Identification of three novel antisense RNAs in the fur locus from unicellular cyanobacteria

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The interplay between Fur (ferric uptake regulator) proteins and small, non-coding RNAs has been described as a key regulatory loop in several bacteria. In the filamentous cyanobacterium Anabaena sp. PCC 7120, a large dicistronic transcript encoding the putative membrane protein Alr1690 and an α-furA RNA is involved in the modulation of the global regulator FurA. In this work we report the existence of three novel antisense RNAs in cyanobacteria and show that a cis α-furA RNA is conserved in very different genomic contexts, namely in the unicellular cyanobacteria Microcystis aeruginosa PCC 7806 and Synechocystis sp. PCC 6803. Syx-fur RNA covers only part of the coding sequence of the fur orthologue sll0567, whose flanking genes encode two hypothetical proteins. Transcriptional analysis of fur and its adjacent genes in Microcystis unravels a highly compact organization of this locus involving overlapping transcripts. Max-fur RNA spans the whole Mafur CDS and part of the flanking dnaJ and sufE sequences. In addition, Mafur seems to be part of a dicistronic operon encoding this regulator and an α-sufE RNA. These results allow new insights into the transcriptomes of two unicellular cyanobacteria and suggest that in M. aeruginosa PCC 7806, the α-fur and α-sufE RNAs might participate in a regulatory connection between the genes of the dnaJ–fur–sufE locus.

INTRODUCTION

In eubacteria, most regulatory RNAs identified to date are non-coding RNAs (ncRNAs) smaller than 300 nt that are located far away from their target genes. The roles of ncRNAs are diverse; they can be involved in the control of several stress responses, virulence and motility, among other functions (Waters & Storz, 2009). In particular, the interplay between Fur (ferric uptake regulator) proteins from different organisms and regulatory RNAs seems to play a major role in the control of iron homeostasis (Večerek et al., 2007). It is assumed that, in vivo, Fur works as a classical repressor using Fe(II) as a cofactor to negatively regulate expression of their target genes through binding to Fur recognition sites (Bagg & Neilands, 1987). Cross-talk with other transcriptional regulators involved in carbon and nitrogen metabolism (Zhang et al., 2005; Lópe-Gomollón et al., 2007) and with other members from the Fur superfamily (Fuangthong et al., 2002; Hernández et al., 2004) also contribute to the modulation of Fur activity. In addition, reciprocal regulation between Fur and several ncRNAs, as well as co-regulation of iron-responsive genes by Fur and ncRNAs, has been described (Waldbeser et al., 1995). Identification of RhyB in Escherichia coli cells provided the answer to the mechanism of activation of the sodB gene under iron-rich conditions (Massé & Gottesman, 2002) and linked the regulation of a set of iron storage and iron-containing proteins to this small ncRNA, involving its own Fur as part of a back-up mechanism.

In cyanobacteria, two cis-encoded regulatory RNAs linked to iron metabolism have been characterized. In the unicellular cyanobacterium Synechocystis, the regulatory RNA IsiR is expressed specifically during iron-rich conditions and, with Fur, co-represses the synthesis of IsiA under iron-replete conditions (Dühring et al., 2006). In the filamentous, nitrogen-fixing cyanobacterium Anabaena (Nostoc) sp. PCC 7120 an α-furA is transcribed in the same message as the membrane protein Alr1690 and covers the complete coding sequence of furA (Hernández et al., 2006). Disruption of the α-furA–alr1690 message leads to an increased expression of FurA and the resulting mutant exhibits an iron-deficient phenotype (Hernández et al., 2010). An alr1690 homologue has also been identified in the context of the fur gene from other species, though this is not a fully conserved arrangement in cyanobacteria. In the Synechocystis sp. PCC...
6803 genome, the furA orthologue sll0567 is flanked by genes encoding hypothetical proteins, while in Microcystis aeruginosa PCC 7806 and NIES-843 strains, the corresponding furA genes are flanked by dnaJ and cysteine desulphurase activator sufE genes (Martín-Luna et al., 2006, http://genome.kazusa.or.jp/cyanobase/).

