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The manganese-responsive regulator MntR represses transcription of a predicted ZIP family metal ion transporter in Corynebacterium glutamicum

Meike Baumgart and Julia Frunzke*

Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany

*Corresponding author: Institut für Bio- und Geowissenschaften, IBG-1: Biotechnologie, Forschungszentrum Jülich, 52425 Jülich, Germany. Tel: +49-2461-613294; Fax: +49-2461-612710; E-mail: j.frunzke@fz-juelich.de

One sentence summary: The DtxR-type regulator MntR of Corynebacterium glutamicum ATCC 13032 acts as a manganese-dependent repressor of a predicted ZIP family metal ion transporter.

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ABSTRACT

Manganese is an important trace element required as an enzyme cofactor and for protection against oxidative stress. In this study, we characterized the DtxR-type transcriptional regulator MntR (cg0741) of Corynebacterium glutamicum ATCC 13032 as a manganese-dependent repressor of the predicted ZIP family metal transporter Cg1623. Comparative transcriptome analysis of a ΔmntR strain and the wild type led to the identification of cg1623 as potential target gene of MntR which was about 50-fold upregulated when cells were grown in glucose minimal medium. Using electrophoretic mobility shift assays, a conserved 18 bp inverted repeat (TGTTCAATGCGTTGAACA) was identified as binding motif of MntR in the cg1623 promoter and confirmed by mutational analysis. Promoter fusion of Pcg1623 to eyfp confirmed that the MntR-dependent repression is only abolished in the absence of manganese. However, neither deletion of mntR nor cg1623 resulted in a significant growth phenotype in comparison to the wild type—strongly suggesting the presence of further manganese uptake and efflux systems in C. glutamicum. The control of cg1623 by the DtxR-type regulator MntR represents the first example of a predicted ZIP family protein that is regulated in a manganese-dependent manner in bacteria.

Key words: DtxR; metal ion homeostasis; Gram-positive bacteria; iron homeostasis

INTRODUCTION

The DtxR/MntR family of metalloregulators represents a central class of transcriptional regulators being involved in the control of metal ion homeostasis in a wide range of Gram-positive and Gram-negative bacteria (Hantke 2001; Andrews, Robinson and Rodriguez-Quinones 2003; Guedon and Helmann 2003). One of the earliest reports is about the iron-dependent expression of the diphtheria toxin in Corynebacterium diphtheriae, mediated by DtxR (Pappenheimer and Johnson 1936; Tao et al., 1994). However, besides virulence genes, DtxR controls the expression of a variety of different genes involved in iron uptake, siderophore synthesis or iron storage in this species (Boyd, Oza and Murphy 1990; Schmitt and Holmes 1991). The active form of DtxR is a homodimer, with each monomer consisting of two domains connected by a flexible tether. The N-terminal domain contains the helix-turn-helix motif, responsible for DNA binding as well as two binding sites for Fe^{2+} ions (Spiering et al., 2003; D’Aquino et al., 2005). The C-terminal domain shares structural similarity with eukaryotic SH3 domains.

MntR of Bacillus subtilis was the first manganese-responsive DtxR-type regulator that has been characterized. It was shown to repress the two manganese uptake systems mntH (proton-coupled NRAMP transporter) and mntABCD (ABC transporter)
under conditions of sufficient manganese supply (Que and Helmann 2000). Furthermore, it also activates the mntABC operon when manganese is limited to increase manganese uptake. A B. subtilis mntR deletion mutant shows a significantly increased sensitivity towards manganese compared to the wild type (Que and Helmann 2000). MntR of C. diphtheriae (DIP0619) represses a five-gene operon in a manganese-dependent manner that contains, besides its own gene, a potential ABC metal ion transporter (mntABC) (Fig. 1A) (Schmitt 2002). Deletion of this transporter had no effect on growth even under Mn\(^{2+}\) limiting conditions (Schmitt 2002), which is not unexpected as many bacteria possess more than one manganese uptake system (Que and Helmann 2000; Andrews et al., 2013).

The genome of the non-pathogenic Gram-positive soil bacterium Corynebacterium glutamicum encodes three DtxR-type regulators. One of them, DtxR (cg2103), has been characterized as the master regulator of iron homeostasis controlling the transcription of more than 60 target genes in an iron-dependent manner (Brune et al., 2006; Wennerhold and Bott 2006). Although manganese has been shown to be crucial for the function of the superoxide dismutase, (El Shafey et al., 2008) nothing is known regarding the control of manganese homeostasis in C. glutamicum to date. One prime candidate is the DtxR-type regulator encoded by cg0741, which shares 52% amino acid sequence identity with MntR of C. diphtheriae.

