NrrA directly regulates expression of the fraF gene and antisense RNAs for fraE in the heterocyst-forming cyanobacterium Anabaena sp. strain PCC 7120

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The heterocystous cyanobacterium Anabaena sp. strain PCC 7120 grows as linear multicellular filaments that can contain hundreds of cells. Heterocysts, which are specialized cells for nitrogen fixation, are regularly intercalated among photosynthetic vegetative cells, and these cells are metabolically dependent on each other. Thus, multicellularity is essential for diazotrophic growth of heterocystous cyanobacteria. In Anabaena sp. strain PCC 7120, the fraF gene, which is required to limit filament length, is induced by nitrogen deprivation. The fraF transcripts extend to the fraE gene, which lies on the opposite DNA strand and could possess dual functionality, mRNAs for fraF and antisense RNAs for fraE. In the present study, we found that NrrA, a nitrogen-regulated response regulator, directly regulated expression of fraF. Induction of fraF by nitrogen deprivation was abolished by the nrrA disruption. NrrA specifically bound to the promoter region of fraF, and recognized an inverted repeat sequence. Thus, it is concluded that NrrA controls expression of mRNAs for fraF and antisense RNAs for fraE in response to nitrogen deprivation.

INTRODUCTION

Cyanobacteria are a large group of bacteria characterized by O2-evolving photosynthesis. Anabaena sp. strain PCC 7120 (hereafter Anabaena) is a filamentous cyanobacterium in which certain vegetative cells differentiate into heterocysts with a semi-regular spacing of 10–15 cells upon limitation of combined nitrogen in the medium (Flores & Herrero, 2010; Kumar et al., 2010). Heterocysts are terminally differentiated cells specialized for nitrogen fixation. The intracellular environment of heterocysts is micro-oxic for protection of the oxygen-labile nitrogenase as a result of increased respiration, inactivation of O2-evolving photosystem II and formation of a thick envelope outside of the cell wall (Wolk et al., 1994). Since heterocysts are unable to carry out photosynthesis, vegetative cells supply heterocysts with carbohydrate required for nitrogen fixation. In return, vegetative cells receive nitrogen fixation products from heterocysts. Thus, multicellularity and division of labour between two cell types are essential for heterocyst-forming cyanobacteria to grow.

In Anabaena, genes influencing filament integrity have been identified (Ehira & Ohmori, 2012; Flores et al., 2007; Merino-Puerto et al., 2010, 2013; Nayar et al., 2007). Inactivation of genes in the fraC operon, which is composed of three genes, fraC, fraD and fraE, produces filament fragmentation under conditions of nitrogen deprivation and impairs diazotrophic growth (Merino-Puerto et al., 2010). Deletion of the fraF gene, which is located downstream of fraE in the opposite orientation, results in a large increase of filament length and impairs diazotrophic growth on solid medium (Merino-Puerto et al., 2013). Thus, FraF and products of the fraC operon have an opposite effect on filament length. Expression of fraF is induced by nitrogen deprivation, while the fraC operon is constitutively expressed. Induction of fraF is dependent on NtcA, the global nitrogen regulator of cyanobacteria (Herrero et al., 2004), but no putative NtcA-binding site is found within its promoter region. The fraF transcripts extend as antisense RNAs (asRNAs) to the fraE gene, and prevention of asRNA...
production results in an increased length of filaments, suggesting that the asRNAs could affect the expression of the fraF operon. Thus, the fraF transcripts are likely to possess dual functionality, mRNAs for fraF and asRNAs for fraE (Merino-Puerto et al., 2013).

