Target Genes, Consensus Binding Site, and Role of Phosphorylation for the Response Regulator MtrA of *Corynebacterium glutamicum*[†]

Melanie Bocker, Christina Mack, and Michael Bott*

Institut für Bio- und Geowissenschaften, IBG-1: Biotechnologie, Forschungszentrum Jülich, D-52425 Jülich, Germany

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The two-component signal transduction system consisting of the sensor kinase MtrB and the response regulator MtrA is highly conserved in corynebacteria and mycobacteria. Whereas *mtrA* of *Mycobacterium tuberculosis* was reported to be essential, we recently succeeded in creating $\Delta mtrAB$ and $\Delta mtrA$ deletion mutants of *Corynebacterium glutamicum* and provided evidence that *mepA* and *nlpC*, both encoding putative cell wall peptidases, are directly repressed by MtrA, whereas *proP* and *betP*, both encoding carriers for compatible solutes, are directly activated by MtrA. In the present study, novel MtrA target genes were identified, including *mepB*, encoding another putative cell wall peptidase. The repressor or activator functions of MtrA correlate with the distance between the MtrA binding site and the transcriptional start site. From the identified binding sites within 20 target promoters, a 19-bp MtrA consensus motif was derived which represents a direct repeat of 8 base pairs separated by 3 base pairs. Gene expression of a strain containing MtrA with a D53N mutation instead of wild-type MtrA resembled that of a $\Delta mtrA$ mutant, indicating that MtrA is active in its phosphorylated form. This result was confirmed by electrophoretic mobility shift assays with phosphorylated MtrA which showed an increased binding affinity.

In order to survive, bacteria must be able to adapt to environmental changes. This adaptive response is often accomplished by two-component signal transduction systems, which are composed of a sensor kinase and a response regulator. In most cases, the membrane-bound sensor kinase responds to specific stimuli by autophosphorylation of a conserved histidine residue; this phosphoryl group is subsequently transferred to the cognate soluble response regulator mediating changes in gene expression or cell behavior (12, 37). In the nonpathogenic Gram-positive soil bacterium *Corynebacterium glutamicum*, 13 two-component systems were identified (20), several of which have already been characterized (4, 5, 27, 35). Whereas some are specific for *C. glutamicum* or the genus *Corynebacterium*, others are conserved also in other genera. One of these is the two-component system MtrAB, which was first described in *Mycobacterium tuberculosis* (MtrAB_{Mt}) (39). Homologs of MtrA and MtrB are present in many, if not all, species of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Thermomonospora*, *Streptomyces*, *Leifsonia*, *Propionibacterium*, and *Bifidobacterium*. The presence of a DNA-binding helix-turn-helix motif in the C-terminal part of MtrA indicated that this response regulator functions as a transcriptional regulator. Whereas *mtrA* was reported to be essential in *M. tuberculosis* (41), we recently succeeded in constructing a *C. glutamicum* $\Delta mtrAB$ deletion mutant (27). This mutant had a pleiotropic phenotype. $\Delta mtrAB$ cells were elongated and sometimes showed irregular septum formation. The mutant was more sensitive to penicillin, vancomycin, and ly-

sozyme but more resistant to ethambutol. The changes in cell morphology and antibiotic susceptibility indicated that the MtrAB system might be involved in cell wall metabolism and/or cell division. Using DNA microarrays, dot blot experiments, and primer extension studies, genes with an altered mRNA level in the $\Delta mtrAB$ mutant were identified (27). Some of them showed increased expression in the mutant, such as *mepA* and *nlpC*, both encoding putative cell wall peptidases, *lpqB*, encoding a putative lipoprotein with unknown function, *ppmA*, encoding a putative membrane-bound protease modulator, and *mscL*, encoding a mechanosensitive channel. Other genes displayed decreased mRNA levels in the $\Delta mtrAB$ mutant, in particular, *betP*, *proP*, and *lcoP*, encoding uptake carriers for compatible solutes, cg0282 (NCgl0226), encoding a protein of unknown function, or cg2095 (NCgl1837), encoding a hypothetical membrane protein. By DNA-protein interaction studies, we provided evidence for the binding of MtrA to the promoter regions of *nlpC*, *mepA*, *betP*, and *proP* and to the intergenic region between *nrdH* and *nadE* (encoding a glutaredoxin-like protein and NAD⁺ synthetase, respectively) (4). Thus, these genes most likely represent direct target genes of MtrA, which therefore probably acts both as a repressor (*mepA* and *nlpC*) and as an activator (*betP* and *proP*). The influence of MtrA on the expression of *nrdH* and/or *nadE* is not yet known since the mRNA levels of these genes were unaltered in the DNA microarray studies (27).

Previous studies analyzed whether the sensor kinase MtrB of *C. glutamicum*, which contains two putative transmembrane helices and an extracytoplasmic domain of ~151 amino acids, functions as an osmosensor. After MtrB had been purified and reconstituted into proteoliposomes, the protein showed autophosphorylation activity and served as a phosphoryl donor to MtrA. Moreover, MtrB was found to possess phospho-MtrA phosphatase activity. In proteoliposomes, MtrB autophosphorylation was stimulated by potassium ions, but these monova-

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

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>C. glutamicum</i> ATCC 13032	Biotin-auxotrophic wild-type strain	1
<i>C. glutamicum</i> $\Delta mtrA$ strain	Derivative of ATCC 13032 with an in-frame deletion of the <i>mtrA</i> gene	4
<i>E. coli</i> DH5 α	λ^- $\phi 80d/lacZ\Delta M15 \Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^-)$ <i>supE44 thi-1 gyrA relA1</i>	Invitrogen
<i>E. coli</i> BL21(DE3)	<i>ompT hsdS_B (r_B^- m_B^-)</i> <i>gal dcm</i> (DE3)	38
Plasmids		
pET28b	Kan ^r ; vector for overexpression of genes in <i>E. coli</i> , adding an N-terminal hexahistidine affinity tag to the synthesized protein (pBR322 <i>oriV_{Ec}</i> and <i>P_{T7} lacI</i>)	Novagen, Merck KGaA
pET28b- <i>mtrA</i> -NHIS ₆	Kan ^r ; pET28b derivative for overproduction of MtrA with an N-terminal hexahistidine tag	This work
pET16b-PhoR	Amp ^r ; pET16b derivative for overproduction of the <i>C. glutamicum</i> response regulator PhoR with an N-terminal decahistidine tag	35
pMalc	Amp ^r ; <i>P_{tac} lacI^q</i> ColE1 <i>oriV</i> ; <i>E. coli</i> expression vector for construction and overproduction of fusion proteins containing the maltose binding protein of <i>E. coli</i> (MalE) without its signal peptide	New England Biolabs
pMalE-MtrBk	Amp ^r ; pMalc derivative for overproduction of a fusion protein consisting of the kinase domain (amino acids 250 to 503) of MtrB (MtrBk) from <i>C. glutamicum</i> and the maltose-binding protein of <i>E. coli</i>	This work
pMalE-CitAC	Amp ^r ; pMalc derivative for overproduction of a fusion protein consisting of the kinase domain (amino acids 357 to 547) of CitA (CitAC) from <i>K. pneumoniae</i> and the maltose-binding protein of <i>E. coli</i>	18
pMBP-PhoS Δ 1-246	Amp ^r ; pMalc derivative for overproduction of the kinase domain of the <i>C. glutamicum</i> sensor kinase PhoS (amino acid residues 247 to 485) fused to the C-terminus of the <i>E. coli</i> maltose-binding protein without the signal peptide (MBP-PhoS Δ 1-246)	35
pEKEx2	Kan ^r ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression (<i>P_{tac} lacI^q</i> pBL1 <i>oriV_{Cg}</i> pUC18 <i>oriV_{Ec}</i>)	7
pEKEx2- <i>mtrA</i>	Kan ^r ; pEKEx2 derivative containing the <i>mtrA</i> gene from <i>C. glutamicum</i> under the control of the <i>tac</i> promoter	This work
pEKEx2- <i>mtrA</i> (D53N)	Kan ^r ; pEKEx2 derivative containing the <i>mtrA</i> (D53N) gene from <i>C. glutamicum</i> under the control of the <i>tac</i> promoter	This work

