The manganese-responsive regulator MntR activates expression of a predicted ZIP family metal ion transporter in *Corynebacterium glutamicum*

Meike Baumgart and Julia Frunzke*

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

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

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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 Δ*mntR* 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 (EMSAs), 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 P_{cg1623} 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.

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, *et al.*, 2003, Guedon & Helmann, 2003). One of the earliest reports is about the iron-dependent expression of the diphtheria toxin (*tox*) in *Corynebacterium diphtheria*, mediated by DtxR (Pappenheimer & 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, *et al.*, 1990, Schmitt & 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 (HTH), 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 & Helmann, 2000). Furthermore, it also activates the *mntABCD* 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 & 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 (*mntABCD*) (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 & 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 & 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 $FeSO_4$, 59µM $MnSO_4$, 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, ten 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 & 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 & Russell, 2001). The oligonucleotides used in this study were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1. DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). The Δ*mntR* and Δcg1623 mutants of *C. glutamicum* were constructed *via* a two-step homologous recombination protocol as described previously (Niebisch & Bott, 2001). For further details regarding plasmid and mutant construction, see supplemental material.

DNA microarrays. Comparative transcriptome analysis was performed as described previously (Vogt, *et al.*, 2014). Briefly, *C. glutamicum* wild type and Δ*mntR* cells were grown in 5 ml BHI (Brain Heart Infusion, Difco) for about 6 hours 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 OD_{600} of 0.5 in CGXII minimal medium with 2% (w v^{-1}) glucose. At an OD_{600} 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 Δ*mntR* strain were pooled and the prepared two-color samples were hybridized at 65°C while rotating for 17 hours 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 (GenePix Pro 7.0). For background correction of spot intensities, ratio calculation and ratio normalization, GPR-files were processed using the BioConductor R-packages limma and marray (http://www.bioconductor.org).

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 6-8 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 $MnCl_2$ or 1 mM EDTA.

Electrophoretic mobility shift assays (EMSAs). EMSAs were performed as described previously (Wennerhold & 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 $MgCl_2$) 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 minutes (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 Δ*mntR* cells were transformed with the resulting plasmid. Using a Biolector system (m2p labs), production of biomass was measured as the backscattered light (620 nm) and the eYFP fluorescence was measured at an excitation of 485 nm and an emission of 520 nm. The specific fluorescence for the cells is defined as eYFP fluorescence per scattered light intensity (given in a.u.). Trace elements were added as indicated in the figure legends. For the complementation experiment the two strains were additionally transformed with plasmid pEC-mntR or the empty plasmid pEC-XC99E as control. The strains were cultivated as described above, without or with 100 µM IPTG to induce *mntR* transcription.

Results & Discussion

Genomic and transcriptional organization of *mntR*

In the genome of *C. glutamicum* ATCC 13032, *mntR* is organized in an operon with two predicted membrane proteins of unknown function (Fig 1A). Recent RNAseq data revealed, that two separate leaderless transcripts are formed, one containing all three genes and one encoding just *mntR* (Pfeifer-Sancar, *et al.*, 2013) (Fig. 1B). The homolog of MntR in *C. diphtheriae* (DIP1969) is the terminal gene of an operon including, besides *mntR*, four genes (*mntABCD)* encoding an ABC transporter responsible for manganese uptake (Schmitt, 2002) (Fig 1A). This operon is repressed by DIP1969 in the presence of manganese (Schmitt, 2002). However, a homolog of this ABC transporter is missing in *C. glutamicum* and *Corynebacterium efficiens* and consequently the genomic organization is different here.

