Chromosomally encoded small antisense RNA in Corynebacterium glutamicum

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Abstract

The first observation of chromosomally encoded small antisense RNA in Corynebacterium glutamicum is reported. Transcription oriented in the reverse direction to the transcription of the genes cg1934 and cg1935 was demonstrated within the chromosomal cg1934–cg1935 intergenic region. The transcription was found to be increased after heat shock. The transcriptional start point of this RNA designated ArnA was localized 21 bp upstream of the cg1935 translational start point by primer extension analysis, when the total RNA was isolated from cells grown at 30 °C. After heat shock, the transcriptional start point of an additional species of ArnA RNA was detected 19 bp upstream of the cg1935 translational start point. The stress-response σ factor SigH was found to be involved in the synthesis of ArnA RNAs. The 3′ end of the ArnA RNAs was identified using the 3′-rapid amplification of cDNA ends technique. The length of the two ArnA RNA species was thus determined to be 129 and 131 nt, respectively. The ArnA RNAs were found to overlap the 5′-untranslated region of the transcript of the cg1935 gene coding for a transcriptional regulator of the GntR family. These results suggest that the noncoding ArnA RNAs have a regulatory function.

Introduction

The first prokaryotic regulatory antisense RNAs were found to be encoded by plasmids and other accessory DNA elements more than 20 years ago (for a review, see Brantl, 2002). More recently, chromosomally encoded small regulatory RNAs (sRNAs) were also detected in various bacterial species. In Escherichia coli, almost 80 sRNAs have been identified so far (Storz et al., 2006); however, their biological functions and the molecular mechanisms of their activities have only been elucidated in a minority of cases (for a review, see Gottesman, 2005). In Gram-positive bacteria, chromosomally encoded small noncoding RNAs were found e.g. in Clostridium acetobutylicum (Fierro-Monti et al., 1992), Staphylococcus aureus (Pichon & Felden, 2005) and Bacillus subtilis (Licht et al., 2005; Silvaggi et al., 2005, 2006). Many small noncoding RNAs have been identified as crucial regulatory elements in bacterial stress responses and in bacterial virulence (Gottesman, 2005).

Unlike the antisense RNAs of plasmids and phages, which are encoded on the opposite strand in close proximity to the target gene (cis-encoded) (Brantl, 2002), many of the chromosomally encoded sRNAs characterized so far act as antisense RNAs on the mRNAs of distant genes (trans-encoded), having only imperfect complementarity with their targets (Gottesman, 2005). In E. coli, the cis-encoded antisense sRNAs are mainly associated with repetitive sequences (Pedersen & Gerdes, 1999; Kawano et al., 2002). Of the other cis-encoded antisense sRNAs of E. coli, the GadY regulatory RNA, complementary to the 3′-untranslated region of mRNA encoding the GadX transcriptional regulator (Opdyke et al., 2004) and the SymR regulatory RNA complementary to the 5′ end of the mRNA of the symE gene coding for an SOS-induced toxin (Kawano et al., 2007), have been characterized in detail. Recently, the 280-nt sRNA SurA, overlapping the 5′ end of the yndL gene, was identified in B. subtilis (Silvaggi et al., 2006).

Corynebacterium glutamicum is a Gram-positive asporogenous bacterium widely used as a producer of amino acids (Hermann, 2003). Determination of the complete genome sequence of C. glutamicum ATCC 13032 by two groups (Ikeda & Nakagawa, 2003; Kalinowski et al., 2003) has
Materials and methods

Bacterial strains, plasmids and culture conditions

The basic bacterial strains used in this study were E. coli DH5a (Hanahan, 1985) and C. glutamicum ATCC 13032. The ΔsigH derivative of C. glutamicum ATCC 13032, containing 140-bp internal deletion within the sigH gene on the chromosome, was constructed in the authors’ laboratory by the gene replacement technique (Schäfer et al., 1997). The ΔsigM derivative of C. glutamicum (Nakunst et al., 2007) was kindly provided by A. Tauch (University Bielefeld, Germany). The plasmids used are listed in Table 1. \textit{Escherichia coli} strains were grown at 37°C in Luria–Bertani (LB) medium. \textit{C. glutamicum} strains were grown at 30°C or at 37°C in 2× YT medium (Sambrook et al., 1989) with 0.5% glucose. Incubation at 40°C for 1 h was used as heat-shock conditions. Selective conditions in the media were obtained using antibiotics at the following concentrations (µg mL\(^{-1}\)): ampicillin (Ap) — 100, kanamycin (Km) — 20 and chloramphenicol (Cm) — 10 (for \textit{C. glutamicum}) and 20 (for \textit{E. coli}).

