**GGTACC**GCCATTATGACTACTGAGCCAGAAG	
hetRcf-Nde1	**CATATG**AGTAACGACATCGATCTGATC	
hetR6H-r	TTAGTGATGGTGATGGTGATGATCTTCTTTTCTACCAAACACCATTTG	
PhetR-KpnI-F	**GGTACC**CCTGCCAATGCAGAAGGTTAAAC	
PhetR-NdeI-R	**CATATG**ACAAATAGTTGAATAGCAGCGTTATTAG	

*Nucleotides in bold indicate a restriction site engineered into the sequence.

To determine mean filament lengths, the number of cells in 125 filaments was determined microscopically and averaged. Growth of *Escherichia coli*, and concentrations of antibiotics were as previously described (Borthakur *et al.*, 2005).

Plasmid and mutant constructions. The transposon used in this study was a derivative of Tn*1058* constructed by Wolk *et al.* (1991). pRL1058, which bears the original transposon on a suicide plasmid, was digested with *Msl*I to remove genes conferring resistance to kanamycin, bleomycin and streptomycin. An Ω cassette conferring resistance to streptomycin and spectinomycin was inserted as a *Sma*I fragment from pDW9 (Golden & Wiest, 1988) to create pRR106, which was used to mutagenize *Anabaena* sp. strain PCC 7120.

pAN120 is the suicide plasmid that was used to replace a 592 bp internal fragment of *fraG* with an Ω cassette conferring resistance to streptomycin and spectinomycin. A region of DNA corresponding to nucleotides +12 to +817, relative to the start of *fraG*, was amplified using the primers alr2338 UF and alr2338 UR, and a downstream region of *fraG* corresponding to nucleotides +1409 to +2213 was amplified using the primers alr2338 DF and alr2338 DR. The two fragments were cloned into pGEM-T (Promega) and then moved into pHY101, which consists of pBluescript SK(+) (Stratagene) with an Ω cassette conferring resistance to streptomycin and spectinomycin (Fellay *et al.*, 1987) in the *Hin*dIII site, on either side of the Ω cassette. A fragment consisting of the upstream and downstream regions of *fraG* flanking the Ω cassette was moved into pRL278 (Black *et al.*, 1993) using *Xho*I and *Spe*I. The *fraG* mutant UHM127 was created using plasmid pAN120, as previously described (Orozco *et al.*, 2006).

Two GFP transcriptional fusions were made, resulting in plasmid pAN116, which contains nucleotides located at −352 to −19 relative to the translational start site of *fraG*, and pAN129, which contains nucleotides located at −760 to −19. Plasmid pAN116 was constructed by amplifying the promoter region of *fraG* using the primers alr2338promoterUp and alr2338promoterDown, and cloning into pGEM-T. The fragment was then moved to pAM1956 (Yoon & Golden, 2001) using *Sac*I and *Kpn*I. For plasmid pAN129, a larger region upstream of *fraG* was amplified using primers UpPalr2338SacIA and Rev 2338. This region was cloned directly into pAM1956 by using *Sac*I and *Kpn*I. Results with both plasmids were identical. To complement the Δ*fraG* mutant, pAN130 was constructed. Plasmid pAN130 contains the promoter and coding region of *fraG*. This region was amplified using UpPalr2338SacIA and alr2338 end KpnI, and was moved into pAM505 (Wei *et al.*, 1994) by using the restriction sites introduced by the primers.

Plasmid pDR138 was used to express *hetR* from the native *hetR* promoter. A 846 bp fragment upstream of the *hetR* coding region was amplified via PCR using primers PhetR-KpnI-F and PhetR-NdeI-R, and cloned into pGEM-T. P_{hetR} was subsequently cloned into pBluescript as an *Apa*I–*Pst*I fragment to create pDR133. *hetR* was amplified from genomic DNA using primers hetRcf-NdeI and hetR6H-r, cloned into pGEM-T and then moved to pDR133 as an *Nde*I–*Pst*I fragment by using an *Nde*I site introduced by one of the primers. The subsequent P_{hetR}–*hetR* fragment was cloned into pAM504 to create pDR138.