Construction and growth of a Δ*mntR* strain

To gain insight into the function and possible target genes of MntR, an in-frame deletion mutant was constructed and analyzed. Growth rate and final OD_{600} 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 Mn^{2+} in Fig. 1C, data for Zn^{2+} and Fe^{2+} 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). 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 Δ*mntR* 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 allophanate hydrolase 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 Δ*mntR* 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 (Zn^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+}) 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 suggests 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 Δ*mntR* 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). 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) (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). 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). 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 basepairs 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 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 & Helmann, 2003). This seems to be the case also for MntR as 100 µM of Mn^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+} strengthened complex formation whereas the addition of Cu^{2+} inhibited binding (Fig. S4). Together, *in vitro* protein-DNA interaction studies and *in vivo* promoter fusion experiments provided convincing evidence that Mn^{2+} 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 activating 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, *et al.*, 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 Mn^{2+} (growth rates: wt: 0.53±0.03, Δcg1623: 0.53±0.02) or Zn^{2+} (growth rates: wt: 0.48±0.04, Δcg1623: 0.44±0.03). Furthermore, we tested the influence of cg1623 overexpression in the presence of Mn^{2+}, Zn^{2+}, or Fe^{2+} excess (Fig. S5). 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 Fe^{2+}, Mn^{2+}, Cd^{2+} and Co^{2+} across cellular membranes (Guerinot, 2000, Eide, 2005, Taudte & 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 & Grass, 2010) or bicarbonate (Gaither & 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, Grass, *et al.*, 2005). *E. coli* ZupT has a rather broad substrate spectrum and was shown to transport Zn^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} (Grass, *et al.*, 2005, Taudte & 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 Δcg1623.

Different regulatory mechanisms have been described for ZIP homologs - both on transcriptional and posttranscriptional level. In *S. cerevisiae* the transcriptional activator Zap1 triggers the transcription of zinc uptake systems under zinc limited conditions (Zhao & Eide, 1997). Several ZIP transporters of *Arabidopsis 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 endocytosis 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 Mn^{2+} 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 Δ*mntR* and the Δcg1623 mutants of *C. glutamicum*, in contrast to what is described for some other organisms (Que & Helmann, 2000). This suggests that a different manganese uptake system as well as a manganese efflux system are 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, et al., 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 no obvious candidate, as no homologs of MntABCD can be found in the *C. glutamicum* genome. Interestingly, a MntH homolog was identified in the *C. glutamicum* R strain (cgR_0158), but not in ATCC 13032. Hence, there are two options: i) *C. glutamicum* possesses a currently unknown manganese uptake system or ii) manganese is taken up as side activity for example by the potential ABC-type zinc transport systems Cg0041-Cg0043 and Cg2911-Cg2913 (Schröder, *et al.*, 2010). The third DtxR regulator of *C. glutamicum*, Cg2784, is very likely involved in the regulation of additional manganese homeostasis components, as its ligand binding residues (D12, D104) strongly suggest Mn^{2+} responsiveness (Guedon & Helmann, 2003) (Fig. 5). As an additional point of evidence, the cg2784 gene is located between the two components of the manganese-ribonucleotide reductase NrdEF (Abbouni, *et al.*, 2009), which could be a hint for the regulation of manganese containing proteins by this regulator. In summary, our study has revealed a first insight into the manganese regulatory network in *C. glutamicum*, but several further components remain to be elucidated.

Additional Material

Additional File 1: Additional methods, figures and tables.