^a *oriV_{Ec}*, *E. coli oriV*; *oriV_{Cg}*, *C. glutamicum oriV*.

lent cations were also found to increase the activity of another reconstituted histidine kinase (DcuS), indicating that they do not serve as a specific stimulus for MtrB to sense hyperosmotic stress (28). In subsequent studies membrane shrinkage was also ruled out as a stimulus for MtrB activation. Instead, various compounds such as amino acids, sugars, and polyethylene glycols were found to stimulate MtrB activity. Due to the diverse chemical nature of these substances, binding to a specific binding site was assumed to be unlikely. Rather, they were proposed to act via a change of the hydration state of MtrB, which shifts the protein into its active state. As the effect of the solutes was independent of the periplasmic loop and the HAMP domain, the kinase domain of MtrB was proposed to be responsible for sensing hypertonicity (29).

Here, we focused on the MtrA-DNA interaction. The MtrA binding sites in the promoter regions of *mepA*, *nlpC*, *betP*, and *proP* were localized, and 18 additional promoters were identified to which MtrA binds *in vitro*. An MtrA consensus motif was deduced from the alignment of the identified binding sites. The function of MtrA as an activator or repressor was shown to correlate with the position of its binding sites relative to the transcriptional start sites. Furthermore, evidence is provided that MtrA is active *in vivo* in its phosphorylated state.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used or constructed in the course of this work are listed in Table 1. *C. glutamicum* strains were routinely cultivated aerobically in 500-ml shaking flasks with 60 ml of CGXII medium (19) with 2% or 4% (wt/vol) glucose on a rotary shaker (150 rpm) at 30°C. *Escherichia coli* DH5 α was used as host for cloning purposes and for overproduction of the proteins MalE-MtrBK and MalE-CitAC. *E. coli* BL21(DE3) (38) was used as host for overproduction of the MtrA protein with an N-terminal His₆ tag (MtrA-NHIS₆). The *E. coli* strains were cultivated aerobically in LB medium at 37°C or at 30°C for protein overproduction. When appropriate, kanamycin was added to a concentration of 50 μ g/ml (*E. coli*) or 25 μ g/ml (*C. glutamicum*), and ampicillin was added to a concentration of 100 μ g/ml (*E. coli*).

Recombinant DNA work. The enzymes for recombinant DNA work were obtained from Roche Diagnostics or New England BioLabs. Oligonucleotides used in this study were synthesized by Eurofins MWG Operon (see Table S1 in the supplemental material). Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (34). Plasmids were isolated from *E. coli* with a QIAprep spin miniprep kit (Qiagen). *E. coli* was transformed by the RbCl method (13). DNA sequencing was performed with a Genetic Analyzer 3100-Avant (Applied Biosystems). Sequencing reactions were carried out with a BigDye Terminator Kit, version 3.1 (Applied Biosystems).

For isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible expression of *mtrA*, the coding region of this gene including 33 bp upstream of the start codon was amplified using chromosomal DNA of *C. glutamicum* ATCC 13032, the oligonucleotide pair OE_mtrA-fw/OE_mtrA-rv, and an Expand High Fidelity PCR kit (Roche Diagnostics). The PCR product was digested with BamHI and KpnI and cloned into pEKEx2 (7) cut with the same enzymes. DNA sequence

analysis of the resulting plasmid pEKEx2-*mtrA* revealed that the cloned *mtrA* fragment was identical to the published wild-type sequence.

For IPTG-inducible production of a phosphorylation-defective MtrA, plasmid pEKEx2-*mtrA*(D53N) was constructed, which encodes an MtrA protein with an Asp53Asn exchange [MtrA(D53N)]. The D53 codon GAC was changed to AAC using the oligonucleotide pair *mtrA*_Asp→Asn_1/*mtrA*_Asp→Asn_2 and a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) in accordance with the instruction manual. Three of the resulting plasmids were sequenced, all of which carried the desired exchange and no additional mutations within the *mtrA* gene.

For overproduction and purification of MtrA with an amino-terminal histidine tag, the *mtrA* coding region was amplified using oligonucleotides that introduced an NdeI restriction site overlapping the start codon (*mtrA*-NdeI) and an XhoI restriction site after the stop codon (*mtrA*-stop-rv). The 698-bp PCR product was digested with NdeI and XhoI and cloned into the expression vector pET28b cut with the same enzymes. The MtrA protein encoded by the resulting plasmid pET28b-*mtrA*-NHis₆ (246 amino acids; 27.1 kDa) contains 20 additional amino acids at the amino terminus (MGSSHHHHHHSSGLVPRGSH) including a hexahistidine tag and a thrombin cleavage site. DNA sequence analysis revealed that the cloned *mtrA* gene contained no mutations.

For overproduction and purification of the kinase domain of the *C. glutamicum* sensor kinase MtrB, the expression plasmid pMalE-MtrBK was constructed. The coding region for the kinase domain of MtrB (amino acids 250 to 503 of an MtrB protein consisting of 503 residues) (27) was amplified using oligonucleotides that introduced a BamHI restriction site (*mtrB*-pMal-BamHI-fw) upstream of codon 250 and a BamHI restriction site after the stop codon (*mtrB*-pMal-BamHI-rv). The purified 795-bp PCR product was digested with BamHI and cloned into the expression vector pMalc cut with BamHI. Recombinant plasmids containing the insert in the desired orientation were identified by restriction analysis and termed pMalE-MtrBK. The pMalc vector contains a modified *E. coli malE* gene encoding a maltose-binding protein (MBP) without signal peptide (amino acids 2 to 26). The cloning strategy resulted in a protein in which the kinase domain of MtrB (amino acids 250 to 503) was fused to the carboxy terminus of MalE. The synthesis of this protein (631 amino acids; 69.2 kDa) was controlled by the *tac* promoter upstream of *malE*. For overproduction and purification of the kinase domain of the *Klebsiella pneumoniae* sensor kinase CitA, the expression plasmid pMalE-CitAC was used (18). Purified MBP-PhoSΔ1–246 (the kinase domain of the *C. glutamicum* PhoS fused to the C terminus of the *E. coli* MBP lacking the signal peptide) and PhoR were provided by Sarah Schaaf (for details, see reference 35).