Here, we characterized Cg0741 (in the following designated as MntR) and we were able to show that this DtxR-type transcriptional regulator functions as a manganese-dependent repressor of a predicted ZIP metal transport system in C. glutamicum.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth media**

The bacterial strains and plasmids used in this study are listed in Table 1. The C. glutamicum type strain ATCC 13032 was used as wild type. Growth experiments were performed at 30°C and 1200 rpm in a BioLector system (m2p-labs, Baesweiler, Germany) in 48-well FlowerPlates containing 750 μL CGXII minimal medium (Keilhauer et al., 1993) supplemented with 3,4-dihydroxybenzoate (30 mg L\(^{-1}\)) and 2% (w v\(^{-1}\)) glucose as carbon source. If appropriate, 25 μg mL\(^{-1}\) kanamycin or 10 μg mL\(^{-1}\) chloramphenicol were added. The standard concentrations for metals in CGXII are as follows: 36 μM FeSO\(_4\), 59 μM MnSO\(_4\) and 3.48 μM ZnSO\(_4\). For growth experiment with metal starvation conditions, the relevant metal salt was omitted from the trace element solution. For growth experiments with metal-excess conditions, 10 times the standard concentration of the relevant metal was used (e.g. 360 μM for FeSO\(_4\)). All cloning
was performed in Escherichia coli DH5α cultivated at 37 °C in lysogeny broth (LB) (Sambrook and Russell 2001) with 50 µg mL⁻¹ kanamycin or 34 µg mL⁻¹ chloramphenicol.

**Recombinant DNA work and construction of deletion mutants**

Routine methods such as PCR, DNA restriction and ligation were performed using standard protocols (Hanahan 1983; van der Rest et al., 1999; Sambrook and Russell 2001). The oligonucleotides used in this study were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1 (Supporting Information). DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). The ΔmntR and Δcg1623 mutants of C. glutamicum were constructed via a two-step homologous recombination protocol as described previously (Niebisch and Bott 2001). For further details regarding plasmid and mutant construction, see the supplemental methods in Supplementary Information.

**DNA microarrays**

Comparative transcriptome analysis was performed as described previously (Vogt et al., 2014). Briefly, C. glutamicum wild-type and ΔmntR cells were grown in 5 mL brain–heart infusion (BHI, Difco) for about 6 h at 30 °C. A second precultivation was performed overnight in CGXII minimal medium containing 2% (w/v⁻¹) glucose as carbon source. The main cultures were inoculated to an OD₆₀₀ of 0.5 in CGXII minimal medium with 2% (w/v⁻¹) glucose. At an OD₆₀₀ of 5, the cells were harvested by centrifugation (4120 x g, 10 min and 4 °C). The cell pellet was subsequently frozen in liquid nitrogen and stored at −70 °C. The preparation of total RNA was performed using the RNeasy Kit from Qiagen (Hilden, Germany). Synthesis of fluorescently labeled cDNA was carried out using SuperScript III reverse transcriptase (Life Technologies, Darmstadt, Germany). Purified cDNA samples of the wild-type and the ΔmntR strain were pooled and the prepared two-color samples were hybridized at 65 °C while rotating for 17 h using Agilent’s Gene Expression Hybridization Kit, hybridization oven and hybridization chamber. After hybridization, the arrays were washed using Agilent’s Wash Buffer Kit according to the manufacturer’s instructions. Fluorescence of hybridized DNA microarrays was determined at 532 nm (Cy3) and 635 nm (Cy5) at 5 µm resolution with a GenePix 4000B laser scanner and GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA). Fluorescence images were saved to raw data files in TIFF format (GenePix Pro 7.0). Quantitative TIFF image analysis was carried out using GenePix image analysis software and results were saved as GPR-file.
Overproduction and purification of MntR

E. coli BL21(DE3) carrying the expression plasmid pET24b-mntR-strep was grown in LB medium at 37°C and 120 rpm. MntR overproduction of MntR with a C-terminal Strep-tag was induced by addition of 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by cultivation at 20°C for 4-6 h before the cells were harvested by centrifugation. StrepTactin affinity chromatography was performed as described previously (Niebisch et al., 2006). The protein was frozen in 20 μL aliquots and stored at −20°C. For determination of the molecular weight, gel filtration was performed using a Superdex™ 200 10/300 GL column (GE Healthcare, Munich, Germany) at a flow rate of 0.5 mL min⁻¹ in gel filtration buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl and 1 mM DTT) containing either 1 mM MnCl₂ or 1 mM EDTA.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Wennerhold and Bott 2006). Briefly, purified MntR was incubated with DNA fragments (30–500 bp, final concentration 0.027–1 μM) in binding buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 5 mM MgCl₂) and metal ions, as indicated in the figure legends, in a total volume of 20 μL. Electrophoresis was performed using 10–15% native polyacrylamide gels at room temperature and 150 or 180 V for 45–60 min (depending on the size of the DNA fragments) and the gels were subsequently stained with SYBR green.

