Bacillus clausii and Bacillus halodurans lack GlnR but Possess Two Paralogs of glnA

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Abstract

Bacillus clausii and Bacillus halodurans lack GlnR but possess a single TnrA regulator of nitrogen assimilation and two paralogs of glnA. Bacillus clausii contains two paralogs of the gene encoding the glutamine synthetase (GS), glnA1 (ABC3940) and glnA2 (ABC2179). The glnA1 gene contains a TnrA site. This TnrA site is located downstream of the -10 region of the promoter. However, the glnA2 gene does not contain the TnrA site at its regulatory region. Bacillus halodurans possesses two paralogs of glnA, both with TnrA-binding sites. The glnA1 (BH2360) gene contains a TnrA site, which overlaps the -10 region of the glnA1 promoter, and the glnA2 (BH3867) gene contains a TnrA site downstream of the -10 region of its promoter. Also, the Bacillus subtilis dicistronic glnRA operon, which encodes GlnR and GS, contains two TnrA sites (glnRAo1 and glnRAo2) in its promoter region. The glnRAo2 site, which overlaps the -35 region of the glnRA promoter, was shown to be required for regulation by TnrA. These results indicate that TnrA regulates the expression of GS, through binds to TnrA sites which overlaps the -35 and -10 promoter elements or lies downstream of the theirs of the glnA or glnRA promoters.

Keywords: Gene regulation; Glutamine synthetase; Transcription factors; TnrA; GlnR

Introduction

The MerR family is a group of transcriptional activators, which regulates gene expression and controls transcription in response to diverse physiological signals [1], such as nitrogen availability [2]. The GlnR and TnrA belong to the MerR family of DNA-binding regulatory proteins. This group of activators contains a conserved N-terminal DNA-binding domain that is approximately 70 amino acids in length [1,3]. The Bacillus clausii and Bacillus halodurans TnrA transcription factors have been found to contain 100 amino acids. Also, the Bacillus subtilis TnrA, as one of the best understood members of the MerR family, composed of 110 amino acids.

In B. subtilis, Bacillus licheniformis, Geo bacillus Kaustophilus and Oceanobacillus iheyensis, the two transcription factors TnrA and GlnR control many genes for utilization of glutamine and other nitrogen-containing compounds. Bacillus clausii and B. halodurans lack GlnR but possesses a single TnrA regulator of nitrogen assimilation and two paralogs of glnA. Other Gram-positive bacteria, such as Streptococcus, Listeria and Staphylococcus lack TnrA but possess the highly conserved GlnR regulon, which mainly contains genes of glutamine transport and utilization [4].

The B. subtilis TnrA is a global regulator that responds the availability of nitrogen sources and both activates and represses many B. subtilis genes during nitrogen limitation. It is involved in the direct and indirect regulation of many genes, which are involved in the transport and catabolism of nitrogen-containing compounds [5-7]. The B. subtilis GlnR regulates the expression of the glnRA operon, which encodes glutamine synthase (GlnA). Under conditions of a nitrogen excess, GlnR functions as a repressor of the glnRA operon [4,7]. When nitrogen sources are in excess, the B. subtilis glutamine synthetase (GS), a key enzyme in nitrogen metabolism, becomes subject to feedback inhibition by glutamine and adenosine monophosphate (AMP). The feedback-inhibited GS forms a complex with TnrA via its C-terminal domain, thereby preventing TnrA from interacting with specific operators and regulating gene expression (6). In contrast, under conditions of nitrogen-limited growth, TnrA is released from the GS–TnrA complex and then binds to the TnrA sites of specific operators, consequently regulating transcription [3].

The TnrA of B. subtilis, as well as activating its own expression also activates transcription of the gabP, nasA, nasB, nasDEF, nrgAB, and the puc genes, and represses that of the glnRA, glnAB, and alsT operons [7,8]. A genome-wide analysis for TnrA-regulated genes of B. clausii associated with a TnrA box was shown that there were some transcription units containing a putative TnrA box, such as tnrA, glnA, nrgA, nasFDEB, and puc genes [9]. It has recently been suggested that the TnrA is also involved in expression of the extracellular alkaline protease (aprE), with a link existing between aprE expression and the B. subtilis GlnA-TnrA system [10]. It has previously been found that aprE expression increases when the GS gene, glnA, is disrupted. This increase in expression has been attributed to a decrease in the expression of socC, which encodes a negative regulator of aprE expression. It has also been observed that the effect of glnA on socC expression is abolished by the further disruption of tnrA, thus indicating that aprE expression is under global regulation through TnrA [10].