Acknowledgements

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458 # Table

459 **Table 1: Strains and plasmids used in this study**

Strain or plasmid	Relevant characteristics	Source or Reference
E. coli		
DH5α	F$^-$ Φ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_K^- , m_K^+) *deoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*; strain used for cloning procedures	(Hanahan, 1983)
BL21(DE3)	F- *ompT hsdS*$_B$ (r_B-, m_B-) *gal dcm* (DE3); host for protein production	(Studier & Moffatt, 1986)
C. glutamicum		
ATCC13032	Biotin-auxotrophic wild type	(Kinoshita*, et al.*, 1957)
ATCC13032 Δ*mntR*	ATCC13032 with an in-frame deletion of cg0741	This work
ATCC13032 Δcg1623	ATCC13032 with an in-frame deletion of cg1623	This work
Plasmids		
pK19*mobsacB*	Kan^R.; plasmid for allelic exchange in *C. glutamicum*; (pK18 *ori*V$_{E.c.}$, *sacB*, *lacZ*α)	(Schäfer*, et al.*, 1994)
pK19*mobsacB*-Δ*mntR*	Kan^R; pK19*mobsacB* derivative containing a PCR product covering the up- and downstream regions of *mntR* (cg0741)	This work
pK19*mobsacB*-Δcg1623	Kan^R; pK19*mobsacB* derivative containing a PCR product covering the up- and downstream regions of cg1623	This work
pET24b	Kan^R; vector for overexpression of genes in *E. coli*, with optional C-terminal hexahistidine affinity tag (pBR322 *ori*$V_{E.c.}$ P_{T7} *lacI*)	Novagen
pET24b-mntR-Strep	Kan^R; pET24b derivative for overproduction of MntR (Cg0741) with a C-terminal STREP-tag	This work
pJC1-venus-term	Kan^R, pJC1 derivative carrying the venus coding sequence and additional terminators	(Baumgart*, et al.*, 2013)
pJC1-Pcg1623-eYFP	Kan^R; pJC1-venus-term derivative carrying the promoter of cg1623 fused to *eyfp* for promoter activity studies	This work
pEC-XC99E	Cm^R, *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression using the P_{tac} promoter	(Kirchner & Tauch, 2003)
pEC-mntR	Cm^R, pEC-XC99E-derivative for expression of *mntR* unter control of the P_{tac} promoter	This work
pAN6	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression using the P_{tac} promoter	(Frunzke*, et al.*, 2008)
pAN6-cg1623	Kan^R; pAN6-derivative for expression of cg1623 under control of the P_{tac} promoter	This work