Promoter fusion studies

In order to analyze the regulation of the cg1623 promoter by MntR in vivo, a DNA fragment covering the cg1623 promoter region was fused to the eyfp-coding sequence (pJC1-Pcg1623-eyfp). Wild-type and ΔmntR cells were transformed with the resulting plasmid. Using a BioLector system (m2p labs), production of MntR with a C-terminal Strep-tag was induced by addition of 1 mM IPTG followed by cultivation at 20°C for 3–7 h before the cells were harvested by centrifugation. Growth rate and final OD₆₀₀ of the wild-type and the deletion mutant were identical when grown in standard CGXII minimal medium (Fig. 1C). The morphology was analyzed by microscopy, but revealed no differences between the strains (data not shown). Even cultivation in the presence of excess manganese, zinc or iron (10 x standard concentrations) or under metal limitation disclosed no significant growth phenotype of the mutant (data for Mn²⁺ in Fig. 1C, data for Zn²⁺ and Fe²⁺ not shown).

Transcriptome analysis of the mntR mutant

In order to elucidate the transcriptional changes caused by the deletion of mntR, DNA-microarrays were performed of cells grown in CGXII minimal medium with glucose as carbon source. In total, 11 genes showed an altered mRNA level of ≥ 2-fold (Table S2, Supporting Information). The mRNA level of mntR was 28-fold reduced, confirming the successful deletion of the corresponding gene. The transcription of the other genes of the mntR operon, cg0739 and cg0740, was unchanged in comparison to the wild-type reference (ratio 1.12 and 0.89, respectively). Remarkably, the gene cg1623, annotated as a zinc transporter of the ZIP family, exhibited an about 50-fold increased mRNA level in the ΔmntR strain. Among the other regulated genes were several, but not all, members of the arg operons (cg1580-85 and cg1586-1580) responsible for arginine biosynthesis, a glutamine 2-oxoglutarate aminotransferase, a putative allopolyhydrolyase and the operon cg3226-27, encoding a lactate permease and a lactate hydrolase. The latter is an operon which very often shows an altered mRNA level in DNA microarray experiments and was therefore not treated as putative target (≥ 2-fold regulated in about 40% of all microarray experiments in our in-house database).

Promoter fusion studies with cg1623

To study the influence of MntR on the expression of cg1623 in vivo, we fused the cg1623 promoter to eyfp and monitored the fluorescence output in the wild-type and the ΔmntR strain in CGXII medium with different trace element substitutions (Fig. 2A). The growth of the two strains did not differ significantly under the tested conditions (data not shown). When grown in standard CGXII minimal medium, the specific fluorescence of the mntR deletion mutant carrying the promoter fusion plasmid was about 20-fold higher compared to the wild type harboring the same plasmid, which indicates that MntR functions as a repressor of cg1623 transcription. Remarkably, the specific fluorescence is almost identical in the two strains when the trace elements (Zn²⁺, Cu²⁺, Mn²⁺ and Ni²⁺) were omitted from the medium. In the following, we tested the impact of these four trace elements separately (Fig. 2A, last four media). Here, only manganese starvation resulted in a comparable increase of fluorescence in the wild type. This indicates that the MntR-dependent regulation of cg1623 is responsive to manganese and
suggestion: Cg1623 as a novel transport system involved in manganese uptake in C. glutamicum. Under iron starvation conditions (second medium), the growth of both strains is strongly decreased. Therefore, the increased fluorescence of both strains is probably due to the higher contribution of the autofluorescence of the cells to the total fluorescence in these samples.

To further confirm the specificity of the regulation of cg1623 by MntR, we performed a complementation experiment with plasmid encoded MntR under control of the IPTG-inducible P_tac promoter (Fig. 2B). Under standard manganese conditions, the basal expression of mntR by the leaky P_tac promoter is already sufficient to suppress transcription of cg1623 in the ΔmntR strain. We also tested the complementation with induced MntR (100 μM IPTG), but these strains showed a growth defect and the specific fluorescence was not further reduced (data not shown).