Comparative genome analysis of several cyanobacterial strains has allowed a reliable biocomputational prediction of a plethora of ncRNAs (Axmann et al., 2005), and these are found to occur at a much higher rate in Microcystis species than in other unicellular cyanobacteria (Voß et al., 2009). More recently, the use of differential RNA sequencing of the Synechocystis sp. PCC 7806, together with complementary microarray-based RNA profiling, showed that a quarter of all chromosomal genes are subject to transcription of three novel antisense RNAs in unicellular cyanobacteria (Voß et al., 2005). These data also reveal that the majority of small RNAs are located on the complementary strand of mRNAs.

In this work, we provide experimental evidence for the transcription of three novel antisense RNAs in unicellular cyanobacteria. Two of them have been identified in the fur–sufE locus of M. aeruginosa. The first is transcribed in the same message as fur and covers the complete CDS of the sufE gene. The second antisense RNA is an x-fur RNA that spans beyond fur and covers part of the flanking sufE and dnaJ genes. The occurrence of a smaller x-fur RNA has also been corroborated in Synechocystis PCC 6803, indicating that the presence of x-fur RNAs is not restricted to Anabaena PCC 7120 and seems to be independent of the fur gene context.

**METHODS**

**Growth conditions.** M. aeruginosa PCC 7806 was grown at 25 °C and 10 μmol photons m⁻² s⁻¹ in modified BG11 medium with 2 mM NaNO₃ and 10 mM NaHCO₃ (Rippka et al., 1979). Synechocystis PCC 6803 was grown in the same conditions using standard BG11 medium.

**Sampling and RNA isolation.** Cells (25 ml) were centrifuged and pellets were resuspended in 600 μl 50 mM Tris/HCl (pH 8), 100 mM EDTA and 130 μl chloroform and incubated on ice for 3 min. After centrifugation, cell pellets were frozen and kept at −70 °C until RNA isolation. Cells were disrupted in the Fastprep Instrument using four cycles of 20 s at a setting of 6 m s⁻¹. Total RNA was extracted using the FastRNA Pro Blue kit (Qbiogene), resuspended in 50 μl DEPC- H₂O and treated with 40 units DNase (Pharmacia).

**RT-PCR analysis.** RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen) and the specific oligonucleotide (Table 1). Residual DNA in RNA preparations was eliminated by digestion with RNase-free DNase I (Roche). The absence of DNA was checked by PCR. The RT-PCR mixture contained 1 μg RNA and 20 pmol of the oligonucleotide in the annealing buffer [10 mM Tris/HCl (pH 8), 1 mM EDTA, 150 mM KCl] in a final volume of 10 μl. Samples were heated for 10 min at 85 °C and incubated for 60 min at 65 °C. Afterwards, 5 pmol each dNTP, 0.2 mmol DTT and 200 U Superscript Reverse Transcriptase were added to the buffer [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂ and 20 mM DTT] to a final volume of 20 μl. Samples were incubated at 47 °C for 1 h and finally heated at 75 °C for 15 min. The products were amplified by PCR using 2 μl of each cDNA as template and analysed in a 1 % (w/v) agarose gel with a Gel Doc 2000 (Bio-Rad).

**Transcription start point (tsp) determination.** Potential Maz-fur and Mafur tsps were determined by primer extension. The Maz-fur promoter region was amplified using oligonucleotides 10 (ATTS) and 11 (AsufE). To identify the tsp of Mafur, oligonucleotides 6 (pFurN) and 13 were used for amplification of the promoter. The resulting fragments were cloned in pGEMT and introduced into E. coli JM109 (Promega). The recombinant plasmids were purified and the correct integration of the inserts was confirmed by restriction analysis with EcoRI. These plasmids were used as templates in the sequencing reaction using the Thermo Sequenase Fluorescent Primer Cycle Sequencing kit (Amersham), where the labelled nucleotides primed

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**Table 1. Oligonucleotides used in this work**