NrrA is a nitrogen-regulated response regulator of cyanobacteria belonging to the OmpR family. In *Anabaena*, expression of *nrrA* is induced by nitrogen deprivation and is directly regulated by NtcA (Ehira & Ohmori, 2006a; Muro-Pastor et al., 2006). NrrA is involved in regulation of heterocyst differentiation. NrrA binds specifically to the region upstream of the nitrogen-responsive transcription initiation sites of the *hetR* gene, the master regulator of heterocyst differentiation (Buikema & Haselkorn, 1991, 2001). Inactivation of *nrrA* diminishes the induction of *hetR* and delays heterocyst differentiation, and overexpression of *nrrA* results in enhanced NRR activity and delays heterocyst differentiation, and overexpression of fraF gene, leading to depressed nitrogen fixation (Ehira & Ohmori, 2006b). Induction of fraF by nitrogen deprivation was abolished by nrrA inactivation and an NrrA-binding site within the fraF promoter region was identified. Thus, NrrA directly regulates expression of fraF and asRNAs for fraE in response to nitrogen deprivation.

**METHODS**

**Bacterial strains and culture conditions.** *Anabaena* sp. strain PCC 7120 and its derivative were grown in BG-11 medium (containing NaN3 as a nitrogen source) as described previously (Ehira & Ohmori, 2006a). The mutant strain DR4312S (Ehira & Ohmori, 2006a) was used as the nrrA disruptant in this study. Liquid cultures were bubbled with air containing 1.0 % (v/v) CO2. For nitrogen deprivation experiments, cells grown in BG-11 medium until they reached an OD750 of 0.4–0.5 were washed twice with nitrogen-free BG-11 medium (BG-110) and then resuspended in BG-110 medium. Spectinomycin was added to the medium at a final concentration of 10 μg ml−1 when required. Genome sequence and annotation were based on Cyanobase (Fujisawa et al., 2014).

**DNA microarray analysis.** Total RNA was extracted from whole filaments according to Pinto et al. (2009) and was treated with DNase I (Takara Bio). Global gene expression was analysed using the *Anabaena* oligonucleotide microarray as described previously (Ehira & Ohmori, 2006a). Microarray analyses were carried out with three sets of RNA samples isolated from independently grown cultures. Two hybridization reactions were performed with different combination of Cy-dyes for each set of RNA samples. Thus, a total of six replicates per gene were available to determine changes in gene expression. Genes with significantly different transcript levels (*P*<0.05; Student’s t-test) by at least a factor of two were determined.

**Quantitative reverse transcription PCR (qRT-PCR).** All primers used in this study are listed in Table 1. cDNA was synthesized from 1 μg total RNA with random hexamer primers using a PrimeScript 1st strand cDNA synthesis kit (Takara Bio). qRT-PCR was performed with a Thermal Cycler Dice Real-time System (TP9000; Takara Bio) in a 20 μl reaction mixture containing 10 μl SYBR Premix Ex Taq II (Takara Bio), 0.2 μM each gene-specific forward and reverse primer and cDNA. Relative ratios were normalized with the value for 16S rRNA and are represented as means of triplicate experiments.

**Gel mobility shift assay.** His–NrrA protein was overproduced and purified from *Escherichia coli* as described previously (Ehira & Ohmori, 2006b). The promoter region of fraF (P2395), corresponding to the region from −307 to +3 with respect to the translation start site of the fraF gene, was amplified by PCR using the primer pair P2395-F and P2395-R and cloned into the HindIII site of pBluescript II SK+ (Agilent Technologies) to construct pBP2395. A digoxigenin-labelled probe was prepared by PCR using a digoxigenin-labelled T7-promoter primer in combination with P2395-F and pBP2395 as a template. Gel mobility shift assay was carried out using 1 fmol of the digoxigenin-labelled probe as described previously (Ehira & Ohmori, 2006b). Deletion probes D1, D2, D3 and D4 were prepared by PCR using the forward primers P2395-F4, P2395-F3, P2395-F5 and P2395-F2 in combination with the reverse primer P2395-R, respectively. Deletion probes of each side of the inverted repeat sequence, DL and DR, were prepared with the PrimeSTAR mutagenesis basal kit (Takara Bio) using the primer pairs P2395D3-F and P2395D3-R, and P2395D4-F and P2395D4-R, respectively.