Bacillus clausii is also known to produce a commercially important extracellular alkaline serine protease (AprE) [11,12]. Elucidating of the molecular mechanisms of the metabolism and gene regulatory networks could thus be used to design metabolic engineering strategies.
for maximizing alkaline serine protease production in *B. clausii*. The aim of this work was to distinguish and analyze the potentially TnrA sites of *glnA* promoter regions of *B. clausii* and *B. halodurans*, responsible for metabolism of nitrogen, and thus have an insight into the nature of the regulation of this metabolic system and reveal the similarities and differences of the associated transcriptional regulatory networks, present in *B. clausii*, *B. halodurans* and *B. subtilis*.

**Materials and Methodology**

**Bacterial strains and nucleotide sequences**

The complete genome sequence of *B. clausii* KSM-K16 and *B. halodurans* C-125, were obtained from GenBank (accession number, AP006627.1 and NC_002570, respectively) (http://www.ncbi.nlm.nih.gov) [13]. The nucleotide sequences of the promoter and the coding region of the *tnrA* belonging to *B. clausii* EHY L2 deposited previously in GenBank were also used in this study (accession number, HM488959). Various TnrA protein sequences applied to this investigation are as follows: YP_175256 (*B. clausii* KSM-K16), HM488959 (*B. clausii* EHY L2), NP_242360 (*B. halodurans* C-125), YP_001813196 (Exiguobacterium sibiricum 255-15), YP_078674 (*B. licheniformis* ATCC 14580), NP_389214 (*B. subtilis* subsp. *subtilis* str. 168), YP_001420907 (*B. amyloliquefaciens* FZB42), YP_001486473 (*B. pumilus* SAFR-032), NP_691871 (*Oceanobacula* thesensis HTE831), YP_002886828 (Exiguobacterium sp. AT1b) and YP_002949574 (Geobacillus sp. WCH70).

**Prediction of the TnrA boxes of *tnrA* and *glnA* promoter regions of *B. clausii* and *B. halodurans***

For prediction of the TnrA boxes of *tnrA* and *glnA* promoter regions of *B. clausii* and *B. halodurans*, the entire genomic nucleotide sequence of *B. clausii* KSM-K16 and *B. halodurans* C-125 obtained from GenBank were analyzed [13]. The 17-bp-long conserved DNA motif represented by the consensus sequence 5′-TGTTAN7TNACA-3′ for the TnrA box, was then entered into the nucleotide basic local alignment search tool (blastn) at the NCBI site (http://www.ncbi.nlm.nih.gov) [13] as a query sequence. If the putative TnrA binding site was located upstream of the transcription start site (TSS) and -35 and -10 promoter elements, the position was considered as a TnrA site, which overlaps the -10 region of the promoter, TnrA could act as a repressor that blocks the transcription of the gene. The -10 region of the promoter, TnrA could act as a repressor that blocks the transcription of the gene [17].

**Phylogenetic analysis of TnrA**

The TnrA and *glnA* sequences of *B. clausii* and *B. halodurans* were compared with its ortholog sequences in the NCBI database using BLAST and aligned using the molecular evolutionary genetics analysis (MEGA) software, version 4.0 [18]. Phylogenetic trees were subsequently constructed by the neighbor-joining (NJ) method.

**Results**

**Nucleotide sequence of the *glnA* promoter regions of *B. clausii* and *B. halodurans***

A genome analysis for *glnA* promoter regions of *B. clausii* and *B. halodurans* associated with a TnrA box was performed. *Bacillus clausii* contains two paralogs of the gene encoding the GS, *glnA1* (ABC3940) and *glnA2* (ABC2179). The *glnA1* gene, whose product has 452 amino acids, contains a TnrA site, 87 bp upstream of the translation start site. This TnrA site is located downstream of the -10 region of the promoter (Figure 1). Comparison of the deduced amino acid sequences of this *B. clausii* GS with other bacteria revealed that the *B. halodurans* GS (BH3867) has a high degree of similarity (91%) with that of *B. clausii*. However, the *B. clausii* *glnA2* (ABC2179) gene, whose product has 449 amino acids, does not contain the TnrA site at its regulatory region. In fact, the *GlnA1* and *GlnA2* proteins of *B. clausii* were found to have only 69% similarity sequence (Figure 2).

It was also observed that *B. halodurans*, as in *B. clausii*, possesses two paralogs of *glnA*, with TnrA sites. The *glnA1* (BH2360) gene contains a TnrA site, which overlaps the -10 region of the *glnA1* promoter, and the *glnA2* (BH3867) gene contains a TnrA site downstream of the -10 region of its promoter. Considering the position of the TnrA box of GS genes in *B. clausii* and *B. halodurans*, downstream or overlapping the -10 region of the promoter, TnrA could act as a repressor that blocks initiation and/or elongation during the transcription process.