Global gene expression analysis. DNA microarray analysis was used to compare the genome-wide mRNA levels of the Δ *mtrA* strain versus the wild-type strain and the Δ *mtrA*/pEKEx2-*mtrA*(D53N) strain versus the Δ *mtrA*/pEKEx2-*mtrA* strain. RNA was prepared as described before (27). All DNA microarray analyses were performed with custom-made DNA microarrays based on 70-mer oligonucleotides obtained from Operon Biotechnologies. The comparisons were performed from four independent biological replicates. The experimental details for handling of these microarrays were described previously (11).

Primer extension analysis. Nonradioactive primer extension analyses were performed as described previously (8, 9) using IRD800-labeled oligonucleotides (see Table S1 in the supplemental material, where the oligonucleotides for primer extension [PE] are identified by the name of the gene of interest as PE_“gene”) and 10 μ g of RNA from *C. glutamicum* the wild-type or Δ *mtrA* strain as a template. The length of the primer extension products was determined by running the four lanes of a DNA sequencing reaction mixture set up using the same oligonucleotide as that used for the reverse transcription alongside the primer extension product. The templates for DNA sequencing covered the whole promoter regions and were obtained by PCR using chromosomal DNA of *C. glutamicum* ATCC 13032 and the oligonucleotide pairs “gene”_prom_fw and “gene”_prom_rv (see Table S1, where “gene” indicates the name of the gene of interest).

Overproduction and purification of MtrA. *E. coli* BL21(DE3) carrying the plasmid pET28b-*mtrA*-NHis₆ was grown at 30°C in 100 ml of LB medium with 50 μ g/ml kanamycin to an optical density at 600 nm (OD₆₀₀) of ~1 before 1 mM IPTG was added. After cultivation for another 4 h, cells were harvested by centrifugation, washed once, and stored at –20°C. Cell extract preparation and protein purification were performed as described previously for the response regulator CitB (5). In order to remove the N-terminal histidine tag, the purified protein MtrA-His was digested with thrombin using a Thrombin Cleavage/Capture kit (Novagen, Merck KGaA) according to the supplier's instructions. After the cleavage reaction, the biotinylated thrombin was removed, and the buffer was again exchanged against BS buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 10% [vol/vol] glycerol). The resulting MtrA protein

(229 amino acids; 25.2 kDa) contains three additional amino acid residues (GSH) at the N terminus. Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard. The purity of the protein preparations was assessed by SDS-PAGE, and subsequent protein detection was performed with Gel Code blue stain reagent (Pierce, Rockford, IL). For determination of the protein mass, a Precision Plus Protein Prestained Standard (Bio-Rad) was used.

Overproduction and purification of MalE-MtrBK and MalE-CitAC. *E. coli* DH5 α carrying plasmid pMalE-MtrBK or pMalE-CitAC was grown at 30°C in 100 ml of LB medium with 100 μ g/ml ampicillin to an OD₆₀₀ of ~1.5 before the addition of 1 mM IPTG. After cultivation for another 3 h, cells were harvested by centrifugation, washed once, and stored at –20°C. Cell extract was prepared as described by Brocker et al. (5) with one alteration: the thawed cells were resuspended in 10 ml of TKMD buffer (50 mM Tris-HCl, 200 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol [DTT; freshly added], pH 7.5). MalE-MtrBK and MalE-CitAC were purified by affinity chromatography on amylose resin (New England BioLabs). The soluble protein fraction was loaded onto a column containing a 5-ml bed volume of amylose resin, which had been equilibrated with TKMD buffer. After a washing step with 40 ml of this buffer, MtrBK and MalE-CitAC were eluted with TKMD buffer containing 10 mM maltose. Fractions containing MalE-MtrBK or MalE-CitAC were pooled, and the elution buffer was exchanged by gel filtration using PD-10 columns (GE Healthcare) against BS buffer. Overproduction and purification of MBP-PhoSΔ1–246 were performed as described previously (35).

Phosphorylation assays. The *in vitro* phosphorylation of MtrA by the sensor kinase derivatives MalE-MtrBK, MalE-CitAC, and MBP-PhoSΔ1–246 was performed as described previously (35). For phosphorylation of MtrA with phosphoramidate, ammonium hydrogen phosphoramidate was synthesized according to Sheridan et al. (36). The resulting crystalline product was dissolved to 0.5 M in BS buffer and immediately used to phosphorylate MtrA. Phosphorylation reactions were carried out in BS buffer containing 50 mM phosphoramidate for 1 h at 30°C.

Electrophoretic mobility shift assays (EMSAs). For testing the binding of MtrA to putative target promoters, purified MtrA protein (0 to 22.5 μ M) was mixed with 100-ng DNA fragments (26 to 570 bp; final concentration, 13 to 300 nM) in a total volume of 20 μ l. Both MtrA and the DNA fragments were dissolved in BS buffer. The reaction mixture was incubated at room temperature for 30 min and then loaded onto a 15% native polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V using cooled TBE (89 mM Tris base, 89 mM boric acid, 2 mM Na₂EDTA) as the electrophoresis buffer. The gels were subsequently stained with SYBR Green I according to the instructions of the supplier (Sigma-Aldrich) and photographed (40). The DNA fragments used in this assay were generated either by PCR or by annealing two complementary oligonucleotides by incubating them in an equimolar ratio for 5 min at 95°C, followed by cooling at room temperature. The resulting products were purified with a PCR purification kit (Qiagen) and eluted in 10 mM Tris-HCl, pH 8.5.