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Figure legends

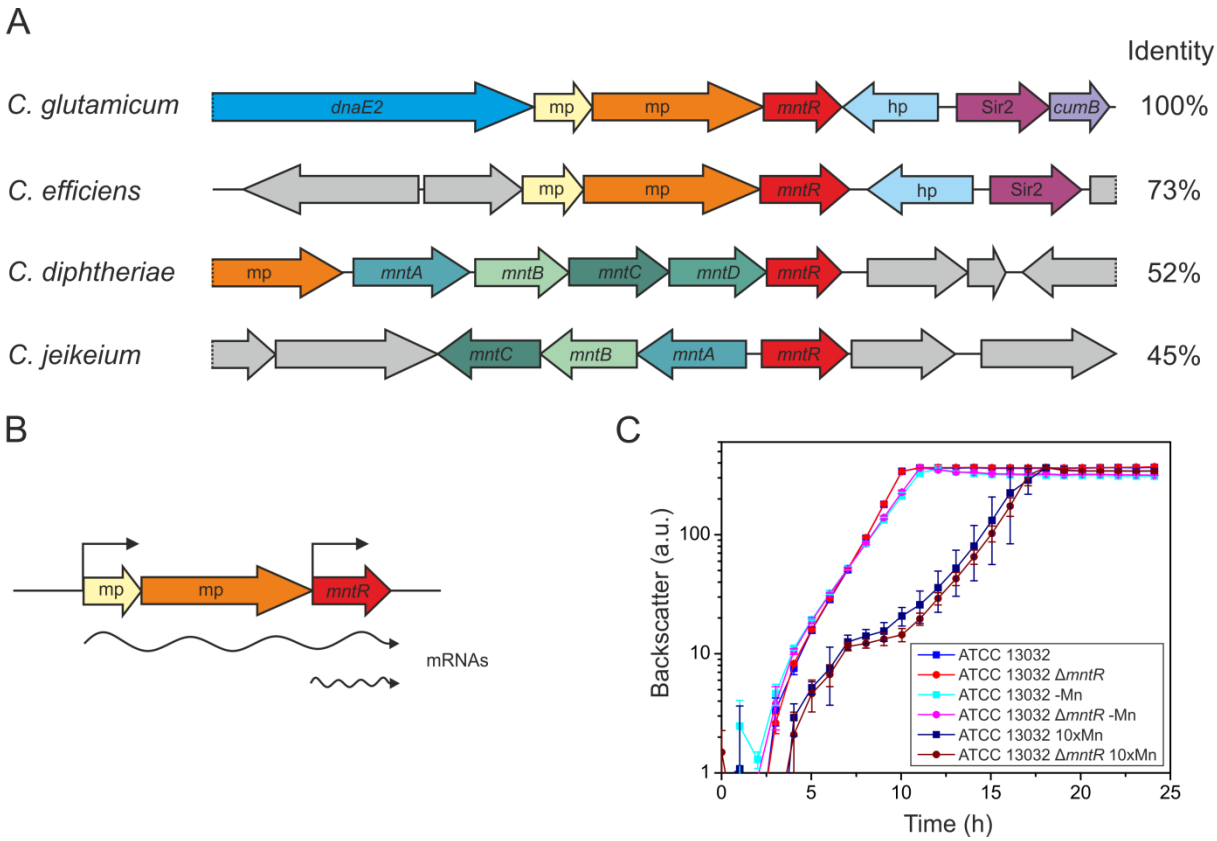
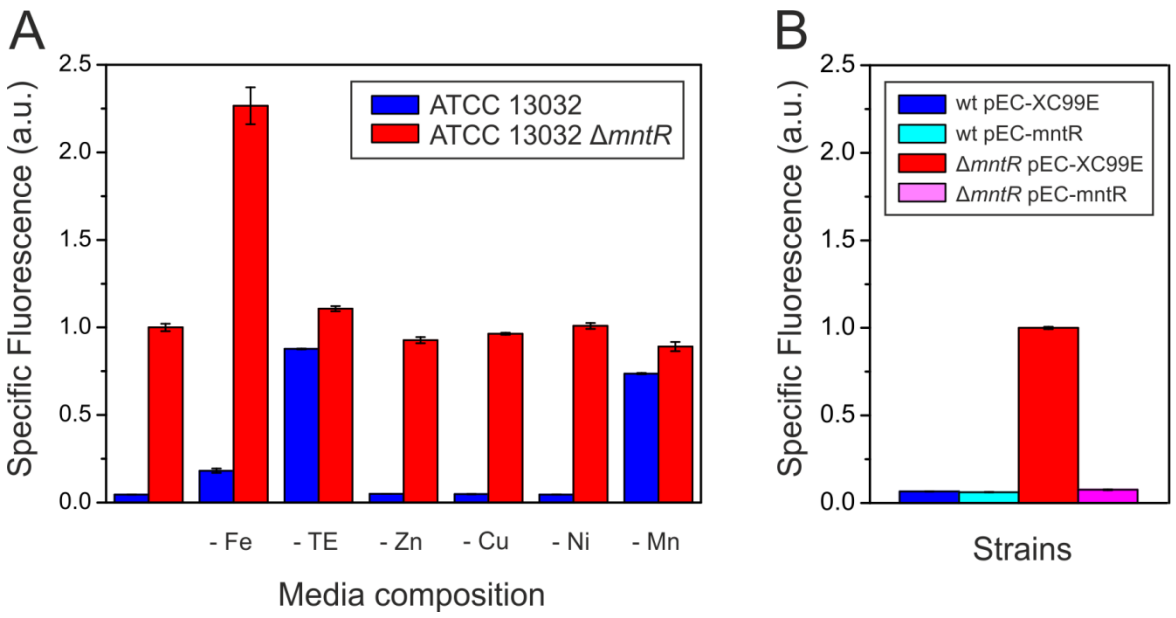


Fig. 1: Phylogenetic conservation of cg0741 (*mntR*) among different *Corynebacteria* and growth of a *mntR* deletion mutant. A, Comparison of the organization of the genomic locus of *mntR* in *C. glutamicum* and related species. *mntR* and homologous genes are highlighted in red. hp, hypothetical protein; mp, membrane protein of unknown function; *dnaE2*, error-prone DNA polymerase; Sir2, Sir2-type NAD-dependent protein deacetylase; *cumB*, cytidine and deoxycytidylate deaminase. Data were taken from MicrobesOnline (Alm, *et al.*, 2005). B, transcriptional organization of *mntR*. According to (Pfeifer-Sancar, *et al.*, 2013) two different transcripts are formed. C, Cultivation of ATCC 13032 and the *mntR* deletion mutant on standard CGXII minimal medium with 2% glucose (w v^{-1}), without manganese (-Mn) and in the presence of manganese excess (10xMn). Presented is the average and standard deviation of three biological replicates.