DNA manipulations and transformation of \textit{E. coli} and \textit{C. glutamicum}

Plasmid DNA from \textit{E. coli} was isolated using the method of Birnboim & Doly (1979). Plasmid DNA from \textit{C. glutamicum} was isolated via a modified alkaline extraction procedure (Birnboim & Doly, 1979). Plasmid DNA from \textit{E. coli} and \textit{C. glutamicum} was used for DNA manipulations and transformation of bacterial strains with the ligated 3'-leader region of the mRNA transcript of the gene coding for a transcriptional regulator of the GntR family.

RNA isolation and primer extension analysis

Total RNA from \textit{C. glutamicum} (pET2-PT11-C) or \textit{C. glutamicum} (pET2-F0) was isolated according to Eikmanns et al. (1994). Reverse transcription was performed with Superscript II reverse transcriptase and the fluorescein-labeled primer CM4 (5'GAAATCTCGTGAAGCGTCG-3'), covering positions +21 to +41 relative to the translational start site of the cat gene in plasmid pET2, as described previously (Pátek et al., 2003a). The reverse transcript was run in an automatic ALF DNA Sequencer (Pharmacia Biotech) with the DNA sequencing reactions generated using the same labeled primer.

3'-Rapid amplification of cDNA ends (RACE) technique

Total RNA was treated with DNAsel and dephosphorylated with calf intestinal phosphatase. Dephosphorylated RNA was ligated with the 3’ RNA adapter [5’-P-UUACUGUUUCUACAGGUGUUCGCGGGC-3'] (Dharmacon Research); 3’-inverted deoxythymidine (3’idT') at 17°C overnight as described by Licht et al. (2005). Further treatment of the RNA with the ligated 3’-RNA adapter and reverse transcription using the primer K3RNAAD2 (5’-CGAACCTGTAAGGAGCAGTGGAA-3’) complementary to the 3’-adapter and ThermoSuperScript reverse transcriptase (Invitrogen) were also performed according to Licht et al. (2005). The products of the reverse transcriptions were amplified by two consecutive PCR reactions, using the adapter-specific primer K3RNAAD2 and mRNA-specific primers GNTR5 (5’-AAGTGATATGGAGTTTGTGA-3’) and GNTR6 (5’-TGGTAATTCCATT

Table 1. Plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics*</th>
<th>Sources/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>Vector for TA-cloning (3.0 kb, Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Promega</td>
</tr>
<tr>
<td>pET2</td>
<td>E. coli–C. glutamicum promoter-probe vector (7.5 kb, Km&lt;sup&gt;+&lt;/sup&gt;, promoterless cat gene)</td>
<td>Vasićová et al. (1998)</td>
</tr>
<tr>
<td>pET2-PT11</td>
<td>Three chromosomal Sau3AI fragments in pET2</td>
<td>This work</td>
</tr>
<tr>
<td>pET2-PT11-C</td>
<td>Chromosomal fragment (1 815 285–1 815 185 nt) in pET2</td>
<td>This work</td>
</tr>
<tr>
<td>pET2-R5</td>
<td>Chromosomal fragment (1 815 238–1 815 117 nt) in pET2</td>
<td>This work</td>
</tr>
<tr>
<td>pET2-F0</td>
<td>Chromosomal fragment (1 815 093–1 815 321 nt) in pET2</td>
<td>This work</td>
</tr>
<tr>
<td>pET2-F5</td>
<td>Chromosomal fragment (1 815 039–1 815 236 nt) in pET2</td>
<td>This work</td>
</tr>
</tbody>
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*Ap<sup>+</sup>, ampicillin resistance; Km<sup>+</sup>, kanamycin resistance; coordinates of \textit{C. glutamicum} chromosome are from the GenBank Database (Accession no. NC_006958).
ATGTATAATA-3'), respectively. The resulting PCR fragment was cloned into the pGEM-T vector and sequenced.

Enzyme assay and determination of minimal inhibitory concentration

The specific activity of chloramphenicol acetyltransferase (CAT) in C. glutamicum cell-free extracts was determined using the method of Shaw (1975). The values reported represent the means from at least three independent experiments, and the values of SD are shown. The minimal inhibitory concentration (MIC) of chloramphenicol for C. glutamicum strains was determined on 2×YT plates using the method of Ozaki et al. (1984).