RESULTS

Identification of the MtrA binding sites in the promoter regions of *nlpC*, *mepA*, *betP*, and *proP*. In previous studies evidence was provided that at least four genes of *C. glutamicum* are directly controlled by MtrA and that this response regulator functions as both a repressor and an activator (4, 27). The genes *mepA* and *nlpC*, which encode putative cell wall peptidases, are repressed by MtrA, while *betP* and *proP*, encoding carriers for uptake of compatible solutes, are activated by MtrA. In order to localize the binding site(s) of MtrA in the promoter regions of these genes, electrophoretic mobility shift assays (EMSAs) were performed. For this purpose, the MtrA protein containing an amino-terminal hexahistidine tag was overproduced in *E. coli*, purified to apparent homogeneity by affinity chromatography, and digested with thrombin to remove the histidine tag (see Fig. S1 in the supplemental material). The resulting MtrA protein contains only three additional amino acids (GSH) at the N terminus. In a first series of EMSAs, DNA fragments covering the entire promoter regions of *mepA*, *nlpC*, *betP*, and *proP* were incubated with two differ-

FIG. 1. Search for MtrA-binding sites within the promoter region of *nlpC* (A), *mepA* (B), *proP* (C), and *betP* (D). EMSAs were performed with subfragments of the promoter regions and purified MtrA. The DNA fragments (13 to 95 nM) were incubated for 30 min at room temperature with a 0-, 25-, or 75-fold molar excess of MtrA. Subsequently, the reaction mixtures (20 μ l) were separated on a native 15% polyacrylamide gel, and the gels were stained with SYBR Green as described previously (40). The numbers shown above the DNA fragments indicate the positions of the fragment ends relative to the transcriptional start sites (+1). The plus and minus symbols left of the fragments indicate whether they are shifted or not, respectively.

ent concentrations of MtrA and separated by native PAGE. As shown in Fig. 1, all four fragments were shifted by a 75-fold molar excess of MtrA. In a second series of EMSAs, subfragments (labeled 2 to 8 in Fig. 1A to D) of the promoter regions were tested for binding. By this approach, the MtrA binding sites were located between -41 and +25 and between -69 and -44 in the case of *nlpC*, between -62 and +31 in the case of *mepA*, between -133 and -54 in the case of *proP*, and between -177 and -91 in the case of *betP* (positions are relative to transcription start site [27]).

Analysis of the five binding regions with the software tool MEME (<http://meme.sdsc.edu/meme/meme.html>) revealed the presence of a common sequence motif. The relevance of this motif in the five binding regions was tested by performing

EMSAs with DNA fragments in which 2 to 4 nucleotides of the motif were exchanged simultaneously. As shown in Fig. 2, most of the mutations had a negative impact on MtrA binding, and several mutations led to a complete inhibition of binding. In the case of the promoter-proximal MtrA binding site of *nlpC* (in *nlpC* subfragment 2 [*nlpC*₂]), the actual binding site appears to be displaced by 5 bp compared to the predicted consensus motif.

Genome-wide search for additional MtrA target genes. To identify additional MtrA target genes, two MtrA consensus binding motifs were derived from the binding sites identified thus far of *mepA*, *nlpC*, *proP*, and *betP* and used to search the whole *C. glutamicum* genome using the ERGO bioinformatics suite (Integrated Genomics, IL). The first consensus motif

nlpC1

	CCGCGATAACGGTTCGATAACGACCAA	++
1)	---ATCG-----	--
2)	-----CCA-----	--
3)	-----TTG-----	-
4)	-----GAT-----	+
5)	-----CGCC-----	--
6)	-----ATCA-----	+
7)	-----ACC-----	++

nlpC2

	TGTTSCGGTTTCGTAACCTTATTGAGAC	++
1)	GTG-----	++
2)	---GTAT-----	+
3)	-----TGGG-----	-
4)	-----ATG-----	--
5)	-----CCA-----	--
6)	-----AGG-----	++
7)	-----CGGT-----	++
8)	-----CTC-----	++

prop

	GGAAATGTGACCAATTGTAAACCCACC	++
1)	TTCC-----	++
2)	---CGTG-----	--
3)	-----TCAA-----	--
4)	-----AACC-----	--
5)	-----GGG-----	--
6)	-----TGCC-----	--
7)	-----AAAC-----	--
8)	-----AAA-----	++

mepA

	AGGTGGTCACAACCTTGATAACGGACT	++
1)	CTT-----	++
2)	---GTT-----	+
3)	-----GAC-----	+
4)	-----ACC-----	+
5)	-----AG-----	+
6)	-----GT-----	+
7)	-----CG-----	--
8)	-----CC-----	--
9)	-----AT-----	-
10)	-----TC-----	+
11)	-----AG-----	++

betP

	ATTAATCGTTATGTCATTGTGATGCTC	++
1)	CGGC-----	++
2)	---CGAT-----	-
3)	-----GGCG-----	--
4)	-----GACG-----	+
5)	-----GTGT-----	--
6)	-----CGT-----	--
7)	-----AGA-----	++

FIG. 2. Mutational analysis of the putative MtrA-binding motifs within the promoter regions of *nlpC*, *mepA*, *betP*, and *prop* suggested by using the MEME software. To determine the importance of the predicted DNA sequence motif (underlined sequences) for MtrA binding, fragments with different mutations were tested in EMSAs (for details, see the legend of Fig. 1). According to the results of the shifts, the fragments were divided into four categories, as follows: ++, mutated fragment shifted like wild-type (wt) fragment; +, mutated fragment shifted with lower affinity than wild-type fragment; -, mutated fragment hardly shifted, and --, mutated fragment not shifted at all. Bases written in white on a black background are important for MtrA binding.

(VRTHASRDHDHSRWMMCR) was found 27 times in the entire genome when mismatches, deletions, and insertions were not allowed. In eight cases, the motif was located in intergenic regions up to 500 bp upstream of the start codon of the neighboring genes. As expected, these hits included the five experimentally determined binding sites used to derive the motif. The other three hits are located between the divergently orientated genes cg2936-cg2937 and cg0646-cg0647, and one hit is located upstream of *rpf2* (cg1037). The search for the second motif (GATCACAATTNGATAACG) revealed 146 hits when four mismatches were allowed but not insertions or deletions. Thirty-five of these hits were located in intergenic regions up to 500 bp upstream of the start codon of the neighboring genes. Only candidate genes that showed altered

mRNA levels in the DNA microarray experiments comparing the $\Delta mtrAB$ or $\Delta mtrA$ deletion mutant with the wild type (for details, see reference 27 and below) were tested in EMSAs: cg0858-cg0859, both encoding proteins with unknown function, cg0980, encoding a putative metalloproteinase, and *rpf2*, encoding a growth factor (14), showed altered mRNA levels in at least one of the mutants (Table 2). Taking together the transcriptome analyses and the binding of MtrA to the promoter regions of these genes (Fig. 3A) suggests that the protein acts as repressor of cg0858 and/or as activator of cg0859 and as repressor of cg0980 and *rpf2*. All MtrA target genes identified so far and the corresponding mRNA ratios observed in the DNA microarray experiments are listed in Table 2.