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477 **Fig. 2: cg1623 promoter fusion studies with *C. glutamicum* ATCC 13032 wild type (wt)**
478 **and Δ*mntR* in CGXII glucose minimal medium.** All strains used in these experiments carry
479 the plasmid pJC1-Pcg1623-eYFP containing the promoter fusion of cg1623 to *eyfp*. A,
480 specific fluorescence of wild type and Δ*mntR* in CGXII glucose minimal medium lacking one
481 or several metal ions. The media composition is given below the x-axis. TE stands for a
482 combination of the four trace element salts $ZnSO_4$, $CuSO_4$, $NiCl_2$, and $MnSO_4$. B,
483 complementation of the fluorescence with plasmid-encoded MntR. *C. glutamicum* wild type
484 and Δ*mntR* carrying the reporter plasmid were additionally transformed with a plasmid
485 encoding MntR under control of a leaky, IPTG inducible promoter (or the empty plasmid
486 pEC-XC99E as control). The first preculture was grown in BHI medium and the second
487 preculture in CGXII minimal medium with glucose, both with either kanamycin (A) or
488 kanamycin and chloramphenicol (B). Presented is the specific fluorescence in the stationary
489 growth phase after 24h of cultivation (average and standard deviation of three biological
490 replicates, the specific fluorescence of the Δ*mntR* strain in standard CGXII-medium was set to
491 1).

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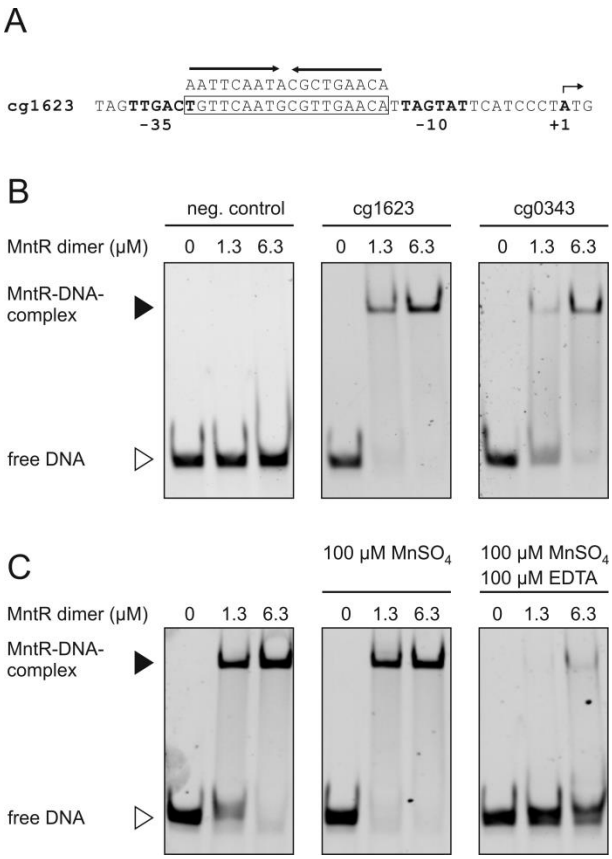

Fig. 3: Manganese-dependent binding of MntR to its target genes. A, localization of the MntR binding motif in the cg1623 promoter (box) and comparison with the MntR binding site of *C. diphtheriae* (above the box), -10 and -35 region as indicated in bold letters. The transcript is leaderless, +1 therefore indicates the transcriptional and translational start. B, EMSAs of purified MntR binding its target promoters. A 30 bp oligonucleotide pair located in the promoter region of cg1918 was used as negative control. C, Manganese dependency of MntR binding. Oligonucleotides (30 bp, 1 µM) were incubated with MntR, $MnSO_4$ and EDTA in the given concentrations, analyzed using 15% native polyacrylamide gels and stained with SybrGreen I.