Acetylene reduction, glycolipid and exopolysaccharide assays. For acetylene reduction assays, strains were grown in BG-11 medium to exponential phase. The cultures were then induced by transferring to BG-11_0 and grown under standard conditions for 120 h. Reduction of acetylene was measured immediately after nitrogen step-down, and every 24 h thereafter, using a gas chromatograph, as previously described (Borthakur *et al.*, 2005). Reported values of acetylene reduction are means of measurements from three independent cultures. TLC and staining of exopolysaccharides were performed as previously described (Callahan & Buikema, 2001; Nichols & Wood, 1968).

Microscopy. Cells were viewed through a Nikon Diaphot 300 inverted microscope, using either a ×60 oil or a ×40 objective, and images were captured with an Olympus DP70 digital camera. For fluorescent images, a Chroma Technologies 41001 filter set, with an excitation of 480±20 nm and an emission of 535±25 nm, was used to monitor fluorescence specific to the GFP. Images were processed in Adobe Photoshop version 7.0.

RESULTS

fraG is necessary for diazotrophic growth and filament integrity

In a screen for mutants that could not grow in the absence of a source of fixed nitrogen, a transposon used to increase the rate of mutagenesis, and mark the site of the mutation, had inserted into the same gene in four of the mutants isolated. The gene designated *alr2338* during annotation of the genome (Kaneko *et al.*, 2001), and named *fraG* (fragmentation, glycolipid) by ourselves, was interrupted at nucleotide positions +124, +158, +176 and +1770 relative to the predicted translational start site. To confirm a cause-and-effect relationship between the insertions and the mutants' inability to grow diazotrophically, an internal region of *fraG* in the wild-type strain *Anabaena* sp. strain PCC 7120 was replaced by an Ω cassette. The resulting strain UHM127 was unable to grow diazotrophically. Because *fraG* is located immediately upstream of *hetR*, which is the master regulator for heterocyst differentiation, *hetR* and its promoter region were introduced into the mutant on a shuttle vector to see if the phenotype was the result of a polar effect on *hetR*. The wild-type phenotype was not restored to the mutant by the addition of *hetR*, whereas addition of a wild-type copy of *fraG* restored the wild-type phenotype, indicating that the phenotype of UHM127 was caused by inactivation of *fraG*.

The gene *fraG* encodes a putative protein of 751 aa. One complete and one partial DUF6 domain were detected at the carboxy-terminal end of the protein. DUF6 domains are indicative of integral membrane proteins and it is common for proteins to contain two of these domains. The carboxy-terminal half of the protein is predicted to contain 10 α-helical transmembrane domains (Marchler-Bauer *et al.*, 2003).

The rate of growth of the mutant strain UHM127 was similar to that of the wild-type in medium containing nitrate as a fixed nitrogen source. However, mean filament length was substantially reduced to about 50 cells, compared with more than 200 cells for the wild-type. Unlike the wild-type, which forms a pattern of single heterocysts along the filament (Fig. 1a), UHM127 fragmented when deprived of fixed nitrogen. Fragmentation started between 16 and 18 h after nitrogen deprivation, and at 24 h, single cells and filaments with a mean filament length of about nine cells were present together (Fig. 1b), compared with intact filaments with a mean length of 150 cells for the wild-type.