TABLE 2. mRNA ratios of direct MtrA target genes as determined by DNA microarray analyses

Protein type and locus	Alternate designation	Gene name	Known or predicted function of gene product	mRNA ratio ^a	
				$\Delta mtrAB$ strain/ wt strain	$\Delta mtrA$ strain/ wt strain
Proteins with transport functions					
cg0133	NCgl0100	<i>abgT</i>	Secondary transporter of the AbgT family (2.A.68)	0.59	0.03*
cg0134	NCgl0101	<i>abgB</i>	Peptidase	0.55*	0.17*
cg1016	NCgl0856	<i>betP</i>	Glycine betaine transporter	0.17*	0.03*
cg3395	NCgl2961	<i>proP</i>	Proline/ectoine carrier	0.12*	0.03*
Proteins predicted to be proteases/peptidases					
cg0980	NCgl0824	<i>mepB</i>	Putative secreted metalloprotease	1.43*	1.35*
cg2402	NCgl2108	<i>nlpC</i>	Putative secreted cell wall peptidase	2.05*	2.10
cg2747	NCgl2411	<i>mepA</i>	Putative cell wall peptidase	14.51*	8.16*
Proteins with other predicted functions					
cg0282	NCgl0226	<i>csbD</i>	Stress protein of unknown function	0.33*	0.03*
cg1037	NCgl0872	<i>rpf2</i>	Resuscitation promoting factor	2.25	2.15*
cg1612	NCgl1368		Putative acetyltransferase	0.50	0.23*
cg2789	NCgl2445	<i>nrdH</i>	Putative glutaredoxin	0.91	0.61*
cg2792	NCgl2446	<i>nadE</i>	NAD synthetase	1.21	1.15
cg3138	NCgl2737	<i>ppmA</i>	Putative membrane-bound protease modulator	3.90*	0.82
Putative secreted proteins of unknown function					
cg0715	NCgl0592		Putative secreted protein	0.63	0.05*
cg0858	NCgl0717		Putative secreted protein	2.76*	1.75*
cg1517	NCgl1291		Putative secreted protein	0.36*	0.45*
cg1665	NCgl1418		Putative secreted protein	0.41*	0.04*
cg1942	NCgl1656		Putative secreted protein	0.31	0.28*
cg2052	NCgl1756		Putative secreted protein	0.53	0.08*
Putative membrane proteins of unknown function					
cg0896	NCgl0752		Putative membrane protein	1.09	0.01*
cg2096	NCgl1838		Putative membrane protein	0.64*	0.10*
cg2777	NCgl2434		Putative membrane protein	0.39	0.19*
cg3254	NCgl2841		Putative membrane protein	0.75	0.01*
Proteins of unknown function					
cg0859	NCgl0718		Uncharacterized protein	0.67*	0.41*
cg1943			Uncharacterized protein		0.09*

^a The mRNA ratios shown represent mean values from three or four independent DNA microarray experiments starting from independent cultures (for details, see text). The strains were cultivated in CGXII minimal medium with 2% (wt/vol) glucose, and mRNA was prepared from cells in the exponential growth phase. Values with a *P* of ≤ 0.05 are indicated with an asterisk. The data for the comparison $\Delta mtrA$ strain versus the wild type (wt) were obtained in this work, and values for the comparison of the $\Delta mtrAB$ strain versus the wild type were reported previously (27).

Binding of MtrA to additional putative target promoters. In our previous work on the MtrAB two-component system of *C. glutamicum*, additional genes besides *nlpC*, *mepA*, *betP*, and *proP* were identified as putative targets of MtrA since their mRNA levels were also increased or decreased in the $\Delta mtrAB$ mutant (27) or because their promoter regions were enriched in chromatin immunoprecipitation (ChIP)-to-chip experiments with MtrA-specific antibodies (4). In this work, additional DNA microarray experiments were performed in which the mRNA levels of the $\Delta mtrA$ mutant were compared to those of the wild type. The results of this comparison are summarized in Table 3.

Twenty-five promoter regions were chosen to be tested for MtrA binding in EMSAs due to the following reasons. The genes or putative operons cg0133-cg0134, *csbD* (cg0282), cg0715, cg0896, cg1517-cg1516, cg1612, cg1665, cg1942, cg1943, cg2052, cg2096-cg2095, cg2777, and cg3254 showed at least

3-fold decreased mRNA levels in the $\Delta mtrAB$ and/or the $\Delta mtrA$ mutant, whereas the genes *lpqB* (cg0865), *mscL* (cg1001), and *ppmA* (cg3138) had at least 3-fold increased mRNA levels in one or both deletion mutants. The intergenic region between *nrdH* (cg2789) and *nadE* (cg2792) and the promoter region of cg0135-cg0136 were enriched by chromatin immunoprecipitation with anti-MtrA antibodies (4). The region upstream of *mtrA* was chosen in order to test for a potential autoregulation. As shown in Fig. 3B to E, all tested promoter regions were shifted by MtrA except those of *lpqB*, *mscL*, *lcoP*, cg0136-cg0135, and *mtrA*. Accordingly, 18 genes were identified that most likely represent further direct MtrA targets.

Determination of an MtrA consensus binding motif. A search for a common sequence motif within the upstream regions of all identified target genes using the MEME software

FIG. 3. Binding of MtrA to promoter regions of putative direct target genes. DNA fragments (120 to 400 bp in size; final concentration, 20 to 60 nM) covering the promoter regions were amplified by PCR, purified, and incubated for 30 min at room temperature without MtrA (left lanes) or with a 25- or a 75-fold molar excess of purified MtrA. Subsequently, the samples were separated by native polyacrylamide (15%) gel electrophoresis, and the DNA was stained with SYBR Green I. (A) EMSAs with promoter regions containing a putative MtrA-binding motif and showing an altered mRNA level in the DNA microarray experiments. (B and C) EMSAs with genes that showed decreased or increased mRNA levels in the DNA microarray experiments comparing the $\Delta mtrAB$ strain or $\Delta mtrA$ strain with the wild type, respectively. (D) EMSAs with genes that were enriched in ChIP-to-chip experiments. (E) EMSA with the promoter region of *mtrA* to test for putative autoregulation. For details of these experiments, see Results.

(<http://meme.sdsc.edu/meme/meme.html>) revealed the 19-bp sequences shown in Fig. 4A. To verify their importance for MtrA binding, each of the 19-bp motifs was fused via PCR to a DNA fragment (control) that is not bound by MtrA, and the resulting DNA fragments were tested in EMSAs with purified MtrA (data not shown). Except for the control and four fragments containing putative binding sites, all of the other 19 PCR products were bound by MtrA, showing that each of these 19-bp motifs represents an MtrA binding site. From the 19 binding sites a second-generation MtrA consensus binding motif was deduced using the Weblogo tool (<http://weblogo.berkeley.edu/>). As shown in Fig. 4B, the MtrA binding motif represents a loosely conserved direct repeat of 8 bp separated by a 3-bp linker.