<table>
<thead>
<tr>
<th>Oligonucleotide (number)</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Description</th>
<th>5’ Position relative to the fur ORF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFurN (1)</td>
<td>GTCGATTGCGCCATGCTGCTTCTAC</td>
<td>Forward primer for fur gene in Microcystis</td>
<td>−11</td>
</tr>
<tr>
<td>MFurC (2)</td>
<td>CAGTTAGGAAATCCGGCATTAGTG</td>
<td>Reverse primer for fur gene in Microcystis</td>
<td>553</td>
</tr>
<tr>
<td>Mintfw (3)</td>
<td>CGACGATTACCGCATG</td>
<td>Forward primer for internal region of fur gene in Microcystis</td>
<td>176</td>
</tr>
<tr>
<td>Mintrev (4)</td>
<td>CACACTGTTTGGAGACTTG</td>
<td>Reverse primer for internal region of fur gene in Microcystis</td>
<td>351</td>
</tr>
<tr>
<td>Ndnal (5)</td>
<td>CGGGGAAATTTGGAGATTCTC</td>
<td>Reverse primer N-terminal region of dnaJ gene</td>
<td>−140</td>
</tr>
<tr>
<td>pFurN (6)</td>
<td>CTCTTTAGCCCTGATTGCATC</td>
<td>Internal dnaJ gene and forward primers for cloning fur promoter</td>
<td>−381</td>
</tr>
<tr>
<td>FurSyneRev (7)</td>
<td>CATGTCTATACCCGGCATT</td>
<td>Forward primer for fur gene in Synechocystis</td>
<td>−1</td>
</tr>
<tr>
<td>FurSyneInt (8)</td>
<td>CAGACAGTGATTGTTAATCTCTTC</td>
<td>Reverse primer for fur gene in Synechocystis</td>
<td>−144</td>
</tr>
<tr>
<td>FurSyneFor (9)</td>
<td>CCTAGGCGCCGAAAATCTGCC</td>
<td>Internal primer fur gene Synechocystis</td>
<td>503</td>
</tr>
<tr>
<td>ATTS (10)</td>
<td>CGGGGAAAAACCCGGG</td>
<td>Forward primer for cloning x-fur promoter region</td>
<td>451</td>
</tr>
<tr>
<td>AsufE (11)</td>
<td>CTGCGAATATGCTTCCGGCTTAATCTC</td>
<td>Reverse primer for cloning x-fur promoter region</td>
<td>961</td>
</tr>
<tr>
<td>ATTS-Cy5 (12)</td>
<td>CGGGGAAATCCGGATTTGG-Cy5</td>
<td>Forward primer for x-fur primer extension</td>
<td>451</td>
</tr>
<tr>
<td>Tsp fur (13)</td>
<td>CTGAGATGATTGCCTTCGG</td>
<td>Reverse primer for cloning fur promoter region</td>
<td>102</td>
</tr>
<tr>
<td>Tsp fur-Cy5 (14)</td>
<td>CTGAGATGATTGGCTTGG-Cy5</td>
<td>Reverse primer for fur primer extension</td>
<td>102</td>
</tr>
</tbody>
</table>
the sequencing reaction which was used as molecular size marker. Reverse transcription was carried out using Expand Reverse Transcriptase (Roche) with 1 μg total RNA as template and the Cy5-labelled oligonucleotide 12 (ATS5-Cy5) for α-fur RNA and 14 (x-CY5) for fur RNA (Table 1). Samples were analysed in an ALF sequencer (Pharmacia Biotech) as described previously (Sola-Landa et al., 2005).

RESULTS

Occurrence of a cis α-fur RNA is conserved in two unicellular cyanobacteria with different gene contexts

Previous identification of an α-furA RNA as part of a dicistronic operon in the nitrogen-fixing Anabaena PCC 7120 led us to seek the existence of this kind of RNA in unicellular cyanobacteria exhibiting different furA gene contexts. The occurrence of α-fur RNA in the toxigenic cyanobacterium M. aeruginosa PCC 7806 was demonstrated by performing RT-PCR analysis using two pairs of deoxoylgonucleotides (Fig. 1a). Oligonucleotides 1 and 2 cover the whole fur gene (588 bp) and oligonucleotides 3 and 4 amplify an internal region of fur (195 bp). Fig. 1(b) shows a retrotranscription experiment with oligonucleotide 1 followed by a PCR using oligonucleotides 1 and 2, which yields a 588 bp band, which likely corresponds with an antisense RNA of the Mafur gene. The sample treated with RNase did not show amplification, removing the possibility that the amplification was due to chromosomal DNA contamination (Fig. 1b). The presence of Max-fur RNA