Results and discussion

Promoter activity within the cg1934–cg1935 intergenic region of C. glutamicum chromosome

The C. glutamicum ATCC 13032 promoter library was constructed by cloning Sau3AI chromosomal fragments, ranging in size from 100 to 500 bp, in the promoter-probe vector pET2 carrying the promoterless cat reporter gene coding for CAT. During the screening of heat-shock promoters in this library, a transformant (designated PT11) resistant to a higher level of chloramphenicol at 37°C (MIC of chloramphenicol: 90 µg mL⁻¹) than at 30°C (MIC of chloramphenicol: 70 µg mL⁻¹) was obtained. It was found that the plasmid pET2 with three Sau3AI fragments (PT11-A, PT11-B and PT11-C), corresponding to the regions of the C. glutamicum chromosome with coordinates 1011230–1011395, 66729–66875 and 1815185–1815285 respectively, is harbored by the PT11 strain. The separate cloning of PCR fragments identical to fragments PT11-A, PT11-B and PT11-C into pET2 proved that only the PT11-C fragment possessed promoter activity. CAT activity in the cell extract of C. glutamicum (pET2-PT11-C) grown at 30°C was 1.34 ± 0.13 µmol min⁻¹ mg of protein⁻¹. A substantially higher CAT activity (2.30 ± 0.43 µmol min⁻¹ mg of protein⁻¹) was measured in the same strain when it was incubated for 1 h at 40°C before cell lysis, suggesting that promoter activity was induced by the heat shock. The PT11-C fragment (101 bp) contained the 5’ end of the cg1935 gene (coding for a transcriptional regulator of the GntR family according to the GenBank Database data) and part of the untranslated intergenic cg1934–cg1935 region. Surprisingly, expression of the cat reporter gene was only observed when the cloned fragment was oriented in such a way that the transcription of the cg1935 gene proceeded divergently from cat. These results suggested that antisense RNA, induced by the heat shock, was synthesized within the cg1934–cg1935 intergenic region.

Evidence for antisense transcription within the cg1934–cg1935 intergenic region and determination of the length of the transcripts

To verify the transcription in the direction opposite to the transcription of the cg1934 and cg1935 genes and to map the 5’ end of the transcript, a primer extension analysis of the total RNA isolated from C. glutamicum (pET2-PT11-C) was performed. As shown in Fig. 1a, a single peak was detected, representing the product of reverse transcription from oligonucleotide primer CM4 complementary to the vector pET2, when total RNA was isolated from cells grown at 30°C. The start site of transcription in the direction opposite to the transcription of the cg1935 gene was mapped as the base G located 21 bp upstream of the cg1935

![Fig. 1. Determination of transcriptional start points (TSPs). (a) Determination of ArnA TSPs. The bottom peaks (PEX) represent cDNA synthesized in reverse transcription (primer extension) using RNA from Corynebacterium glutamicum. The peaks generated by the automatic sequencer represent the products of sequencing reactions (A, C, G, T) performed with the same fluorescein-labeled primer as that used for primer extension. A portion of the nucleotide sequence derived from the sequencing signals is shown below. Note that the sequence is complementary to that shown in Fig. 2. The TSPs (underlined) were determined by a comparison of the positions of the primer extension products and the sequencing signal. (b) Determination of the cg1935 TSP.](https://academic.oup.com/femsle/article/279/2/195/514067)
translational start site and the putative promoter hexamers (−10 TTCTTT, −35 GGTAAA) were determined (Fig. 2). The −10 hexamer shows moderate similarity to the *C. glutamicum* housekeeping promoter consensus sequence TA(c/t)aaT (Pátek et al., 2003b), while no apparent −35 consensus motif was found. However, the absence of a distinct −35 motif had been observed in many *C. glutamicum* promoters (Pátek et al., 2003b). The essential role of this region in transcriptional activity was confirmed by the observation that a cell extract of *C. glutamicum* harboring the plasmid pET2 containing the fragment with deletion of the putative −10 and −35 hexamers (pET2-R5) showed no promoter activity (CAT activity < 0.001 μmol min⁻¹ mg of protein⁻¹).