To elucidate the importance of the length and the sequence of the 3-bp linker, mutated versions of the MtrA binding site in

the *mepA* promoter region were tested in EMSAs (see Fig. S2 in the supplemental material). The corresponding 26-bp to 32-bp DNA fragments were created by annealing complementary oligonucleotides. As shown in Fig. S2, both the extension and the reduction of the 3-bp linker by only a single base pair strongly inhibited the binding of MtrA, whereas the sequence exchanges had no significant effect on MtrA binding. Thus, the distance between the 8-bp repeats is critical for binding.

Position of the MtrA binding sites with respect to the transcriptional start sites. The results reported previously and in this work revealed that *C. glutamicum* MtrA can act as both a repressor and an activator. Therefore, it was of interest to search for correlations between the position of the MtrA binding sites in relation to the transcriptional start sites and the activating or repressing effects of MtrA. For this purpose, the transcriptional start sites of *csbD*, *cg1517*, *nlpC*, and *rpf2* were

TABLE 3. Transcriptome comparison of the *C. glutamicum* $\Delta mtrA$ strain and the wild-type strain and of the *C. glutamicum* $\Delta mtrA/pEKEx2-mtrA(D53N)$ strain and the $\Delta mtrA/pEKEx2-mtrA$ strain using DNA microarrays

Locus	Alternate designation	Gene name	Known or predicted function of the gene product	mRNA ratio ^a	
				$\Delta mtrA$ strain/wt strain	MtrA(D53N)/MtrA
cg0133	NCgl0100	<i>abgT</i>	Secondary transporter of the AbgT family (2.A.68)	0.03*	0.01*
cg0134	NCgl0101	<i>abgB</i>	Peptidase	0.17*	0.14*
cg0135	NCgl0102		Putative inner membrane protein	0.56	0.43*
cg0136	NCgl0103		Protein of unknown function	0.44*	0.48*
cg0282	NCgl0226	<i>csbD</i>	Stress protein of unknown function	0.03*	0.02*
cg0689	NCgl0571		Protein of unknown function	2.18*	1.07
cg0715	NCgl0592		Putative secreted protein	0.05*	0.15*
cg0859	NCgl0718		Protein of unknown function	0.41*	0.62*
cg0862	NCgl0721	<i>mtrA</i>	Response regulator	0.00*	0.66*
cg0896	NCgl0752		Protein of unknown function	0.01*	0.82
cg0909	NCgl0764		Protein of unknown function	0.39*	1.47*
cg1016	NCgl0856	<i>betP</i>	Glycine betaine transporter	0.03*	0.04*
cg1037	NCgl0872	<i>rpf2</i>	Resuscitation promoting factor	2.15*	1.85*
cg1085			Protein of unknown function	0.14	0.50*
cg1210			Protein of unknown function	0.49*	0.89*
cg1213		<i>tnp1a</i>	Transposase	2.46*	0.56
cg1227	NCgl1033		Protein of unknown function	0.46*	0.85
cg1279	NCgl1083		Putative secreted protein	2.11*	0.95
cg1457	NCgl1240	<i>dnaQ2</i>	DNA polymerase III, putative ϵ subunit	2.33*	1.14*
cg1476	NCgl1253	<i>thiC</i>	Thiamine biosynthesis protein	0.41*	0.81
cg1513	NCgl1287	<i>tnp23a</i>	Transposase fragment	0.96	3.51*
cg1516	NCgl1290		Protein of unknown function	0.43*	0.31
cg1517	NCgl1291		Putative secreted protein	0.45*	0.39*
cg1562	NCgl1323		Protein of unknown function	0.61*	0.43*
cg1612	NCgl1368		Putative acetyltransferase	0.23*	0.41*
cg1665	NCgl1418		Putative secreted protein	0.04*	0.01*
cg1671	NCgl1422		Putative membrane-associated GTPase	0.57	2.01*
cg1942	NCgl1656		Putative secreted protein	0.28*	0.30*
cg1943			Protein of unknown function	0.09*	0.07*
cg2052	NCgl1756		Putative secreted protein	0.08*	0.08
cg2054			Protein of unknown function	0.40*	0.71
cg2095	NCgl1837		Protein of unknown function	0.13	0.21*
cg2096	NCgl1838		Protein of unknown function	0.10*	0.07
cg2236	NCgl1961	<i>thiE</i>	Thiamine-phosphate pyrophosphorylase	0.47*	0.88
cg2500	NCgl2199		Bacterial regulatory proteins, ArsR family	2.32*	1.27*
cg2719	NCgl2387		Putative enterochelin esterase	0.40*	0.98
cg2720	NCgl2388	<i>lppS</i>	Secreted lipoprotein, ErfK/YbiS/YcfS/YngG family	0.40*	0.56*
cg2747	NCgl2411	<i>mepA</i>	Putative cell wall peptidase	8.16*	4.60*
cg2777	NCgl2434		Protein of unknown function	0.19*	0.51*
cg3254	NCgl2841		Protein of unknown function	0.01*	0.02*
cg3395	NCgl2961	<i>proP</i>	Proline/ectoine carrier	0.03*	0.04*

^a The mRNA ratios shown represent mean values from four independent DNA microarray experiments starting from independent cultures. The strains were cultivated in CGXII minimal medium with 2% (wt/vol) glucose, and mRNA was prepared from cells in the exponential growth phase. The table includes genes that showed at least a 2-fold change in mRNA levels (increased or decreased) in at least two of the four replicates in at least one of the two comparisons. Values with a *P* of ≤ 0.05 are indicated with an asterisk. The genes are ordered according to the mRNA ratio of the comparison of the $\Delta mtrA$ strain versus the wild type. The comparison of the $\Delta mtrA/pEKEx2-mtrA(D53N)$ strain and the $\Delta mtrA/pEKEx2-mtrA$ strain is expressed as the ratio of MtrA(D53N) to MtrA.

identified by primer extension (Fig. 5A). The transcriptional start sites of *betP*, *proP*, and *mepA* were reported previously (27). In the case of *rpf2*, our results confirmed the transcriptional start site determined by Jungwirth et al. (17). As shown in Fig. 5B (see also Fig. S3 in the supplemental material), the MtrA binding sites in the promoter regions of the genes that are repressed by MtrA are centered at position -63 (*nlpC*₁), -12 (*nlpC*₂), $+4$ (*mepA*), and -14 (*rpf2*). In the case of the genes that are activated by MtrA, the binding sites are centered at position -150 (*betP*), -115 (*proP*), -58 (*csbD*), and -64 (cg1517). These data largely support the assumption that the position of the binding sites with respect to the transcriptional start sites determines whether MtrA acts as a repressor or an activator. Binding of MtrA in the vicinity of the -10 region prevents binding of the RNA polymerase and thus ini-

tiation of transcription, whereas binding of MtrA upstream of the -35 region could promote binding of RNA polymerase to the DNA and thus stimulate initiation of transcription. In the case of the repressed gene *nlpC*, one MtrA binding site overlaps the -10 region (*nlpC*₂), whereas the other MtrA binding site is located upstream of the -35 region (*nlpC*₁). Apparently, the presence of the binding site *nlpC*₂ is enough to prevent binding of the RNA polymerase irrespective of whether MtrA binds to *nlpC*₁.

