DtxR, an iron-dependent transcriptional repressor that regulates the expression of siderophore gene clusters in Thermobifida fusca

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One sentence summary: An iron-dependent transcriptional repressor that regulates the expression of siderophore gene clusters has been found in Thermobifida fusca.

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ABSTRACT

Thermobifida fusca is an aerobic, moderately thermophilic, filamentous soil bacterium. Iron is an essential metal involved in the vital metabolic functions in microorganisms. Thermobifida fusca was found to survive in the iron-deficiency condition and produce siderophores which synthesized to acquire iron. The iron transport was regulated by DtxR, a prototypic member of a superfamily of transition metal ion-activated transcriptional regulators. Tfu\textsubscript{0249} was found to be working as DtxR in T. fusca. The promoter regions of the three gene clusters related to the iron transport bound and regulated by Tfu\textsubscript{0249} were identified by bioinformatics analysis. The gel shift assays confirmed that Tfu\textsubscript{0249} bound the binding sites firmly on the upstream of three gene clusters. By comparing the binding sites of three clusters and gel shift assay, TWAGGTWAGSCTWACCTWA was found to be recognized and bound by Tfu\textsubscript{0249} to control iron transport in T. fusca.

Key words: Thermobifida fusca; DxtR-like repressor; gel shift assay

INTRODUCTION

Iron is an essential metal involved in the vital metabolic functions in microorganisms. The redox of iron plays crucial roles in biocatalysis and the electron transport chain (Miethke and Marahiel 2007). Consequently, the scavenging of iron from the host by pathogenic microorganisms is an important factor in many infections (Merchant and Spatafora 2014). Although iron is abundantly present in the environment, ionic forms of iron are insoluble under physiological conditions and hence are difficult to assimilate for microorganisms. In most microbial habitats, Fe(II) is oxidized to Fe(III) either spontaneously by reacting with molecular oxygen or enzymatically during assimilation and circulation in host organisms; in the environment, Fe(III) forms ferric oxide hydrate complexes ($\text{Fe}_2\text{O}_3 \times n\text{H}_2\text{O}$) in the presence of oxygen and water at neutral to basic pH (Miethke and Marahiel 2007). These complexes are very stable, leading to a free Fe(III)
concentration of $10^{-9}$ to $10^{-18}$ M. In microorganisms, the most ubiquitous and important of these is the biosynthesis and secretion of high-affinity ferric iron chelators called siderophores (Oves-Costales, Kadi and Challis 2009). Siderophore-mediated scavenging of ferric iron from hosts contributes significantly to the virulence of pathogenic microbes (Oves-Costales, Kadi and Challis 2009).

Due to the tight relationship between siderophore utilization and virulence, the strategy of iron-dependent pathogen control is a promising field for future investigations and offers a broad array of possible therapeutic applications. While the siderophore-antibiotic strategy uses Fe-siderophore uptake systems as gateways for cellular infiltration with established antibiotics, the siderophore pathway inhibition strategy tries to abolish siderophore utilization in order to starve the pathogens out for iron. Both concepts proved to be successful with in vitro and ex vivo culture model systems (Miethke and Marahiel 2007).

Thermobifida fusca is an aerobic, moderately thermophilic, filamentous soil bacterium (Fong and Deng 2010). It is a major degrader of plant cell walls in heated organic materials such as compost piles, rotting hay or manure piles (Wilson 2004). Thermobifida fusca contains one gene cluster belonging to the non-ribosomal peptide (NRP) family of natural products: fsca, fsCB, fsCc, fsCd, fsCe, fsCF, fsG, fsch, fscI (Dimise, Widboom and Bruner 2008). The cluster contains the siderophore-producing enzymes, with biosynthetic pathways to the iron-chelating 2,3 dihydroxybenzyl and hydroxamide moieties. Three Non-ribosomal Peptide Siderophores (NRPs) have been identified: fuscachelin A, B, C (Dimise, Widboom and Bruner 2008). Siderophore natural products most often bind to extracellular ferric iron through the formation of a hexa-coordinate, octahedral chelation complex (Oves-Costales, Kadi and Challis 2009). The T. fusca siderophore can achieve this through the production of a heteromeric NRP with two terminal catechols and an internal hydroxamate. However, the growth of T. fusca in the iron-depletion media is very slow. A mutant strain with high yield of NRPs in regular media with iron is extremely important for isolation and industrial application.