Fig. 1. Identification of Ma5-fur RNA. (a) Schematic representation of two regions of the Mafur gene that were amplified. Oligonucleotides are indicated by black arrowheads. (b) RT-PCR analyses of the Ma5-fur RNA. The 1 % agarose gel shows the amplified fragments after the reverse transcription and PCR steps. The sizes (in bp) of the molecular mass markers are indicated on the right. Lanes: 1, molecular mass markers; 2, PCR with oligonucleotides 1 and 2 and chromosomal DNA of Microcystis (positive control); 3, reverse transcriptase step with oligonucleotide 1 and PCR step with oligonucleotides 1 and 2; 4, sample as in lane 3, in the presence of DNase-free RNase; 5, PCR with oligonucleotides 3 and 4 and chromosomal DNA of Microcystis (positive control); 6, RT step with oligonucleotide 3 and PCR step with oligonucleotides 3 and 4; 7, sample as in lane 6 in the presence of DNase-free RNase.

Fig. 2. Identification of Syx-fur RNA. (a) Scheme of the fur locus in Synechocystis PCC 6803 and the oligonucleotides used for identification of Syx-fur RNA. (b) RT-PCR analysis of the Syx-fur RNA. The 1 % agarose gel shows the amplified fragments after the reverse transcription and PCR steps. The size (in bp) of the molecular mass marker are indicated on the right. Lanes: 1, molecular mass markers; 2, reverse transcriptase step with oligonucleotide 7 and PCR with oligonucleotides 7 and 8; 3, sample as in lane 2 in the presence of DNase-free RNase; 4, reverse transcriptase step with oligonucleotide 7 and PCR step with oligonucleotides 7 and 9.
was confirmed by carrying out retrotranscription using oligonucleotide 3 followed by a PCR with oligonucleotides 3 and 4, which yielded a 195 bp amplification product (Fig. 1b). Again, there was no amplification product from the sample treated with RNase.

The expression of the Syz-fur RNA was shown by using RT-PCR (Fig. 2). Retrotranscription with oligonucleotide 7 followed by a PCR using oligonucleotides 7 and 8 yielded a band of 145 bp corresponding to an antisense RNA of the SyfurA orthologue sll0567. There was no amplification product from the sample treated with RNase. This result is in concordance with the recent data taken from the Synechocystis global tsp mapping (Mitschke et al., 2011) which reveals the presence of a tsp within furA (sll0567). The fact that a new retrotranscription experiment with oligonucleotide 7 followed by a PCR using oligonucleotides 7 and 9 did not show any amplification product, indicates that the length of Syz-fur RNA is smaller than those of Anabaena or Microcystis and only covers part of the fur coding sequence.

**Characterization of Max-fur RNA**

With the aim of determining the extent of base-pairing of the Max-fur RNA with the fur locus, we intended to determine their boundaries. Primer extension analysis of Mafur shows that this gene is transcribed from a single tsp that is localized at −102 bp (Fig. 3 and Supplementary Fig. S1, available with the online version of this paper), only 3 bp from the translation start site of dnaJ, indicating that the RNA polymerase-binding site to promote fur transcription has to overlap the dnaJ CDS.

Identification of the 5’ ends of the Max-fur RNA shows three potential transcriptional start sites, whose presence...
was verified in RNA samples from different *Microcystis* cultures (Supplementary Fig. S2, available with the online version of this paper). Localization of the potential Max-*fur* tsps in the sequence of the Mafur locus (Figs 3 and 4) shows a partial overlap between the Max-*fur* transcript starting from tsp1 and the *sufE* gene, which lies tail-to-tail with Mafur and keeps a distance of only 4 bp between both coding sequences, while the RNAs starting from tsp2 and/or tsp3 cover most of the Mafur coding sequence.