		Shift
	GTTGAC**TGTTCAATGCGTTGAACA**TTAGTA	+
M1)	---TCA----------	+
M2)	------GTG----------	-
M3)	---------GAC----------	-
M4)	----------CGT----------	(+)
M5)	----------ATG----------	(+)
M6)	----------GTC---------	-
M7)	----------CAC------	-
M8)	----------GGC---	+

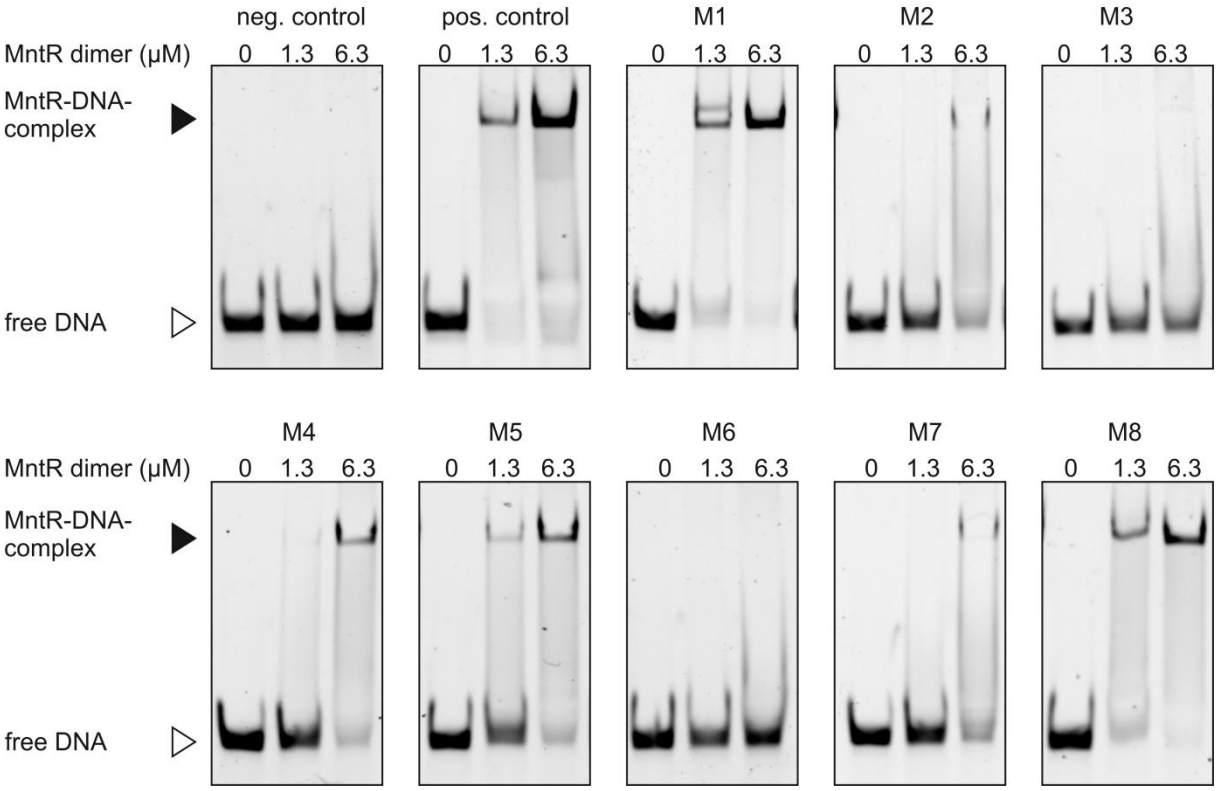

Fig. 4: Mutational analysis and verification of the MntR binding site. The predicted binding site is printed in bold letters. Three nucleotides were exchanged in each oligonucleotide as indicated (M1-M8). + indicates that the mutated fragment was bound with the same affinity as the unaltered wild-type 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.