When the culture of *C. glutamicum* (pET2-PT11-C) was incubated for 1 h at 40 °C before the isolation of total RNA, an additional peak representing another primer extension product was detected (Fig. 1a). This peak corresponds to an additional transcript, longer by two nucleotides, starting at the base G located 19 bp upstream of the cg1935 translational start point (TSP) (Fig. 2). The putative promoter hexamers (−10 TGTCTT−35 GAGGTA), containing motifs that are highly similar to the GTT and GGaA motifs typical for promoters recognized by the alternative extracytoplasmic function (ECF) σ factors SigE and SigH in *Mycobacterium tuberculosis* (Rodrique et al., 2006) and by SigH and SigM in *C. glutamicum* (Engels et al., 2004; Nakunst et al., 2007), might represent an additional promoter, which drives transcription from the heat-shock TSP. As shown in Fig. 2, the distance between the GGTA and GTT motifs is 18 bp, which is the same as the distance between the GGAA and GTT motifs in the *C. glutamicum* *clpC* promoter recognized by SigH under heat stress (Engels et al., 2004).

To test whether an ECF σ factor is involved in the antisense transcription within the cg1934–cg1935 intergenic region, the plasmid pET2-PT11-C was transferred into *C. glutamicum* ΔsigH and ΔsigM strains and CAT activity in the cell extracts of these strains grown at 30 °C or incubated for 1 h at 40 °C before cell lysis was determined. CAT activities in the cell extracts of *C. glutamicum* ΔsigM (pET2-PT11-C) (1.54 ± 0.01 μmol min⁻¹ mg of protein⁻¹) after growth at 30 °C and 1.91 ± 0.10 μmol min⁻¹ mg of protein⁻¹ after incubation for 1 h at 40 °C) were similar to those assayed in the wild-type *C. glutamicum* (pET2-PT11-C) (1.34 ± 0.13 μmol min⁻¹ mg of protein⁻¹ and 2.30 ± 0.43 μmol min⁻¹ mg of protein⁻¹, respectively). On the other hand, CAT activities in the cell extracts of *C. glutamicum* ΔsigH (pET2-PT11-C) (0.86 ± 0.12 μmol min⁻¹ mg of protein⁻¹ after growth at 30 °C and 0.92 ± 0.21 μmol min⁻¹ mg of protein⁻¹ after incubation for 1 h at 40 °C) were significantly lower than those assayed in the wild-type *C. glutamicum* (pET2-PT11-C). Moreover, no positive effect of heat shock on promoter activity was observed in the *C. glutamicum* ΔsigH strain. These results indicate that the ECF σ factor SigH is involved in the synthesis of the *C. glutamicum* small antisense RNAs, especially under heat-shock conditions. The involvement of an ECF σ factor in sRNA synthesis has also been proved recently in *E. coli* where the synthesis of MicA and RybB sRNAs was found to be controlled by the ECF σ factor SigE (Johansen et al., 2006; Udekwu & Wagner, 2007). The residual CAT activity assayed in the cell extracts of *C. glutamicum* ΔsigH (pET2-PT11-C) was caused undoubtedly by the transcription from the other promoter recognized very probably by the primary σ factor SigA. Two overlapping promoters, a SigA-dependent one and a SigH-dependent one, were also found to drive transcription of the *C. glutamicum clpC* gene (Engels et al., 2004).

The 3′ end of the RNA, corresponding to the base T located 149 bp upstream of the cg1935 translational start point and 22 bp downstream of the cg1934 termination codon in the DNA sequence, was determined using the 3′-RACE technique (Fig. 2). The 129-nt long RNA, designated ArnA (antisense RNA), is thus synthesized divergently from the cg1935 gene within the chromosomal cg1934–cg1935 intergenic region.
under standard conditions, while after heat shock, an additional 131-nt ArnA RNA species is also produced.

The computer prediction of secondary RNA structures revealed a hairpin structure with a 14-bp stem ($\Delta G = -18.5$ kcal mol$^{-1}$) at the 3'end of ArnA RNAs, which might act as the rho-independent transcriptional terminator (Fig. 3). As shown in this figure, ArnA RNA species differ in further predicted secondary structures, which are significantly weaker than the putative terminator according to their free energy values.

A search for potential ORFs in the ArnA RNAs revealed an ORF of 24 codons in length. Because this potential ORF lacks an appropriately positioned ribosome-binding site (RBS) and no homolog of its deduced protein product was found in the GenBank Database, it is unlikely that the small ArnA RNAs encode a peptide. Therefore, the small ArnA RNAs, transcribed in the direction opposite to the transcription of the $C.\ glutamicum$ genes cg1934 and cg1935, are very probably noncoding regulatory RNAs.