Several attempts to identify the 3′ end of Max-*fur* by 3′ RACE were unsuccessful. In order to estimate whether the Max-*fur* RNA could work by occluding the Mafur ribosome-binding site, RT-PCR analysis was performed using oligonucleotides at different positions to approximately define the end of the Max-*fur* RNA. Fig. 5 shows that there was an amplification product from Max-*fur* RNA when oligonucleotides 2 and 5 were used, while there was no amplification detected when oligonucleotides 2 and 6 were used. These data allowed us to locate the 3′ end of the Max-*fur* RNA transcript between oligonucleotides 5 and 6 (see Figs 3 and 4), indicating that the antisense extends beyond the putative Mafur ribosome-binding site and covers part of the *dnaJ* gene that lies divergently to Mafur and is located 105 bp upstream of the coding sequence of the regulator.

**RT-PCR analysis suggests the presence of a second antisense RNA complementary to *sufE***

In order to define the Mafur transcript, attempts to identify its 3′ end were performed using RACE without concluding results. Therefore, the ability of several oligonucleotides located at different distances from the Mafur stop codon to allow amplification in RT-PCR assays was tested. Retrotranscription using oligonucleotide 2 (MFurC) followed by PCR with oligonucleotides 1 (MFurN) and 2 (MFurC) yielded one band of 588 bp (Fig. 6). A second retrotranscription experiment performed with oligonucleotide 11 (ASufE) followed by PCR using oligonucleotides 1 (MFurN) and 11 (ASufE) produced a band of 997 bp, suggesting that Mafur is transcribed in a dicistronic message together with a second putative antisense RNA complementary to *sufE*. The identity of this transcript was confirmed by sequencing the corresponding cDNA cloned in pGEMT (data not shown), and performing the corresponding controls using RNase-treated samples (Fig. 6).

**DISCUSSION**

In this paper we report the expression of x-*fur* RNAs in three cyanobacterial strains with important differences in their metabolism and also exhibiting very different *fur* regions (Fig. 4). In the nitrogen-fixing, filamentous
Anabaena PCC 7120, furA is surrounded by the sigma factor sigC and all1690. z-furA is transcribed in the same message as Alr1690 and extends into the 5'-untranslated region of furA mRNA; this is longer than most ncRNAs described to date in euabacteria. In Synechocystis PCC 6803, the furA orthologue sl0567 is flanked by two hypothetical proteins, while the gene from M. aeruginosa PCC 7806 lies between dnaJ and sufE, as does its orthologue from M. aeruginosa NIES843 (http://genome.kazusa.or.jp/cyanobase/; Martin-Luna et al., 2006). These results indicate that the expression of a cis z-fur RNA is not restricted to Anabaena PCC 7120, but it is spread among different cyanobacterial genera and its occurrence is not linked to a defined furA context.

Analysis of the Mafur locus shows that it presents a rather compressed genetic arrangement. A region of the dual promoter overlaps the Mafur 5'-untranslated region and the 3' end of Max-fur extends into the dual CDS. In addition, the Max-fur tsp1 is located within sufE CDS. The prevalence of overlapping genomic signals, such as functional promoters inside CDSs, as well as the overlap of 3' ends of 137 transcript pairs has been reported in the archaea Halobacterium salinarum (Koide et al., 2009). This phenomenon has also been observed in yeast (Nagalakshmi et al., 2008) and has important consequences on the transcriptional regulation of these organisms. In the case of Microcystis, the presence of z-fur and z-sufE RNAs might add new levels of information to the fur–sufE locus, whose physiological implications should be investigated. In E. coli, the sufBCD operon is regulated by Fur and specifically adapted to synthesize Fe–S clusters when iron or sulphur metabolism is disrupted by iron starvation or oxidative stress (Outten et al., 2004). SufE is involved in the assembly of Fe–S clusters and works coordinately with the SufBCD complex to increase the activity of the cysteine desulphurase SufS (Outten et al., 2003). These proteins are particularly important in cyanobacteria, since they are indirectly involved in the regulation of PSI and its activation by a shift to high light conditions links their synthesis to the photosynthetic activity (Seki et al., 2006). The existence of z-fur and z-sufE in Microcystis might relate Fur expression to Fe–S cluster biogenesis providing the possibility of differential regulation of those genes, increasing the versatility of this ubiquitous, potentially toxic, cyanobacterium.

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