In bacteria, gene regulation of siderophore utilization and iron homeostasis in general is mediated mainly at the transcriptional level by the ferric uptake repressor (Fur) or the diphtheria toxin repressor (DtxR). While Fur is the global iron regulator in many Gram-negative and low-GC-content Gram-positive bacteria, DtxR fulfills a comparable role in Gram-positive bacteria with a high GC content (Visca et al., 1992). The DtxR is the prototypic member of a superfamily of transition metal ion-activated transcriptional regulators that have been isolated from Gram-positive prokaryotes (Love et al., 2004). DtxR has been confirmed as the master regulator of iron-dependent gene expression Corynebacterium glutamicum (Love et al., 2004). Based on the sequence of DtxR, by analyzing the genome sequence, it was found that Tfu.0249 had almost the same sequence to DtxR. DtxR protein has been identified to bind sequence TWAGGTWAGSCTWACCTWA (Tao and Murphy 1994) in C. diphtheriae. There might be more binding sites for Tfu.0249 because the binding sites of DtxR-like proteins in different species are different (Wennerhold and Bott 2006). In this study, Tfu.0249 was overexpressed and purified in Escherichia coli. The binding sites of the upstream of the iron-related genes by Tfu.0249 were identified and confirmed by gel shift assay. A master binding sequence by Tfu.0249 were confirmed in T. fusca.

**MATERIALS AND METHODS**

**Growth conditions**

Thermobifida fusca muC was grown in Hagerdahl medium (Ferchak and Pye 1983). For experiments conducted in Erlenmeyer flasks, 50 mL pre-cultures of T. fusca were grown at 55°C and 250 rpm for 12–24 hours in a 500 mL Erlenmeyer flask. Growth cultures for testing were inoculated using 5% of the pre-culture and grown at 55°C and 250 rpm for 42–48 hours. Escherichia coli strains were grown on LB medium with adding different antibiotics to keep plasmids.

**Cell density and product measurement**

Due to the growth physiology of T. fusca (filamentous cells that aggregate), the culture density of T. fusca was determined by measuring cytoplasmic protein content. 1 mL culture was centrifuged at 10 000 × g for 5 min. The pellets were re-suspended in fresh media and centrifuged at 10 000 × g for 5 min again. Sediments were dissolved in 200 μL 50 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 0.1 M DTT and 50% glycerol. Samples were then pulse sonicated at 70% strength for 10 min in an ice bath. After centrifuging at 10 000 × g for 5 min, the proteins in the supernatant were measured by the Bradford protein assay (Bradford 1976). The dry cell weight (DCW) is proportionally related to the overall protein content.

Products were detected using an HPLC system equipped with Bio-Rad HPX-87H Ion Exclusion Column. The mobile phase was 0.005 mol L⁻¹ H₂SO₄ at the rate of 0.6 mL/min and IR and UV detectors were used.

**Recombinant DNA work**

The enzymes for recombinant work were obtained from New England Biolabs. The oligonucleotides used in this study were obtained from IDT dna and they are listed in Table 1 (Supporting Information). Promoter regions were amplified from T. fusca by using the primers listed in Table 1 (Supporting Information). The promoter regions and pET-28a(+) were cut at BamHI-NotI sites and then ligated by ligase.

Plasmids from E. coli were isolated with the QiAprep Spin Miniprep Kit. Escherichia coli was transformed by electroporation.