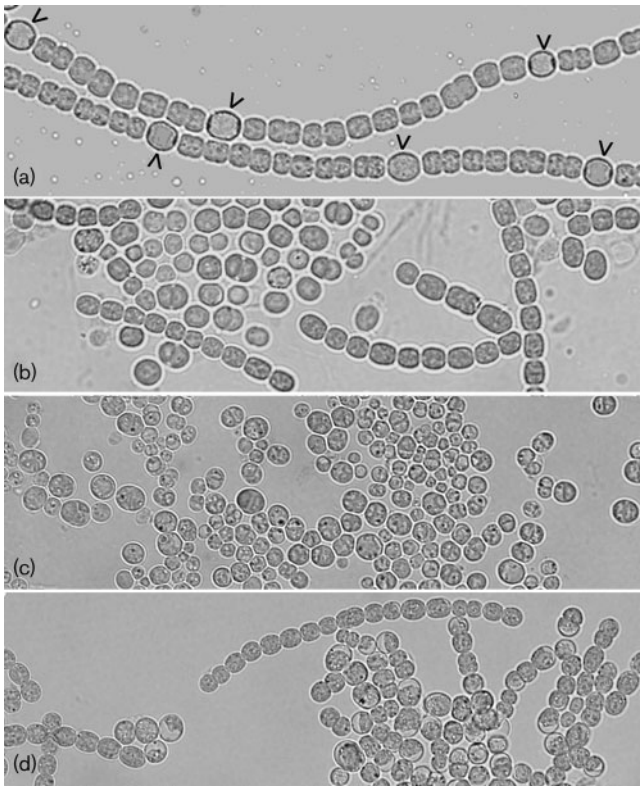

Fig. 1. Phenotype of the *fraG* mutant, UHM127. (a) The wild-type strain *Anabaena* sp. strain PCC 7120 at 24 h post-induction. (b) UHM127 at 24 h post-induction. (c) UHM127 at 72 h post-induction. (d) UHM127, carrying a P_{petE}–*hetR* fusion on plasmid pDR138, at 24 h after transfer from BG-11 lacking copper to BG-11 containing 300 nM $CuSO_4$. Carets indicate heterocysts.

The single cells in the mutant culture were less pigmented than the cells in the filaments, reminiscent of the reduced pigmentation in heterocysts caused by the degradation of phycobilisomes. After 48 h nitrogen deprivation, the mean number of cells for the mutant was five, and for the wild-type it was 145. At 72 h and thereafter, the mutant culture was mostly unicellular (Fig. 1c). No mature heterocysts were observed in the mutant culture at any time.

To determine if UHM127 was a Fox^- (unable to fix in the presence of molecular oxygen) or a Fix^- (unable to fix under any conditions) mutant, acetylene reduction assays were performed. The wild-type reduced acetylene in the presence and absence of oxygen. A *pbp6* mutant (A. S. Nayar and S. M. Callahan, unpublished data), which is known to have a Fox^- phenotype (Leganés *et al.*, 2005), was used as a control for anaerobic conditions and could only fix in the absence of oxygen as expected; UHM127 could not fix under either condition. At 48 h post-induction, rates of acetylene reduction for the wild-type under aerobic and anaerobic conditions, and for the *pbp6* mutant, were 91, 392 and 86 nmol ethylene h^{-1} ml^{-1} $(OD_{750}$ unit$)^{-1}$, respectively. UHM127 was therefore categorized as a Fix^- mutant with developmental defects.

Fragmentation can be elicited by differentiation alone

Deprivation of a fixed source of nitrogen induces a developmental programme in *Anabaena* sp. strain PCC 7120 that culminates in the formation of a pattern of heterocysts along a filament. To examine whether deprivation of fixed nitrogen, or induction of differentiation, was the more direct cause of fragmentation of the mutant strain UHM127, differentiation was induced in medium containing fixed nitrogen and the phenotype of the mutant was observed. Extra copies of *hetR* under the control of the copper-inducible *petE* promoter on plasmid pPetHetR cause differentiation of heterocysts in the wild-type strain in the presence of nitrate or ammonia when copper is present in the medium (Buikema & Haselkorn, 2001). UHM127 carrying pPetHetR, in the presence of nitrate and absence of copper, had filaments similar to the same strain without the plasmid. However, when copper was included in the medium to induce expression of *hetR* and heterocyst formation, the strain fragmented in a manner similar to the mutant strain in a medium lacking fixed nitrogen at 24 h post-induction (Fig. 1d). Fragmentation in the presence of fixed nitrogen when differentiation was induced by over-expression of *hetR* suggested that differentiation, and not a lack of fixed nitrogen in the medium, was the more direct cause of fragmentation by the mutant.

fraG is not necessary for pattern formation

The absence of mature heterocysts in strain UHM127 indicated that *fraG* was necessary for complete differentiation of heterocysts. To determine whether inactivation of *fraG* affects the formation of the heterocyst pattern, a *hetR–gfp* transcriptional fusion on plasmid pSMC127 (Callahan & Buikema, 2001) was introduced on a shuttle vector into both the mutant and the wild-type. In the wild-type, a pattern of expression of *hetR* in single cells that predicted the pattern of cells that differentiated into heterocysts could be visualized with the GFP fusion between approximately 8 and 24 h after removal of combined nitrogen. In UHM127, a pattern of GFP fluorescence was seen in proheterocysts 12 h after induction, before fragmentation started in the mutant (Fig. 2a, b). A pattern of induction of the *hetR* promoter in single cells separated by approximately 10 cells with lower fluorescence, and similar to that in the wild-type, was seen in the mutant, and this suggested that *fraG* was not necessary for proper pattern formation.