**RESULTS**

**NrrA positively regulates expression of the fraF gene**

We carried out DNA microarray analysis to identify genes regulated by NrrA. Since the expression of *nrrA* is induced within 3 h of nitrogen deprivation (Ehira & Ohmori, 2006b), we assumed that NrrA binds to a site within fraF and regulates its expression. Thus, we identified 46 genes downregulated by NrrA. The fraF gene was included in genes under the control of NrrA. Induction of fraF by nitrogen deprivation was abolished by nrrA inactivation and an NrrA-binding site within the fraF promoter region was identified. Thus, NrrA directly regulates expression of fraF and asRNAs for fraE in response to nitrogen deprivation.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTrrn16SF2</td>
<td>GCAAGTCGAACGGTGTCCTCTC</td>
</tr>
<tr>
<td>RTrrn16SR2</td>
<td>GGTATGAGCCAGGTTCCTC</td>
</tr>
<tr>
<td>RT2395-F</td>
<td>TGCCAAATTGTTGGTCTGTA</td>
</tr>
<tr>
<td>RT2395-R</td>
<td>TCGGCACTCTGCTCAATAATAGC</td>
</tr>
<tr>
<td>P2395-F</td>
<td>TTACCTGATAATGCCCACAGC</td>
</tr>
<tr>
<td>P2395-R</td>
<td>CACCTCGAGTGTCAATGAG</td>
</tr>
<tr>
<td>T7promoter</td>
<td>TAATAGCAGCTCIAATAGGG</td>
</tr>
<tr>
<td>P2395-F2</td>
<td>GTGTTTTTCTGACCTTTGACAGC</td>
</tr>
<tr>
<td>P2395-F3</td>
<td>TGTAGTGCTGATTTTCTGCTA</td>
</tr>
<tr>
<td>P2395-F4</td>
<td>GAAAAGTGCAAACTTAACCTTAGC</td>
</tr>
<tr>
<td>P2395-F5</td>
<td>CCCATCACCCTGTCATATT</td>
</tr>
<tr>
<td>P2395D3-F</td>
<td>TTGGTCTCAGTTTCTGACCTT</td>
</tr>
<tr>
<td>P2395D3-R</td>
<td>TGGAAACGAGCAAGGTGATGAGGGA</td>
</tr>
<tr>
<td>P2395D4-F</td>
<td>GTGTCTTCTGACGTCTATGCTTG</td>
</tr>
<tr>
<td>P2395D4-R</td>
<td>GCTGATCTGAAACACTAATAATGAG</td>
</tr>
</tbody>
</table>

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2006a), gene expression patterns of the WT strain and the