**Formation of double-stranded DNA oligonucleotides via self-assembly**

The formation of double-stranded DNA oligonucleotides via self-assembly was described previously (Park et al., 2005). In brief, 10 mM forward strand and reverse strands were mixed in a DNA assembling buffer (40 mM Tris base, pH 8.0, 40 mM acetic acid, 1 mM EDTA and 15 mM Mg (Ac)2) in a molar ratio of 1:4:4. The mixture was heated at 95°C for 5 min, and then slowly cooled down to 20°C in 48 hours to allow self-assembly of the DNA sequences (Zhang and Yadavalli 2012).

**Overproduction and purification of DtxR-like repressor (Tfu.0249)**

The Tfu.0249 protein containing his-tag at the carboxyl terminal was overproduced in E. coli BL21(DE3) using the expression plasmid pET-28a-DtxR and purified by Ni₂⁺-chelate affinity chromatography as described previously by Thermo Scientific. IPTG was added into the LB medium when the OD reached 0.3–0.4
Table 1. The gene clusters regulated by Tfu_0249.

<table>
<thead>
<tr>
<th>Cluster 1</th>
<th>Definition</th>
<th>Orthology</th>
<th>Predicted binding site</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfu_2386</td>
<td>Binding-protein-dependent transport lipoprotein</td>
<td>Iron complex transport system substrate-binding protein</td>
<td>TTAGGGTAGCTGCTAT</td>
<td>2810454–2810471</td>
</tr>
<tr>
<td>Tfu_2385</td>
<td>FecCD-family membrane transport protein</td>
<td>Iron complex transport system permease protein</td>
<td>ATAGGGTAGCTTGTCTA</td>
<td>380961–380944</td>
</tr>
<tr>
<td>Tfu_2384</td>
<td>Hemin importer ATP-binding subunit</td>
<td>Iron complex transport system ATP-binding protein</td>
<td>TAAGGGTAGACTTACCTT</td>
<td>1723288–1723305</td>
</tr>
</tbody>
</table>

with final concentration of 1 mM. The induction time varied in different experiments.

**Gel shift assays**

For testing the binding of Tfu_0249 to putative target promoters, purified Tfu_0249 protein was mixed with DNA fragments in a total volume of 20 μl. The binding buffer contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 40 mM KCl, 12.5% (vol/vol) glycerol. Approximately, 20 nM of a promoter fragment lacking a DtxR binding site (fragment with T7 promoter) was used as negative control. The reaction mixture were incubated at room temperature for 30 min and then loaded onto a gradient polyacrylamide gel. Electrophoresis was performed at room temperature and 200 V using 1x Tris-glycine as an electrophoresis buffer. The gels were subsequently stained with ethidium bromide and photographed. All PCR products used in the gel shift assays were purified with the PCR Purification Kit (Thermo Scientific) and eluted in EB buffer (10 mM Tris-HCl, pH 8.5).

**RNA preparation and real-time PCR**

The gene expressions of the iron-related genes of T. fusca were studied using real-time PCR by harvesting cells at the mid-log growth phase corresponding to experimental points. Cells at the selected points were centrifuged at 10 000 × g for 10 min. The cell pellets were resuspended in RNAprotect bacteria reagent (Qiaagen, Valencia, CA) as prescribed by the manufacturer. After incubation at room temperature for 5 min, the cells were pulsed sonicated at 50% strength for 5 min. RNeasy Midi Kits (Qiagen, Valencia, CA) were then used to isolate RNA using the protocol suggested by the manufacturer. The experiment details were described previously (Merklein, Fong and Deng 2014). The RT-PCR results were log2-transformed.

**RESULTS**

**Growth of T. fusca on the different iron concentrations**

Iron is essential for microorganisms (Dimise, Widboom and Bruner 2008). *Thermobifida fusca* was found to be growing without iron but the growth was extremely slow (Dimise, Widboom and Bruner 2008). Fig. 1 illustrates the effect of iron on the growth of *T. fusca*. When *T. fusca* was grown on iron-deficiency environment, it took 5 days to reach the maximal biomass yield (0.48 g L⁻¹ DCW), while if there was 50 μM of iron, *T. fusca* achieved the maximal density (0.60 g L⁻¹ DCW) in about 39 hours and in excess of iron the maximum (0.65 g L⁻¹ DCW) was reached after 15 hours of growth.
Figure 1. Cell growth of \textit{T. fusca} \textit{muC} on the different iron concentrations.