Expression of *fraG* is enhanced in heterocysts

To determine the temporal and spatial aspects of expression of *fraG* under different culture conditions, the promoter region of *fraG* was fused to *gfp* and the construct was introduced into the wild-type strain on a shuttle vector. With nitrate in the medium, a low level of fluorescence was observed in all vegetative cells of filaments. The level of fluorescence was significantly higher than that of the same strain carrying a promoterless version of the construct,

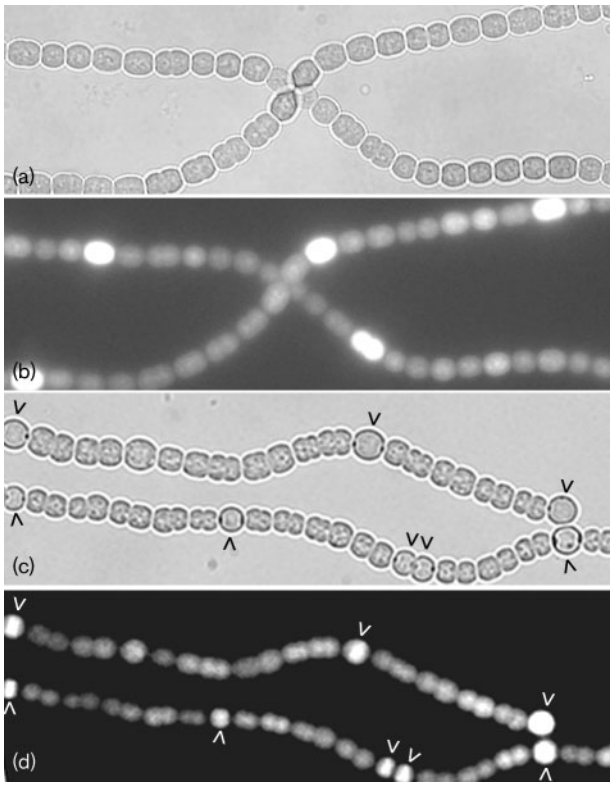

Fig. 2. Patterned transcription from the *hetR* promoter in a *fraG* mutant, and induction of expression of *fraG* in heterocysts. The *fraG* mutant strain UHM127 carrying a P_{hetR}–*gfp* fusion on plasmid pSMC127 at 12 h after induction of heterocyst formation was photographed under visible light (a) and ultraviolet light (b). *Anabaena* sp. strain PCC 7120 carrying a P_{fraG}–*gfp* transcriptional fusion on plasmid pAN116 at 48 h after induction of heterocyst formation was photographed under visible light (c) and ultraviolet light (d). Carets indicate heterocysts.

indicating that levels of expression from the *fraG* promoter were at a low uniform level along the filament in the presence of nitrate. In contrast, in filaments with heterocysts, there was a moderate increase in fluorescence from mature heterocysts compared with that from intervening vegetative cells, which retained the level of fluorescence seen in the presence of nitrate (Fig. 2c, d). From the time of removal of fixed nitrogen from the culture, to the appearance of mature heterocysts, the same low uniform level of fluorescence was observed as seen with filaments grown in medium containing nitrate. Therefore, transcription of *fraG* appeared to be enhanced in heterocysts, or perhaps in proheterocysts just prior to maturation.

fraG is necessary for synthesis of heterocyst glycolipids

Although cells with larger size and thickened cell envelopes, two of the attributes of mature heterocysts, were not seen in the *fraG* mutant, single cells with reduced pigmentation appeared to break off and fragment filaments between 16 and 18 h post-induction. These cells appeared to be proheterocysts that were arrested at a certain stage of development. To determine if heterocyst-specific exopolysaccharides and glycolipids, which are involved in the late stages of heterocyst maturation and necessary for creation of a microaerophilic environment in functioning heterocysts, were produced in the *fraG* mutant, UHM127 was checked for their presence. Alcian Blue, which binds specifically to heterocyst envelope polysaccharides, stained the cells with reduced pigmentation that broke from the filaments between 16 and 18 h post-induction, confirming that they were proheterocysts and indicating that *fraG* was not necessary for exopolysaccharide synthesis or deposition (Fig. 3).