**Antisense RNAs overlap the 5’ untranslated region of the cg1935 mRNA**

To determine whether the ArnA antisense RNAs can interact with cg1935 mRNA, the transcriptional start point of the cg1935 mRNA was determined by primer extension analysis of the total RNA isolated from the $C.\ glutamicum$ harboring plasmid pET2-F0. This plasmid carries a 274-bp chromosomal fragment (coordinates 1815039–1815312) cloned in the direction allowing expression of the cat reporter gene from the promoter of the cg1935 gene. As shown in Fig. 1b, a single peak was detected as a result of reverse transcription with the CM4 primer complementary to the pET2 vector. The TSP was mapped as the base T located 117 bp upstream of the cg1935 translational start site (Fig. 2). The putative promoter hexamers (−10 TCTTTT, −35 ACCACA) show moderate similarity to those of the $C.\ glutamicum$ housekeeping promoter consensus sequences (TA[ct]aT and ttGcca) (Pátek et al., 2003b). Determination of the 5’ ends...
of the oppositely oriented RNA transcripts within the cg1934–cg1935 intergenic region proved that substantial parts (97 nt from 129 nt or 99 nt from 131 nt) of the antisense ArnA RNAs overlap the 5′-untranslated region of the cg1935 mRNA (Fig. 2). The newly discovered small ArnA RNAs of C. glutamicum are thus cis-encoded antisense RNAs whose regulatory role has been suggested. Unlike the widespread cis-encoded antisense regulatory RNAs of plasmids and bacteriophages, the number of chromosomally encoded 5′ antisense sRNAs described so far is rather limited. In E. coli, five different 5′-antisense RNAs were found to be involved in negative control of genes coding for various toxins (Pedersen & Gerdes, 1999; Kawano et al., 2002, 2007). The 280-nt sRNA SurA, overlapping the 5′ end of the yndL gene, was found to be activated during the sporulation process in S. subtilis (Silvaggi et al., 2006).

To find out whether expression of the cg1935 gene is also affected by heat shock, the activity of the cg1935 promoter was analyzed using the transcriptional fusion with the cat gene within the pET2. The CAT activity in a cell extract of C. glutamicum (pET2-F0) grown at 30 °C was found to be 0.124 ± 0.032 μmol min⁻¹ mg of protein⁻¹, while a significantly lower CAT activity (0.069 ± 0.025 μmol min⁻¹ mg of protein⁻¹) was measured in the same strain when its culture was incubated for 1 h at 40 °C before cell lysis. To test whether the observed decrease of cg1935 expression under the heat-shock conditions is caused by the interaction of cg1935-mRNA with the assumed increased amount of antisense ArnA RNA synthesized at 40 °C, the promoter P-cg1935 activity of the 200-bp chromosomal fragment F5 (coordinates 1815039–1815238) lacking the ArnA promoter was also estimated. The CAT activities in the cell extracts of C. glutamicum (pET2-F5) were found to be 0.038 ± 0.007 μmol min⁻¹ mg of protein⁻¹ after growth at 30 °C and 0.032 ± 0.006 μmol min⁻¹ mg of protein⁻¹ after incubation for 1 h at 40 °C. The observed significant decrease of P-cg1935 activity under the conditions of lack of ArnA RNAs suggested that these antisense RNAs exert a positive effect on expression of the cg1935 gene. The decrease of P-cg1935 activity found after heat shock when the ArnA RNAs are synthesized [in C. glutamicum (pET2-F0)] has not thus been caused by interaction of antisense ArnA RNAs with the cg1935-mRNA but by another negative regulatory mechanism. Generally, analysis using the transcriptional fusions in the promoter-probe vector pET2 cannot reveal regulatory effects at the translational level. The suggested positive effect of ArnA RNAs on the cg1935 gene expression could thus be explained by an interference of these RNAs with transcription of the cg1935 gene by still unknown mechanisms or by their positive effects on cg1935-mRNA stability. Stabilization of some mRNAs by the action of chromosomally encoded sRNAs was described in E. coli (Opdyke et al., 2004; Gottesman, 2005). Understanding the mechanisms by which the antisense ArnA RNA species control the cg1935 gene expression requires further extensive analyses.

The cg1935 gene codes for a transcriptional regulator of the GntR family (FadR subfamily). According to the BLAST search, this protein showed the highest similarity with the paralogous protein encoded by the C. glutamicum cg2783 gene (identity 78%). The cg1935 gene is located on the C. glutamicum chromosome within the cluster of 24 genes oriented in the same direction (15 of them have no homologs in other bacteria). These findings suggest that the cg1935 gene product might be unique to C. glutamicum. Elucidation of the function of this transcriptional regulator would be necessary for understanding the biological role of the newly discovered C. glutamicum antisense regulatory RNAs.

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