In vitro phosphorylation of MtrA by different sensor kinase derivatives. The DNA-binding properties of response regulators are controlled by the phosphorylation status of the protein. Therefore, attempts were made to establish an *in vitro* system allowing efficient phosphorylation of MtrA. For this purpose, the kinase domain of the sensor kinase MtrB (amino

FIG. 4. (A) Verified and putative binding sites predicted by the MEME software within the fragments that were shifted by MtrA (Fig. 3). Except for binding sites 1 to 4, all others (5 to 23) were shifted by MtrA in EMSAs (for experimental details, see text) (data not shown). The numbers given indicate the distance from the center of the binding site (10th bp of the motif) to either the transcriptional start site (TC) or, if this is unknown, the proposed translational start site (TL). (B) Consensus motif of MtrA derived from the verified binding sites using the Weblogo software (<http://weblogo.berkeley.edu/>)

acids 250 to 503) was fused to the carboxy terminus of the *E. coli* maltose binding protein MalE lacking its signal peptide. The fusion protein MalE-MtrBK was overproduced in *E. coli* and purified by affinity chromatography with amylose resin. In the case of other sensor kinases, e.g., the citrate sensor CitA from *Klebsiella pneumoniae* (18) or PhoS from *C. glutamicum* (35), such fusion proteins displayed constitutively active autokinase activity. In the case of MtrB, however, only a very weak autophosphorylation was detected when MalE-MtrBK was incubated with [γ - 32 P]ATP (see Fig. S4A in the supplemental material). In contrast, the proteins MalE-CitAC from *K. pneumoniae* and MBP-PhoS Δ 1–246 from *C. glutamicum* showed much stronger autophosphorylation under the same experimental conditions (see Fig. S4B to D). Twenty minutes after the start of the autophosphorylation reaction, purified MtrA was added in 2-fold molar excess to the kinase proteins. As shown in Fig. S4A to C in the supplemental material, MtrA became phosphorylated by all three kinase derivatives, but the kinetics were very slow. The highest level of MtrA phosphorylation after 60 min was achieved with MBP-PhoS Δ 1–246; the lowest was with MalE-MtrBK, presumably due to the very low autophosphorylation activity of the latter protein. As shown in Fig. S4D, the phosphoryl group transfer from MBP-PhoS Δ 1–

246 to its cognate response regulator PhoR was much more efficient than to the noncognate response regulator MtrA. In the absence of a sensor kinase derivative, no phosphorylation of MtrA by ATP was observed (see Fig. S4E in the supplemental material). The extremely weak autophosphorylation activity observed for MalE-MtrBK is in agreement with a previous study showing that a soluble MtrB derivative lacking the first 190 amino acids and carrying a C-terminal StrepTag-II was inactive (29).

In vivo influence of MtrA phosphorylation. The preceding *in vitro* experiments could not answer the question of whether MtrA is active in its phosphorylated or in its unphosphorylated state. The latter possibility has been described for the response regulator HoxA (21). Therefore, the question was addressed using an *in vivo* approach. The *C. glutamicum* Δ mtrA mutant (4) was transformed with expression plasmids encoding either the wild-type MtrA protein or the phosphorylation-defective variant MtrA(D53N). To test the influence of the D53N exchange on the function of MtrA as a transcriptional regulator, the mRNA ratios of the Δ mtrA/pEKEx2-mtrA(D53N) strain versus the Δ mtrA/pEKEx2-mtrA strain were determined using DNA microarrays, and the results were compared with the ratios obtained for the comparison of Δ mtrA mutant versus the

FIG. 5. (A) Primer extension analyses of the genes *nlpC*, *rpf2*, *csbD*, and *cg1517* with the oligonucleotides PE_ *nlpC*, PE_ *rpf2*, PE_ *csbD*, and PE_ *cg1517* and 10 μ g of total RNA from the wild type (for *csbD* and *cg1517*) or from the Δ *mtrA* mutant (for *nlpC* and *rpf2*). The transcriptional start sites are indicated by asterisks. (B) Location of the identified MtrA binding sites within the promoter regions of the repressed genes *mepA*, *nlpC*, and *rpf2* and of the activated genes *betP*, *proP*, *csbD*, and *cg1517*. The transcriptional start points are indicated by an arrow; the MtrA binding sites are shown as black boxes.

wild type. In Table 3, the ratios are listed for all genes that exhibited at least a 2-fold alteration of the mRNA level in one or both sets of experiments. It is evident that all presently identified direct target genes of MtrA (listed in Table 2) show similar results in both sets of experiments, indicating that the strain producing the MtrA(D53N) variant behaves similarly to the Δ *mtrA* deletion mutant. Western blot analysis of Δ *mtrA*/pEKEEx2-*mtrA*(D53N) and Δ *mtrA*/pEKEEx2-*mtrA* with anti-MtrA-antibodies (see Fig. S5 in the supplemental material) shows that MtrA and MtrA(D53N) accumulate in the cells in comparable amounts, indicating that the mutated MtrA variant is stable. Taken together, these results provide strong evidence that MtrA is active in its phosphorylated form.

In vitro influence of MtrA phosphorylation on DNA-binding affinity. To confirm that MtrA is active in its phosphorylated state, comparative EMSAs were performed with the DNA fragments *nlpC*₅ and *proP*₅ (Fig. 1A and C) and unphosphorylated MtrA or MtrA that had been preincubated with MBP-PhoSΔ1–246 and 80 μ M ATP for 30 min. However, no differences in the binding behavior were observed (data not shown), suggesting that only a negligible fraction of MtrA became phosphorylated by MBP-PhoSΔ1–246.

Acetyl phosphate and phosphoramidate are known to serve as low-molecular-weight phosphoryl donors for some response regulators (22, 26). Incubation of MtrA with phosphoramidate led to dimerization of the protein (apparent mass of the dimer, 56.6 kDa) as determined by gel filtration (Fig. 6), indicating that MtrA gets phosphorylated by phosphoramidate. In con-

trast, untreated protein or MtrA preincubated with acetyl phosphate behaved more like a monomer (apparent mass, 35.3 kDa). For MtrA of *M. tuberculosis* it has also been shown that phosphoramidate, but not acetyl phosphate, is able to phosphorylate MtrA_{Mt} (10). However, only part of full-length MtrA from *M. tuberculosis* dimerized after incubation with phosphoramidate, whereas MtrA of *C. glutamicum* was converted into the dimeric form almost completely.