Glycolipids from both the wild-type strain and the mutant were separated by TLC and visualized. Samples of the wild-type harvested at 48 h post-induction contained the two heterocyst-specific glycolipids, whereas those from UHM127 did not, indicating that *fraG* was necessary for their production (Fig. 4). UHM127 complemented with a wild-type copy of *fraG* on a plasmid produced similar types and quantities of glycolipids as the wild-type. The absence of heterocyst glycolipids in the mutant implied that either *fraG* performs a function that may be directly required for glycolipid synthesis or, alternatively, fragmentation arrests development before glycolipid synthesis begins in the wild-type, and their absence in the mutant is an indirect consequence of mutation of *fraG*. To distinguish between these two possibilities, glycolipids from the mutant and the wild-type were visualized at 15 h post-induction, before fragmentation of the mutant. One of the two heterocyst glycolipids was clearly visible in samples from the wild-type 15 h post-induction, indicating that glycolipid synthesis began before the time of fragmentation of UHM127 (Fig. 4). The mutant, however, had not produced heterocyst glycolipid at this time when filaments were still intact; this

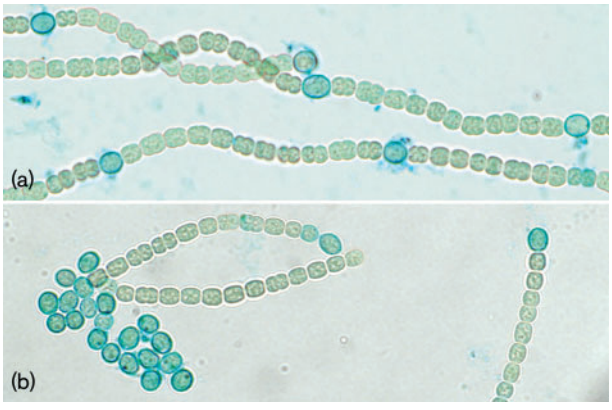

Fig. 3. Alcian Blue staining of cells of the *fraG* mutant strain UHM127. Alcian Blue staining of heterocyst-specific exopolysaccharides in *Anabaena* sp. strain PCC 7120 (a) and UHM127 (b) at 24 h after induction of heterocyst formation. Carets indicate heterocysts.

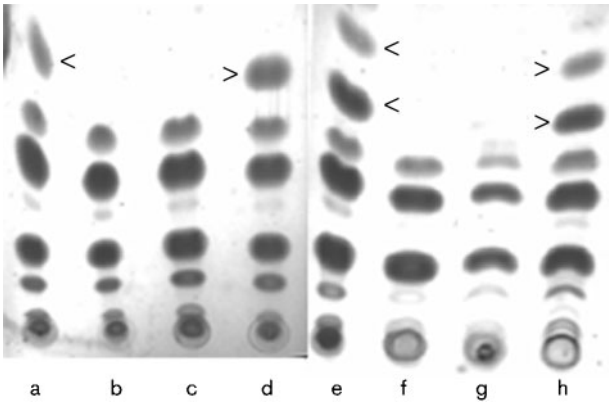

Fig. 4. TLC of glycolipids produced by the wild-type strain *Anabaena* sp. strain PCC 7120, the Δ*hetR* mutant UHM103, and the *fraG* mutant UHM127. Lanes: a–d, samples collected at 15 h post-induction; e–f, samples collected at 48 h post-induction; a and e, *Anabaena* sp. strain PCC 7120; b and f, UHM103; c and g, UHM127; d and h, UHM127 complemented with plasmid pAN130. Carets indicate heterocyst-specific glycolipids.

indicated that fragmentation most likely did not arrest development prior to synthesis of glycolipid to stop its production. Instead, it appeared that *fraG* was necessary for both glycolipid synthesis and maintenance of filament integrity during differentiation.