Figure 2. SDS-PAGE electrophoresis of Tfu\textsubscript{0249}. (A) Tfu\textsubscript{0249} from the cell lysates of \textit{T. fusca} \textit{muC}; (B) Tfu\textsubscript{0249} from the cell lysates of \textit{E. coli} BL-21 with the plasmid pET-28a-DtxR.

Identification of DtxR-like repressor (Tfu\textsubscript{0249}) in \textit{T. fusca}

Based on the sequence of DtxR, our group blasted the genome sequence and found that Tfu\textsubscript{0249} had 94\% similarity to DtxR sequence. DtxR protein has been identified to bind sequence TWAGGTAGSCTWACCTWA (Tao and Murphy 1994), which might be also true for Tfu\textsubscript{0249} in \textit{T. fusca}, and we found that there were at least three iron-related gene clusters having the binding sites for Tfu\textsubscript{0249}.

The expression of diphtheria toxin in lysogenic toxigenic strains of \textit{C. diphtheria} was controlled by the heavy metal ion-activated regulatory protein DtxR (Schmitt and Holmes 1991). DtxR was found to bind a sequence on the promoter regions of the genes related to iron transport. By blasting the amino acid sequence of DtxR against the genome sequence of \textit{T. fusca}, a DtxR-like protein Tfu\textsubscript{0249} with MW 26389.17 Da was found. \textit{Thermobifida fusca} cells grown on iron-excess, iron-deficiency and 50 \textmu M were harvested and the cells were lysed by ultrasound. The cell lysates were checked on the SDS-PAGE gel (Fig. 2A). Tfu\textsubscript{0249} protein was found under all three conditions.

Tfu\textsubscript{0249} was overexpressed \textit{E. coli} BL-21(DE3) and purified. The induction time of pET-28a-DtxR in BL-21 was set 4 hours, 6 hours and overnight. The cell growth with pET-28a-DtxR was significantly slower than the one with pET-28a(+) (data not shown). \textit{Escherichia coli} with pET-28a-DtxR expressed were harvest and washed by the purified water and then lysed by ultrasound. The cell lysates were analyzed by the electrophoresis using SDS-PAGE. In Fig. 2B, Tfu\textsubscript{0249} protein was shown on the SDS-PAGE gel. The expressions of Tfu\textsubscript{0249} under three induction lengths were not significantly different in \textit{E. coli} BL-21(DE3).

Binding sites of DtxR-like repressor: Tfu\textsubscript{0249}

By blasting the binding sequence of \textit{C. diphtheria} by DtxR (Schmitt and Holmes 1991), three binding sites were found to be on the upstream of three ferric iron transport related genes (Table 1): cluster 1: Tfu\textsubscript{2386}, Tfu\textsubscript{2385}, Tfu\textsubscript{2384}; cluster 2: Tfu\textsubscript{0338}, Tfu\textsubscript{0337}, Tfu\textsubscript{0336}, Tfu\textsubscript{0335}; cluster 3: Tfu\textsubscript{1491}, Tfu\textsubscript{1492}, Tfu\textsubscript{1493}, Tfu\textsubscript{1494}, Tfu\textsubscript{1495}. The binding site of cluster 1 was TTAGGTAGCCTAAGCAT; the binding site of cluster 2 was ATAGGTAGCCTTGTCTA; the binding site of cluster 3 was
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Figure 3. Gene expressions of the three gene clusters on iron-deficiency and iron-excess conditions. The RT-PCR results were log2-transformed. (A) gene cluster 1; (B) gene cluster 2; (C) gene cluster 3. The biological triplicates were used in this experiment.