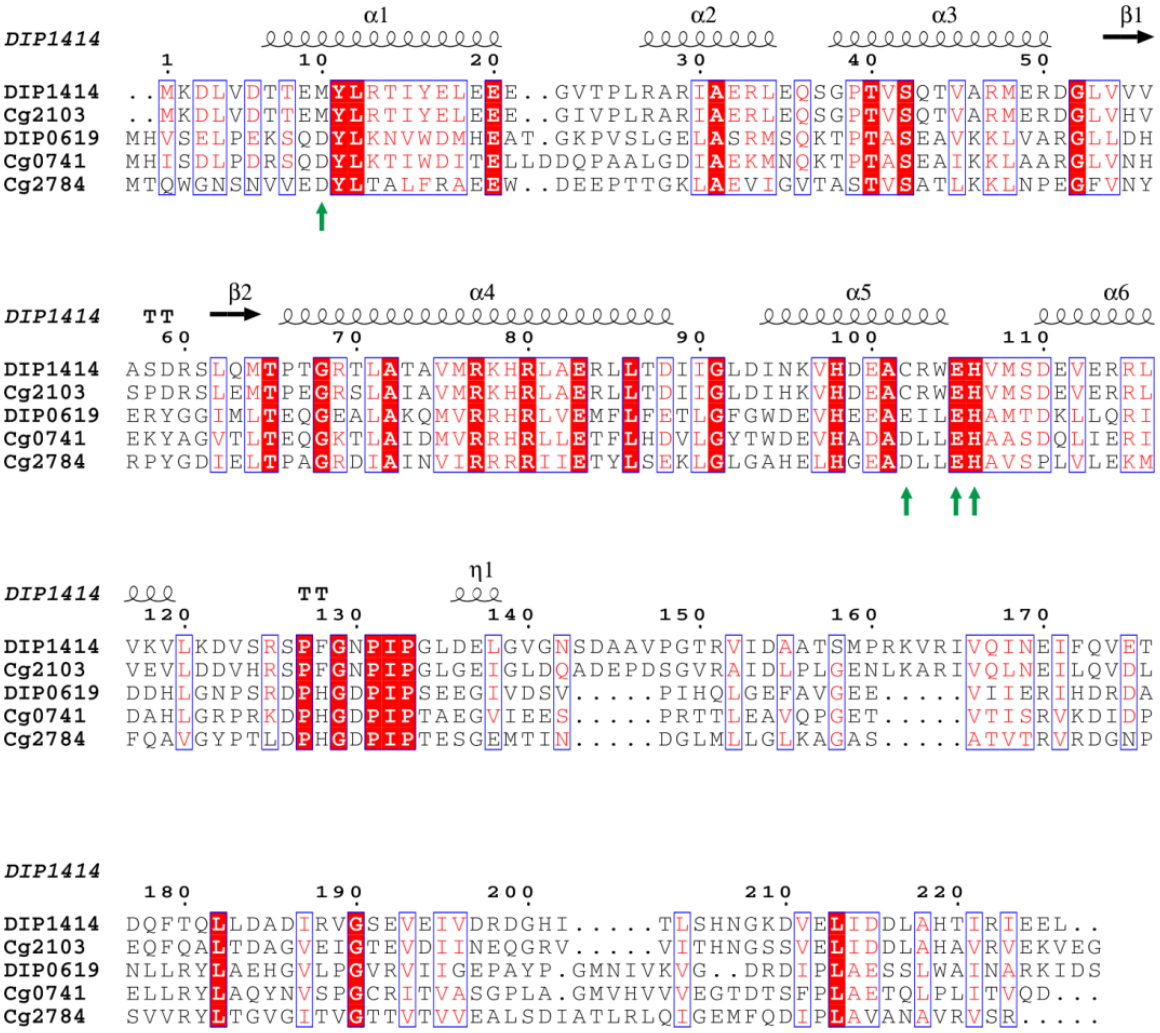

Fig. 5: Alignment of the DtxR-type regulators of *C. diphtheriae* and *C. glutamicum*. Residues highlighted with a red background are highly conserved. Residues printed in red are partially conserved. Residues marked with a green arrow are involved in metal binding. The secondary structure of DIP1414 is shown above the alignment (Qiu, *et al.*, 1996). The alignment presentation was prepared using ESPript (Gouet, *et al.*, 2003).