DISCUSSION

A handful of genes have been described that are necessary for filament integrity in *Anabaena* sp. strain PCC 7120, and that cause an increased fragmentation phenotype under diazotrophic conditions. Buikema & Haselkorn (1991a) described the isolation of four mutants with Fox$^-$ fragmentation phenotypes in a study that also led to the identification of *hetR*. For one mutant, the mutation necessary for the phenotype was found to disrupt the *fraC* gene, which encodes a phenylalanine-rich peptide with four potential transmembrane domains (Bauer *et al.*, 1995). In contrast to the *fraG* mutant UHM127 described here, a *fraC* mutant can fix nitrogen in the absence of molecular oxygen, mature heterocysts are occasionally observed and can be induced by introducing extra copies of *hetR* on a plasmid, and the fragmentation phenotype is more severe in the presence of fixed nitrogen. Similarly, mutation of *fraH*, which encodes a proline-rich peptide, causes fragmentation of filaments, but mature heterocysts are common in the mutant. The genetically uncharacterized fragmentation mutant N5 described by Wolk and coworkers, on the other hand, shows no signs of differentiation (Ernst *et al.*, 1992), unlike UHM127, which develops a normal pattern of proheterocysts. Finally, Golden and coworkers inactivated some group 2 sigma factors from *Anabaena* sp. strain PCC 7120 and found that a *sigE sigD* double mutant fragmented upon removal of fixed nitrogen, but mature heterocysts were formed (Khudyakov & Golden, 2001), suggesting that these two sigma factors are more likely to be involved in transcription of *fraC* or *fraH*, than transcription of *fraG*.

The increased fragility of the majority of fragmentation mutants in both nitrogen-replete and nitrogen-free media suggests that the integrity of heterocyst–vegetative cell junctions may rely on proteins that are also necessary for vegetative cell junctions, rather than being dependent solely on a separate set of proteins specific to heterocyst junctions. A low level of expression of *fraG* in vegetative cells, followed by induction in heterocysts, is consistent with this idea.

The somewhat pleiotropic effect of mutation of *fraG*, which resulted in Fix$^-$ Hgl$^-$ (lacking correctly localized heterocyst glycolipids) Fra phenotypes, suggests that mutation of *fraG* affects both structural and regulatory aspects of heterocyst development, with a defect in one affecting the other. In particular, FraG may have a structural role only, and its effect on glycolipid synthesis and regulation of differentiation could be indirect. Disruption of glycolipid formation, or the accumulation of a glycolipid intermediate, may affect filament integrity. Conversely, as an integral membrane protein, FraG may have primarily a structural role that is necessary for the advancement beyond a certain point in the developmental programme. In this case, regulation of differentiation would involve sensing of membrane or cell-junction structure. The formation of heterocyst-specific glycolipid by the wild-type at a time before fragmentation of the mutant, coupled with the absence of glycolipid synthesis in the mutant, suggests that fragmentation is not the structural change that prevents differentiation in the absence of *fraG*. Finally, the primary function of *fraG* may be in transport or signal transduction that is necessary for development, and disruption of development initiates a genetically programmed response that leads to fragmentation. Fragmentation of the wild-type strain under some environmental conditions, and after the death of heterocysts when filaments are returned to nitrogen-replete conditions, may confer a selective advantage under certain conditions, presumably by facilitating cell dispersion.

ACKNOWLEDGEMENTS

We thank C. Peter Wolk, Center for Microbial Ecology, Michigan State University, USA, for the kind gift of plasmid pRL1058. This work was supported by grant MCB-0343998 from the National Science Foundation.

REFERENCES

Bauer, C. C., Buikema, W. J., Black, K. & Haselkorn, R. (1995). A short-filament mutant of *Anabaena* sp. strain PCC 7120 that fragments in nitrogen-deficient medium. *J Bacteriol* **177**, 1520–1526.

Black, T. A., Cai, Y. & Wolk, C. P. (1993). Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* **9**, 77–84.

Borthakur, P. B., Orozco, C. C., Young-Robbins, S. S., Haselkorn, R. & Callahan, S. M. (2005). Inactivation of *patS* and *hetN* causes lethal

levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Mol Microbiol* **57**, 111–123.

Buikema, W. J. & Haselkorn, R. (1991a). Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **173**, 1879–1885.

Buikema, W. J. & Haselkorn, R. (1991b). Characterization of a gene controlling heterocyst development in the cyanobacterium *Anabaena* 7120. *Genes Dev* **5**, 321–330.

Buikema, W. J. & Haselkorn, R. (2001). Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. *Proc Natl Acad Sci U S A* **98**, 2729–2734.

Callahan, S. M. & Buikema, W. J. (2001). The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. *Mol Microbiol* **40**, 941–950.

Ernst, A., Black, T., Cai, Y., Panoff, J.-M., Tiwari, D. N. & Wolk, C. P. (1992). Synthesis of nitrogenase in mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst development or metabolism. *J Bacteriol* **174**, 6025–6032.

Fan, Q., Huang, G., Lechno-Yossef, S., Wolk, C. P., Kaneko, T. & Tabata, S. (2005). Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120. *Mol Microbiol* **58**, 227–243.