Figure 4. Gel shift assay of Tfu0249 binding to the upstream of three gene clusters. (A) A1, the promoter region of cluster 1 bound by Tfu0249; A2, the promoter region of cluster 1 without Tfu0249 added; A3, T7 promoter region with Tfu0249 added. (B) B1, the promoter region of cluster 2 bound by Tfu0249; B2, the promoter region of cluster 2 without Tfu0249 added; B3, T7 promoter region with Tfu0249 added. (C) C3, the promoter region of cluster 3 bound by Tfu0249; C2, the promoter region of cluster 3 without Tfu0249 added; C1, T7 promoter region with Tfu0249 added.

The gene expressions of three clusters were measured by RT-PCR (Fig. 3). The biological triplicates were used for RT-PCR. The RT-PCR results were log2-transformed [raw data are shown in Table 2 (Supporting Information)]. In cluster 1, gene expressions of Tfu2836, Tfu2385, Tfu2384 in the iron-deficiency condition were significantly higher than those in the iron-excess condition. In cluster 2, the expressions of Tfu0338, Tfu0337, Tfu0336 and Tfu0355 in the iron-deficiency condition were much higher than those in the iron-excess condition. In cluster 3, all genes except Tfu1491 transcribed more in the iron-deficiency condition than those in the iron-excess condition.

Gel shift assays

The binding of Tfu0249 with three promoter regions was checked by gel shift assays (Fig. 4). The DNA fragments bound by Tfu0249 moved slowly than those which were fractionated without Tfu0249 or with non-specific repressor (Spiridonov and Wilson 2000). The results of gel shift assays confirmed that Tfu0249 bound all three predicted binding sites.

After comparing the three binding sites of three clusters, a master binding site was proposed: TWAGGTAGCTACCTATAGGTAGACTTACCTT. The binding of Tfu0249 on the promoter regions containing three binding sites were subjected to Gel shift assay (Spiridonov and Wilson 2000). The promoter regions containing the binding sites of Tfu0249 were amplified by the primers listed in Table 1 (Supporting Information).

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Gel shift assays with synthetic oligonucleotides. Further study of synthetic sequences based on the proposed binding sequence confirmed the binding of Tfu. Synthetic oligonucleotide 2 (TCAGGTAAGACTTACCTGA). E1: binding sequence 2 bound by Tfu with 50 μM iron, E2: binding sequence 2 without Tfu, E3: binding sequence 2 with Tfu added but without iron.

**DISCUSSION**

Iron is an essential metal involved in the vital metabolic functions in microorganisms. The redox of iron plays crucial roles in biocatalysis and the electron transport chain (Miethe and Marahiel 2007). In the absence of iron, bacteria may transcribe a variety of genes in order to acquire iron from their host or environment. In microorganisms, the most ubiquitous and important of these is the biosynthesis and secretion of high-affinity ferric iron chelators called siderophores (Oves-Costales, Kadi and Challis 2009).

**Thermobifida fusca** was found to survive in the iron-deficiency condition and produce siderophores which were synthesized to acquire iron. Based on the sequence of DtxR, it was found that Tfu had 94% similarity to DtxR sequence. DtxR protein has been identified to binding sequence TWAGGTWAGSCTWACCTWA (Tao and Murphy 1994). By blasting the binding sequence of DtxR, three gene clusters were found to have the binding sites on the upstream of them. In order to verify if Tfu could bind those binding sites experimentally, it was expressed and purified in E. coli BL-21(DE3). The pure protein was subjected to the gel shift assays.

The gel shift assays showed that Tfu bound the proposed binding sites firmly. By comparing the binding sites of three clusters, TWAGGTWAGSCTWACCTWA was proposed to be the universal binding sites for Tfu. Further study of the synthetic sequences based on the proposed binding sequence confirmed the binding of Tfu.

**SUPPLEMENTARY DATA**

Supplementary data is available at FEMSLE online.

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**Conflict of interest statement.** None declared.

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