MntR is a dimer, independent of the presence of Mn^{2+}

For in vitro studies, MntR was heterologously expressed in E. coli BL21 (DE3) and purified as a C-terminal strep-tag fusion (Fig. S1A, Supporting Information). In line with the report of Lieser et al. for B. subtilis MntR, size exclusion chromatography revealed that C. glutamicum MntR forms a dimer in the presence or absence (+EDTA) of Mn^{2+} (Fig. S1B, Supporting Information) (Lieser et al., 2003). Upon addition of EDTA the peak shifts to slightly higher molecular weight, possibly because the absence of manganese leads to a conformational change. For B. subtilis, it was described that in the absence of metal ions, the two DNA binding domains are spread farther apart than in the metal bound state (DeWitt et al., 2007).

Identification of MntR target genes

The microarrays and promoter fusion studies suggested cg1623 to be a direct target gene of MntR. Therefore, a DNA fragment covering the promoter region of cg1623 was tested for complex formation with MntR in EMSAs. An obvious shift was observed for this DNA fragment, whereas the promoters of cg0739 and mntR itself were not bound by MntR, in vitro (Fig. S2, Supporting Information). The promoter regions of further putative targets identified in the transcriptome analysis were also tested, but no considerable interaction with MntR was observed in EMSA studies (Fig. S3, Supporting Information). Only the promoter of cg1580 showed a slight shift with the highest protein concentration in vitro. But the genes of this operon (cg1580-cg1585) were regulated in different directions (ratios cg1580-cg1506: 2.0, 1.0, 0.5, 0.2, 0.15, 0.15) and a putative binding motif could not be identified in this region. A potential regulation of this operon by MntR was therefore regarded as unlikely to be physiologically relevant and not further elucidated.

A nearly perfect 18 bp inverted repeat with high sequence identity to the MntR binding motif of C. diphtheriae was identified in the promoter region of cg1623, centering 24 bp upstream of the transcriptional and translational start site (leaderless transcript, personal communication Jörn Kalinowski) (Fig. 3A). A 30 bp fragment containing this motif was indeed bound by MntR with high affinity in the presence of manganese (Fig. 3B). In the following, the high specificity of MntR for its palindromic binding site was confirmed by a mutational analysis revealing that the outer six base pairs of the binding motif are most important for complex formation (Fig. 4). A further MntR motif in the promoter of cg0343 was identified by a genome-wide in silico search and was also bound by MntR in EMSAs but with slightly lower affinity (Fig. 3B). Cg0343 encodes a MarR-type transcriptional regulator.
regulator of unknown function which is not conserved among Corynebacteria and Mycobacteria. The mRNA level of cg0343 was not significantly altered in the comparative transcriptome analysis (average ratio of three experiments: 0.84, P-value: 0.090). A possible reason for this could be that cg0343 is regulated by further regulators or other regulatory mechanisms which counteract the effect of mntR deletion under the tested conditions. Therefore, the relevance of MntR for cg0343 regulation remains to be elucidated.

**MntR binding is dependent on the presence of divalent metal cations**

Addition of the chelating agent EDTA led to the dissociation of MntR-DNA complexes in vitro (Fig. 3C), confirming that the binding is strictly dependent on the presence of divalent metal ions. For DtxR-regulators, it is known that despite their high specificity in vivo they appear to have low ion selectivity in vitro (Guedon and Helmann 2003). This seems to be the case also for MntR as 100 μM of Mn2+, Fe2+, Zn2+, Ni2+ or Co2+ strengthened complex formation whereas the addition of Cu2+ inhibited binding (Fig. 54, Supporting Information). Together, in vitro protein–DNA interaction studies and in vivo promoter fusion experiments provided convincing evidence that Mn2+ is the major metal ion triggering MntR activity in the living organism.

**The putative manganese transporter cg1623**

In this work, we show that MntR, as a manganese-responsive regulator, seems to have a similar function as in related organisms, but mediates response to manganese starvation by
Figure 4. Mutational analysis and verification of the MntR binding site. The predicted binding site is printed in bold letters. Three nucleotides were exchanged in each oligonucleotide as indicated (M1–M8). + indicates that the mutated fragment was bound with the same affinity as the unaltered wild-type fragment (positive control); (+) indicates that the mutated fragment was shifted, but with lower affinity; (−) indicates that the mutated fragment was not shifted or with much lower affinity.