\( \text{nrrA} \) disruptant DR4312S (Ehira & Ohmori, 2006a) after

3 h of nitrogen deprivation were compared to minimize

indirect effects of \( \text{nrrA} \) disruption. The transcript levels of

46 genes in the \( \text{nrrA} \) disruptant were compared to minimize

the WT (Table 2), most of which were upregulated by

nitrogen deprivation (data not shown), suggesting reduced

induction in the \( \text{nrrA} \) disruptant. NrrA is required for

the full induction of \( \text{hetR} \) and facilitates heterocyst

differentiation (Ehira & Ohmori, 2006a). In addition to

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Gene} & \text{Product} & \Delta \text{nrrA}/\text{WT}^* & P^† \\
\hline
\text{asr0064} & \text{Hypothetical protein} & -1.61 & 9.3e-05 \\
\text{asr0098} & \text{Unknown protein} & -1.05 & 2.8e-02 \\
\text{alr0099} & \text{\text{hetZ} protein} & -1.08 & 1.4e-02 \\
\text{asr0100} & \text{Unknown protein} & -1.03 & 1.0e-03 \\
\text{alr0101} & \text{\text{patU5} protein} & -1.01 & 5.1e-05 \\
\text{all0313} & \text{Unknown protein} & -1.29 & 3.9e-02 \\
\text{alr0444} & \text{Unknown protein} & -2.22 & 2.0e-02 \\
\text{alr0445} & \text{Hypothetical protein} & -1.65 & 3.1e-03 \\
\text{alr0446} & \text{Hypothetical protein} & -1.30 & 7.2e-03 \\
\text{alr0447} & \text{ATP-binding protein of ABC transporter} & -1.11 & 4.7e-03 \\
\text{all0565} & \text{Unknown protein} & -1.24 & 1.4e-02 \\
\text{all0955} & \text{Hypothetical protein} & -1.75 & 1.6e-02 \\
\text{all0968} & \text{\text{fabG} protein} & -1.49 & 9.3e-04 \\
\text{all1272} & \text{\text{glgP1} protein} & -1.87 & 1.4e-04 \\
\text{all1454} & \text{\text{niD} protein} & -1.58 & 3.7e-03 \\
\text{all1455} & \text{\text{niH} protein} & -1.11 & 6.5e-04 \\
\text{all1692} & \text{\text{sigC} protein} & -1.57 & 1.1e-03 \\
\text{asr2301} & \text{\text{patS} protein} & -1.70 & 2.1e-03 \\
\text{asr2332} & \text{Unknown protein} & -1.86 & 1.7e-03 \\
\text{alr2339} & \text{\text{hetR} protein} & -1.44 & 9.3e-05 \\
\text{all2395} & \text{\text{fraF} protein} & -1.09 & 2.0e-03 \\
\text{all2397} & \text{Unknown protein} & -1.15 & 2.1e-04 \\
\text{alr2463} & \text{Unknown protein} & -1.36 & 5.1e-04 \\
\text{all2705} & \text{Hypothetical protein} & -1.67 & 5.0e-05 \\
\text{all2747} & \text{Similar to precorrin methylase} & -1.60 & 1.4e-04 \\
\text{alr2817} & \text{\text{hetC} protein} & -1.31 & 2.5e-02 \\
\text{alr2830} & \text{\text{rfbC} protein} & -1.28 & 3.1e-03 \\
\text{alr2831} & \text{Probable NAD(P)-dependent oxidoreductase} & -1.06 & 1.5e-03 \\
\text{alr2833} & \text{Hypothetical protein} & -1.99 & 4.7e-02 \\
\text{alr2837} & \text{Glycosyltransferase} & -2.80 & 4.3e-04 \\
\text{alr2838} & \text{Unknown protein} & -1.24 & 2.2e-02 \\
\text{alr2839} & \text{Glycosyltransferase} & -1.76 & 1.6e-02 \\
\text{alr2840} & \text{Glycosyltransferase} & -2.49 & 6.3e-03 \\
\text{alr3240} & \text{Permease protein of ferrichrome ABC transporter} & -1.06 & 3.0e-04 \\
\text{alr3241} & \text{ATP-binding protein of ferrichrome ABC transporter} & -1.57 & 4.3e-04 \\
\text{alr3676} & \text{Unknown protein} & -1.13 & 1.5e-02 \\
\text{all3696} & \text{Unknown protein} & -1.04 & 1.1e-02 \\
\text{alr3732} & \text{\text{pknE} protein} & -1.33 & 4.7e-03 \\
\text{alr3808} & \text{Nutrient-stress-induced DNA-binding protein} & -1.16 & 6.7e-03 \\
\text{alr4000} & \text{Photosystem II CP43 protein PsbC homologue} & -1.23 & 5.8e-04 \\
\text{asr4638} & \text{Unknown protein} & -2.14 & 2.2e-02 \\
\text{alr4685} & \text{Unknown protein} & -3.86 & 1.7e-03 \\
\text{alr4686} & \text{Cytochrome P450} & -1.81 & 7.9e-05 \\
\text{asr4987} & \text{Unknown protein} & -1.08 & 2.8e-02 \\
\text{alr4991} & \text{\text{desC} protein} & -1.04 & 2.3e-02 \\
\text{all5369} & \text{Unknown protein} & -2.72 & 9.0e-04 \\
\hline
\end{array}
\]

*Relative ratios of transcript level in the \( \text{nrrA} \) disruptant to those in the WT 3 h after nitrogen deprivation are shown in the base 2 logarithm.