**Nucleotide sequence of the *tnrA* promoter regions of *B. clausii* and *B. halodurans***

Alignment of TnrA sites in the promoters was used to compare the *tnrA* promoter sequences in *B. clausii* KSM-K16 and EHY L2, *B. halodurans* and *B. subtilis*. The *tnrA* gene of the two *B. clausii* strains, KSM-K16 and EHY L2, contains two TnrA sites in its promoter region, with a 25 bp interspace. The TnrA site1 overlaps with the -10 region and TnrA site2 lies upstream of the -35 region of the *tnrA* promoter. Comparison of the *tnrA* promoter sequences in *B. clausii* KSM-K16 and EHY L2 revealed that the TnrA boxes of both strains are located in the same positions, have similar sequences and consist of -10 and -35 elements in their TnrA promoter regions. The TnrA gene of the *B. clausii* and *B. subtilis* contains two TnrA sites in its promoter region with a 26 bp interspace. The *tnrA* gene of the *B. halodurans*, contains a TnrA sites in its promoter region (Figure 3).

**Structure and properties of the *B. clausii* and *B. halodurans* TnrA proteins**

The *B. clausii* and *B. halodurans* TnrA proteins are smaller than most MerR family members. It contains 100 amino acids and two domains (Figure 4). A conserved N-terminal DNA binding domain is located between residues 5 and 76. Based on the crystal structures
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Figure 1: Comparison of the nucleotide sequences of the GS promoter regions in B. clausii, B. halodurans and B. subtilis. The proposed -35 and -10 promoter elements are underlined. The TnrA sites are shown in boxes. The translational start site is indicated by bold italic letters.

Figure 2: The chromosomal regions of the B. clausii and B. halodurans glnA are compared with other similar organisms. Sets of genes with similar sequence are grouped with the same number; a) The graphic is centered on the B. clausii glnA1 (ABC3940) and B. halodurans glnA2 (BH3867) genes, which numbered 1. b) The graphic is centered on the B. clausii glnA2 (ABC2179), B. halodurans glnA1 (BH2360) and B. licheniformis and B. subtilis glnA genes, which numbered 1, the number 2 is aluminum resistance protein and the number 3 is GlnR.

Figure 3: Comparison of the nucleotide sequence of the tnrA promoter region in B. clausii KSM-K16, B. clausii EHY L2, B. subtilis and B. halodurans. The proposed -35 and -10 promoter elements are underlined. The TnrA sites are shown in boxes. The translational start site is indicated by bold italic letters.

Figure 4: A graphical view of the secondary structure and domains of the B. clausii and B. halodurans TnrA proteins, where α-helices and β-strands are represented by cylinders and arrows. The coiled-coil domain is indicated by underlined bold letters and interface amino acids are underlined.
of the multidrug-binding transcription regulator BmrR of *B. subtilis* and secondary structure prediction by PSIPRED, this domain was shown to contain a β-strand, a helix-turn-helix motif formed by helices 1 and 2, and a second wing formed by helices 3 and 4. A conserved 15-amino-acid C-terminal region was also found, which like other TnrA orthologs, functions as a signal transduction domain. In fact a similar domain in the TnrA of *B. subtilis* has also been reported to be involved in signal transduction [19-21].

Using the COILS program [17], the C-terminal region of TnrA was predicted to contain coiled-coil structures, arising from the association of amino acid residues (68 to 83) with other similar C-terminal regions of the TnrA. Furthermore, Ile-70, Met-73, Ala-77 and Lys-80 were recognized as interface residues on α-helices 4 and 5 of the *B. clausii* TnrA protein (Figure 4).

**Phylogenetic analysis of tnrA and glnA genes**

Comparison of the deduced amino acid sequences showed that there was strong homology between the TnrA of *B. clausii* and *B. halodurans* (98% similarities at the amino acid levels). The generated phylogenetic tree showed that the TnrA sequences of *B. clausii* and *B. halodurans* were grouped together. Also, *Bacillus pumilus*, *B. subtilis*, *Bacillus amyloliquefaciens* and *B. licheniformis* fell into the same clade (Figure 5). Furthermore, the GlnA sequences of *B. clausii* (ABC2179) and *B. halodurans* (BH2360) were grouped together and *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* fell into the same clade (Figure 5).