Fellay, R., Frey, J. & Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. *Gene* **52**, 147–154.

Golden, J. W. & Wiest, D. R. (1988). Genome rearrangement and nitrogen fixation in *Anabaena* blocked by inactivation of *xisA* gene. *Science* **242**, 1421–1423.

Huang, X., Dong, Y. & Zhao, J. (2004). HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. *Proc Natl Acad Sci U S A* **101**, 4848–4853.

Huang, G., Fan, Q., Lechno-Yossef, S., Wojciuch, E., Wolk, C. P., Kaneko, T. & Tabata, S. (2005). Clustered genes required for the synthesis of heterocyst envelope polysaccharide in *Anabaena* sp. strain PCC 7120. *J Bacteriol* **187**, 1114–1123.

Kaneko, T., Nakamura, Y., Wolk, C. P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K. & other authors (2001). Complete genome sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res* **8**, 205–213, 227–253.

Khudyakov, I. & Golden, J. W. (2001). Identification and inactivation of three group 2 sigma factor genes in *Anabaena* sp. strain PCC 7120. *J Bacteriol* **183**, 6667–6675.

Leganés, F., Blanco-Rivero, A., Fernandez-Pinas, F., Redondo, M., Fernandez-Valiente, E., Fan, Q., Lechno-Yossef, S. & Wolk, C. P. (2005). Wide variation in the cyanobacterial complement of presumptive penicillin-binding proteins. *Arch Microbiol* **184**, 234–248.

Marchler-Bauer, A., Anderson, J. B., DeWeese-Scott, C., Fedorova, N. D., Geer, L. Y., He, S., Hurwitz, D. I., Jackson, J. D., Jacobs, A. R. & other authors (2003). CDD: a curated Entrez database of conserved domain alignments. *Nucleic Acids Res* **31**, 383–387.

Meeks, J. C. & Elhai, J. (2002). Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol Mol Biol Rev* **66**, 94–121.

Murry, M. A. & Wolk, C. P. (1989). Evidence that the barrier to the penetration of oxygen into heterocysts depends upon two layers of the cell envelope. *Arch Microbiol* **151**, 469–474.

Nichols, B. W. & Wood, B. J. B. (1968). New glycolipid specific to nitrogen-fixing blue-green algae. *Nature* **217**, 767–768.

Orozco, C. C., Risser, D. D. & Callahan, S. M. (2006). Epistasis analysis of four genes from *Anabaena* sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst pattern formation. *J Bacteriol* **188**, 1808–1816.

Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M. & Chen, W. S. (1977). Formation of glutamine from [^{13}N]ammonia, [^{13}N]dinitrogen, and [^{14}C]glutamate by heterocysts isolated from *Anabaena cylindrica*. *J Bacteriol* **129**, 1545–1555.

Walsby, A. E. (1985). The permeability of heterocysts to the gases nitrogen and oxygen. *Proc R Soc Lond B Biol Sci* **226**, 345–366.

Wei, T.-F., Ramasubramanian, R. & Golden, J. W. (1994). *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. *J Bacteriol* **176**, 4473–4482.

Winkenbach, F., Wolk, C. P. & Jost, M. (1972). Lipids of membranes and of the cell envelope in heterocysts of a blue-green alga. *Planta* **107**, 69–80.

Wolk, C. P. (2000). Heterocyst formation in *Anabaena*. In *Prokaryotic Development*, pp. 83–104. Edited by Y. V. Brun & L. J. Shimkets. Washington, DC: American Society for Microbiology.

Wolk, C. P., Cai, Y. P. & Panoff, J. M. (1991). Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proc Natl Acad Sci U S A* **88**, 5355–5359.

Yoon, H.-S. & Golden, J. W. (2001). PatS and products of nitrogen fixation control heterocyst pattern. *J Bacteriol* **183**, 2605–2613.

Zhang, C.-C., Laurent, S., Sakr, S., Peng, L. & Bedu, S. (2006). Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Mol Microbiol* **59**, 367–375.

Zhou, R., Wei, X., Jiang, N., Li, H., Dong, Y., Hsi, K.-L. & Zhao, J. (1998). Evidence that HetR is an unusual serine-type protease. *Proc Natl Acad Sci U S A* **95**, 4959–4963.

Edited by: D. J. Scanlan