†\( P \) values for the relative ratios were determined by Student’s \( t \)-test.
hetR, genes involved in heterocyst differentiation, such as hetZ-patU5-patU3, sigC and patS (Khudyakov & Golden, 2001; Yoon & Golden, 1998; Zhang et al., 2007), were downregulated by nrrA disruption (Table 2). However, no interaction between NrrA and promoter regions of hetZ, sigC and patS was observed (data not shown), indicating indirect regulation of these genes by NrrA.

The fraF gene (all2395) was recently shown to negatively influence the length of filaments in Anabaena (Merino-Puerto et al., 2013). Expression of fraF is increased by nitrogen deprivation in a NtcA-dependent manner, but no putative NtcA-binding site could be identified within its promoter region, suggesting regulation by NtcA is indirect (Merino-Puerto et al., 2013). The fraF transcript level was decreased in the nrrA disruptant (Table 2). Changes in expression of fraF after nitrogen deprivation were analysed by qRT-PCR in the WT and nrrA disruptant (Fig. 1). In the WT, the transcript levels of fraF were increased within 3 h of nitrogen deprivation, with increases about 3-fold after 8 h. In the nrrA disruptant, the fraF transcript level was decreased to half of the WT level in the presence of nitrate and slightly increased following nitrogen deprivation, but its level was lower than that of the WT before induction. Thus, NrrA is a positive regulator of fraF.

NrrA binds to the promoter region of fraF

Gel mobility shift assays were carried out with purified His–NrrA and DNA probe P2395, corresponding to the region from −307 to +3 with respect to the translational start site of fraF (Fig. 2). His–NrrA reduced the electrophoretic mobility of probe P2395, and the amount of the NrrA-P2395 complex increased in proportion to the concentration of His–NrrA (Fig. 2, lanes 1–4). The band intensity of the NrrA-P2395 complex was decreased upon addition of a non-labelled P2395 fragment to the assay mixture (Fig. 2, lanes 5 and 6). The addition of a fragment PhetR2, which does not interact with His–NrrA (Ehira & Ohmori, 2006b), did not affect the amount of the NrrA-P2395 complex (Fig. 2, lanes 7 and 8). These observations led to the conclusion that NrrA binds to the region upstream of fraF in a sequence-specific manner.

The 5′ ends of fraF transcripts have been identified by primer extension analysis (Merino-Puerto et al., 2013) and RNA sequencing (Mitschke et al., 2011). The transcription initiation site (TIS) positioned at 137 bp upstream of the translational start site of fraF was detected in both analyses and is likely to be a major nitrogen-regulated TIS. To determine the NrrA-binding site within the fraF promoter region, a deletion series of four probes was used for gel mobility shift assays (Fig. 3a). NrrA bound to probes D1, D2 and D3 in a similar way to P2395, but no interaction between D4 and NrrA was observed (Fig. 3b), indicating that a sequence required for NrrA binding to the fraF promoter was present in the region from −205 to −184. We have previously indicated that the sequence AA (A/T) GTCA is required for NrrA binding to the glgP1 and hetR promoters (Ehira & Ohmori, 2006b, 2011). Eight nucleotides downstream of these sequences, we found incomplete inverted sequences (Fig. 3a). The inverted repeat (IR) sequence (aTTAN6TGAC) was also found within the fraF promoter region, which was located from −187 to −172 (Fig. 3a). Removal of each side of the IR sequence from P2395 decreased the DNA-binding affinity of NrrA, although weak interactions between NrrA and probes DL and DR were still observed (Fig. 3c). These results indicate that NrrA binds to the IR sequence in dimeric form.

Fig. 1. Changes in the transcript level of all2395 after nitrogen deprivation. Relative transcript levels of fraF at the indicated times were determined by qRT-PCR in the WT strain (●) and the nrrA disruptant DR4312S (○). Transcript levels were determined in duplicate using three independently grown cultures. The transcript level of the WT strain at 0 h was taken as 1.