It is important to note that the alkaline protease of *B. clausii*, similar to that of *B. subtilis*, is also expressed in abundance under nitrogen-limited conditions [10]. So it may be possible that the *aprE* (coding for alkaline protease) of *B. clausii* is also under nitrogen regulation through the GlnA-TnrA pathway. On this basis, a nitrogen-replete status in the cell may be a situation where TnrA is captured by complex formation with feedback-inhibited GlnA. Therefore, we propose to construct a potent *B. clausii* for the production of alkaline serine proteases by the disruption of *glnA* or truncation of the C-terminal region of *tnrA*,

![Figure 5: Phylogenetic trees of the TnrA (a) and GlnA (b) homologs from Bacillus species. Numbers above branches represent bootstrap values (1000 replicates) using the neighbor joining (NJ) method.](image)
which will lead to the release of TnrA from the feedback-inhibited GlnA, thus mimicking a nitrogen limited situation.

Discussion

The transcription factor, TnrA, which is involved in the control of nitrogen metabolism is a monocistronic gene that together with its orthologs, has been reported in many Bacillus genomes and related genera, such as *O. iheyensis* orthologs, has been reported in many *Bacillus* species. Nitrogen metabolism is a monocistronic gene that together with its orthologs has been observed to lack the TnrA promoter sequences in *B. clausii*, *B. halodurans* and *B. subtilis*. Revealed that these bacteria have one or two sequences representing the TnrA Box in the promoter region of TnrA. The distance between the two TnrA box sites is approximately equal, containing 25 and 26 nucleotides in *B. clausii* and *B. subtilis*, respectively [22]. In future research, we propose to carry out experimental analysis of all TnrA promoters with regard to the location of the TnrA box in *Bacillus* strains that carry this transcriptional regulator.

Comparison of TnrA promoter sequences in *B. clausii* and *B. subtilis* reveals that the -10 and -35 regions have mismatches with the σ^A^-dependent promoters with a low level of intrinsic transcriptional activity [22]. The TnrAs of *B. clausii*, *B. halodurans*, *Geobacillus* WCH70, *O. iheyensis*, *Exiguobacterium sibiricum* and *Exiguobacterium AT1b* contain 99-101 amino acids, while the TnrAs of *B. subtilis*, *B. amylophiliquefaciens*, *B. licheniformis*, and *B. pumilus* contain 109-103 amino acids [3]. Comparison of the deduced amino acid sequences of *B. clausii* TnrA with other bacteria showed extensive similarity (98%) with *B. halodurans* and high degree (80%) with *B. subtilis*.

*Bacillus clausii* contains two paralogs of *glnA1* (ABC3940) and *glnA2* (ABC2179), encoding GS, of which only *glnA1* has the TnrA box. Also, *B. halodurans* contains two paralogs of *glnA1* (BH2360) and *glnA2* (BH3867), both with a TnrA box. This study proposes to carry out future experimental analysis of genes, *B. clausii* and *B. halodurans* coding for TnrA transcription factors with a low level of intrinsic transcriptional activity [22]. The TnrAs of *B. clausii*, *B. halodurans*, *Geobacillus* WCH70, *O. iheyensis*, *Exiguobacterium sibiricum* and *Exiguobacterium AT1b* contain 99-101 amino acids, while the TnrAs of *B. subtilis*, *B. amylophiliquefaciens*, *B. licheniformis*, and *B. pumilus* contain 109-103 amino acids [3]. Comparison of the deduced amino acid sequences of *B. clausii* TnrA with other bacteria showed extensive similarity (98%) with *B. halodurans* and high degree (80%) with *B. subtilis*.

Considering the TnrA-binding site of the monocistronic glnA operon is preserved in both *B. clausii* and *B. halodurans*, it could be that in the *B. subtilis*, the TnrA-binding sites of the dicistronic glnA operon play an important role in controlling the production of GS rather than that of the GlnR transcription factor. In fact, in *B. subtilis*, GlnR is only involved in the regulation of its own operon (glnRA) and tnrA [22]. Hence, it may be possible that GlnR has a weak regulatory role in the nitrogen metabolism of *B. subtilis*. It proposed that the TnrA-regulated genes, glnA, tnrA and nrgA, play an important role in nitrogen metabolism of most bacilli [9].

Comparison of TnrA regulons, which contain the TnrA sites of *B. clausii* and *B. subtilis* revealed that the general transcription factor, TnrA (tnrA), the glutamine synthetase gene (glnA), oligopeptide ABC transporter operons, the assimilatory nitrate and nitrite reductase operon (nas), the genes of purine catabolism (puc) and ammonium transport (nrgA), are conserved in both *B. clausii* and *B. subtilis* bacteria [4,7,9].

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