Oligonucleotides (30 bp, 1 μM) were incubated with MntR in the given concentrations and analyzed using 15% native polyacrylamide gels. A 30 bp oligonucleotide pair located in the promoter region of cg1918 was used as negative control.

derepression of a target gene not homologous to previously described MntR targets. Cg1623 is an uncharacterized membrane protein which is annotated as a member of the ZIP family of metal transporters. It is the only ZIP protein of C. glutamicum with a rather low conservation among the Corynebacteriales (homologous proteins are only present in the genomes of C. efficiens and Corynebacterium aurimucosum). It consists of 263 amino acids with seven (SMART; Letunic et al., 2012) or eight (PredictProtein; Rost, Yachdav and Liu 2004) predicted transmembrane helices. A deletion mutant of cg1623 was constructed in this study and tested for its behavior under standard and metal starvation conditions. However, no growth phenotype was observed in standard CGXII medium (growth rates: wt: 0.61 ± 0.04, Δcg1623: 0.59 ± 0.02) and without Mn²⁺ (growth rates: wt: 0.53 ± 0.03, Δcg1623: 0.53 ± 0.02) or Zn²⁺ (growth rates: wt: 0.48 ± 0.04, Δcg1623: 0.44 ± 0.03). Furthermore, we tested the influence of cg1623 overexpression in the presence of Mn²⁺, Zn²⁺ or Fe²⁺ excess (Fig. S5, Supporting Information). Basal expression from the pAN6 plasmid with the leaky promoter P_tac has no significant influence on growth compared to an empty plasmid control strain. The induction of cg1623 by 100 μM IPTG leads to a strong growth defect already in standard CGXII medium, which is a rather typical consequence of the overproduction of a membrane protein. Hence, with this experimental setup it is not possible to observe ion specific effects to get further hints regarding the function of cg1623.

Proteins of the ZIP family of metal transporters can be found in a wide range of organisms including bacteria, fungi, plants, insects and mammals (Eide 2005) and are known to translocate, besides zinc, also other metal ions such as Fe²⁺, Mn²⁺, Cd²⁺.
and Co²⁺ across cellular membranes (Guerinot 2000; Eide 2005; Taudte and Grass 2010). The discussion with respect to the driving force is controversial, but there are some hints that transport might be triggered by the proton motive force (Taudte and Grass 2010) or bicarbonate (Gaither and Eide 2000). The best characterized members are the ZIP1-4 zinc transporters of Arabidopsis thaliana (Grotz et al., 1998), whereas the E. coli ZupT represents the first prokaryotic ZIP transporter identified and characterized in more detail (Grass et al., 2002, 2005). E. coli ZupT has a rather broad substrate spectrum and was shown to transport Zn²⁺, Fe²⁺, Co²⁺, Mn²⁺, and Cd²⁺ (Grass et al., 2005; Taudte and Grass 2010). An E. coli zupT single mutant has only a very slight phenotype which can be well explained by the broad substrate spectrum and the fact that there are several other uptake systems for zinc, manganese, and iron in E. coli. This seems to be also the case in C. glutamicum because we did not observe an obvious phenotype for the single deletion mutant Δ1cg1623.

Different regulatory mechanisms have been described for ZIP homologs—both on transcriptional and post-transcriptional level. In Saccharomyces cerevisiae, the transcriptional activator Zap1 triggers the transcription of zinc uptake systems under zinc limited conditions (Zhao and Eide 1997). Several ZIP transporters of A. thaliana are also known to be induced under zinc deficiency conditions (Grotz et al., 1998). Another level of control in yeast is the inactivation of zinc uptake systems by endocyto-sis and degradation in the presence of high zinc concentration (Gitan et al., 1998). In contrast, E. coli ZupT appears to be constitutively expressed (Grass et al., 2005). To our knowledge, cg1623 is the first example of a ZIP family protein that is regulated in a manganese-dependent manner. Whether cg1623 really transports Mn²⁺ and/or other metal ions remains to be elucidated in further experiments.

Control of manganese homeostasis in C. glutamicum

The regulator MntR that was characterized in this study represses transcription of the predicted ZIP family metal transporter cg1623 in the presence of sufficient intracellular concentrations of manganese. In growth experiments, we did not observe any significant phenotype for both the Δ1mntR and the Δ1cg1623 mutants of C. glutamicum, in contrast to what is described for some other organisms (Que and Helmann 2000). This suggests that a different manganese uptake system as well as a manganese efflux system is likely present and regulated by different regulatory system(s) or mechanisms. A good candidate for the efflux system is cg1660, which has a high similarity to MntP (42% identity), a potential manganese efflux pump of E. coli (Waters, Sandoval and Storz 2011). The transcription level for cg1660 did not change upon deletion of mntR (ratio 1.169, p-value: 0.023). For the additional manganese uptake system, there is currently...
SUPPLEMENTARY DATA
Supplementary data is available at FEMSLE online.

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