Fig. 2. Gel mobility shift assays with His–NrrA and the promoter region of fraF. Digoxigenin-labelled probe P2395 (50 pM) including the promoter region of fraF was mixed with His–NrrA in the amounts indicated above each lane, and the mixtures were subjected to electrophoresis. Non-labelled fragments of P2395 (lanes 5 and 6) and PhetR2 (lanes 7 and 8) were added at a final concentration of 1.5 nM (lanes 5 and 7) or 5 nM (lanes 6 and 8). Filled and open arrowheads indicate the NrrA-P2395 complex and free probe, respectively.
In the present study, we indicated that nitrogen-dependent expression of the fraF gene was regulated by NrrA. NrrA bound to the promoter region of fraF (Fig. 2) and upregulated expression of fraF in response to nitrogen deprivation (Fig. 1). It has been shown that expression of fraF is under the control of NtcA (Merino-Puerto et al., 2013). Since NrrA is directly regulated by NtcA (Muro-Pastor et al., 2006), the nitrogen limitation signal induces the expression of fraF via two transcriptional regulators, NtcA and NrrA. Expression of the fraF gene is higher in heterocysts than in vegetative cells (Merino-Puerto et al., 2013), which is consistent with the higher expression of nrrA in heterocysts (Ehira & Ohmori, 2006a). The fraF transcripts possess dual functionality, mRNAs for fraF and asRNAs for fraE (Merino-Puerto et al., 2013). Recently, the excludon, a new paradigm in RNA-mediated gene regulation, has been defined to describe a genetic locus for which a transcript activates expression of one gene or operon while excluding or inhibiting expression of another (Wurtzel et al., 2012). A genetic locus containing the fraF gene and the fraC operon is an example of the excludon in Anabaena. NrrA could influence filament length by regulating expression of fraF and asRNAs for fraE.

The NrrA recognition sequence was determined by analysis of the promoter region of fraF (Fig. 3). In a previous study (Ehira & Ohmori, 2011), we have shown that the sequence AAAGTCA within the glgP1 promoter and the sequence AATGTCA within the hetR promoter, which include the upstream side of the IR sequence, are indispensable for the binding of NrrA (Fig. 3a). Deletion up to the upstream side of the IR sequence within the fraF promoter (probe D4) also abolished the interaction between NrrA and the fraF promoter (Fig. 3b). Moreover, removal of each side of the IR sequence from probe P2395 impaired the binding of NrrA (Fig. 3c), indicating that NrrA recognized the IR sequence. The IR sequence plays a pivotal role in NrrA binding; only a faint shift band was observed using probes DL and DR (Fig. 3c). As the shift bands observed in DL and DR migrated faster than that of P2395, NrrA might bind to half of the IR sequence in monomeric form or be detached from DNA during electrophoresis because of the weak interaction. The NrrA-binding site is centred 42.5 nt upstream from the TIS positioned at −137, indicating that NrrA activates this promoter by the class II mechanism (Browning & Busby, 2004).

Although expression of genes involved in heterocyst differentiation, such as hetZ-patU5-patU3, sigC and patS,
was significantly decreased by the nrrA disruption (Table 2), no interaction between NrrA and the promoter regions of these genes was observed. Since HetR binds to the promoter regions of hetZ and patS (Du et al., 2012; Huang et al., 2004), downregulation of hetR could affect the transcript levels of hetZ and patS in the nrrA mutant. We were unable to detect interaction between the sigC promoter and HetR (data not shown). Thus, mechanisms of sigC regulation by NrrA remain unknown.

Orthologues of NrrA are present in most cyanobacteria except marine picocyanobacteria. NrrA orthologues are also induced by nitrogen deprivation under the control of NtcA, and regulate glycogen catabolism in the unicellular cyanobacterium Synechocystis PCC 6803 (Azuma et al., 2011) and Synechococcus PCC 7002 (unpublished data). Thus, regulation of nrrA expression by NtcA and glycogen catabolism by NrrA are likely to be common features in cyanobacteria. In Anabaena, NrrA regulates hetR and fraF in addition to glpP1. The hetR gene is conserved among filamentous cyanobacteria (Zhang et al., 2009), and the fraF gene is frequently found in filamentous heterocyst-forming cyanobacteria (Merino-Puerto et al., 2013). NrrA of Anabaena seems to have acquired new members of the regulon to regulate filament length and cellular differentiation in response to nitrogen deprivation.

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