Based on these data and the observation that the phosphorylated form of MtrA (MtrA~P) is stable (28), binding of MtrA preincubated with and without 50 mM phosphoramidate to selected promoter regions (*nlpC*₇, *nlpC*₈, *mepA*₄, *proP*₅, and *betP*₄ and *nlpC*₆ as a negative control) (Fig. 1) was compared using EMSAs. As shown in Fig. 7, preincubation with phosphoramidate strongly improved the DNA-binding affinity. Further EMSAs with MtrA~P and the double-stranded oligonucleotides containing a single binding site (either the one upstream of *mepA* or the one upstream of *betP*) flanked by four or six additional base pairs verified that MtrA and MtrA~P bind to the same DNA sequence motif (data not shown). As assumed, incubation of MtrA with acetyl phosphate had no influence on the DNA binding affinity (data not shown).

DISCUSSION

In continuation of our previous studies on the MtrAB two-component system of *C. glutamicum* (4, 27), we now studied the interaction of the response regulator MtrA with its target

FIG. 6. Determination of the native molecular mass of MtrA by size exclusion chromatography. Purified MtrA that had been preincubated with or without phosphoramidate or acetyl phosphate was separated on a HiLoad 26/60 Superdex 200 prep-grade column using BS buffer without glycerol for elution. For calibration, a premixed protein molecular mass marker containing the following proteins was used: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and amylase (200 kDa). V_0 was determined with blue dextran (2,000 kDa). AU, arbitrary units.

DNAs and the role of the phosphorylation status for the activity of MtrA. We identified 18 new direct target genes of MtrA, determined a 19-bp consensus binding site, and provided evidence that MtrA is active *in vivo* in its phosphorylated form. The knowledge of the target genes of a transcriptionally active response regulator is key for understanding the physiological function of the two-component system and for getting clues on the stimulus or stimuli sensed by the cognate sensor kinase. Unfortunately, in the case of MtrA, only a few of its

target genes have an experimentally validated function (*betP*, *proP*, *rpf2*, and *mepA*); for some a function can be predicted (*nlpC*, *mepB*, *cg0133*, *cg0134*, *ppmA*, and *cg1612*), and for 13 other genes the function is completely unknown. The following paragraphs summarize our current knowledge of these genes. *betP* and *proP* encode carriers for the compatible solutes betaine and proline/ectoine, respectively, which are involved in the response to hyperosmotic stress (30, 32). *rpf2* encodes one of two resuscitation promoting factors present in *C. glutami-*

FIG. 7. Binding of unphosphorylated and phosphorylated MtrA to the DNA fragments *nlpC*₇, *nlpC*₈, *mepA*₄, *proP*₅, *betP*₄, and *nlpC*₆ (Fig. 1). The DNA fragments (169 to 246 bp in size; final concentration, 22 to 31 nM) were incubated for 30 min at room temperature without MtrA (left lanes) or with increasing MtrA concentrations (0 nM to 868 nM). In the right panels MtrA was phosphorylated with 50 mM phosphoramidate prior to the binding reaction. Subsequently, the samples were separated by native polyacrylamide (15%) gel electrophoresis, and the DNA was stained with SYBR Green I.

cum (14, 24). Both *rpf* genes are nonessential, but long-stored cells of an *rpf* double mutant displayed an extended lag phase after transfer to fresh medium (14, 24). Recently it has been shown that *rpf2* expression is under the control of the transcriptional regulators RamA, RamB, and GlxR in a carbon source-dependent manner (17). Our finding that *rpf2* is negatively regulated by MtrA demonstrates a complex regulation of this gene.

The genes *mepA* and *nlpC* encode putative secreted cell wall peptidases (2, 4, 27). In the case of MepA the predicted function was verified by the finding that *mepA* expression in *E. coli* resulted in the inability to cope with hypo-osmotic stress (27). Also cg0980 seems to encode a putative secreted cell wall peptidase since *in silico* analysis of the Cg0980 protein revealed a metallopeptidase domain of the M23 family (<http://merops.sanger.ac.uk/>) in its carboxy-terminal half. A domain of the same family is also present in MepA, and therefore we designated Cg0980 as MepB (for metallo-peptidase B). In the N-terminal part, MepB exhibits a hydrophobic region from positions 21 to 43, which may represent either a putative transmembrane helix or part of a signal peptide, with the most likely cleavage site after residue 43 (AVA ↓ AQ). Independent of whether MepB is membrane bound or secreted, the protease domain is most likely located extracytoplasmically.

The genes cg0133 and cg0134 form a putative operon. The Cg0133 protein shows significant similarity to the *p*-aminobenzoyl-glutamate transporter AbgT of *E. coli* (16). Cg0134 contains a peptidase domain of the M20D family (<http://merops.sanger.ac.uk/>) but lacks a signal peptide or transmembrane helices. Its homologs in *E. coli* are AbgA and AbgB, which are involved in *p*-aminobenzoyl-glutamate utilization and presumably are responsible for the cleavage of this compound after its import by AbgT (6). It has been speculated that the true physiological substrate of the Abg proteins might not be *p*-aminobenzoyl-glutamate but a related molecule such as a dipeptide (6).

The *ppm4* gene encodes a putative protease modulator (27), and cg1612 encodes a putative acetyltransferase. With regard to the function of CsbD, it is known only that the homologous gene from *Bacillus subtilis* belongs to the σ^B -dependent general stress regulon (33). For six MtrA targets (cg0715, cg0858, cg1517, cg1665, cg1942, and cg2052), the *in silico* analysis predicts that the corresponding proteins are secreted, and the products of four other MtrA targets (cg0896, cg2096, cg2777, and cg3254) are presumably integral membrane proteins. Two further target genes of unknown function code for soluble proteins (cg0859 and cg1943).

Based on the knowledge of the MtrA target genes summarized above, it is difficult to construct a coherent functional model of the MtrAB system. However, the fact that the large majority of the genes encode secreted or membrane-integral proteins clearly supports the view that MtrAB is primarily involved in the control of processes in the cell envelope, most likely cell wall metabolism and osmoregulation. Although the *in vitro* data with reconstituted MtrB suggest a role of the cytoplasmic kinase domain in sensing hyperosmotic stress (29), we still favor the idea that the periplasmic domain of MtrB is also involved in stimulus recognition, e.g., in sensing the integrity of the peptidoglycan sacculus. Recently it has been shown that in *M. tuberculosis* the MtrAB two-component system is

activated by interaction of the lipoprotein LpqB with the extracellular domain of MtrB. As in *M. tuberculosis*, in *C. glutamicum* the *lpqB* gene is also located directly downstream of *mtrB*. Therefore, it is likely that in *C. glutamicum* MtrA, MtrB, and LpqB also form a three-component system (31).

Within most of the direct target genes an MtrA-binding sequence could be identified. The deduced loosely conserved binding motif represents a direct repeat of 8 bp separated by a 3-bp linker (Fig. 4). Direct repeats are typical binding sites for OmpR-type response regulators (25) and an 8-3-8 structure was also reported as a target sequence for the OmpR-type response regulator PhoR of *C. glutamicum* (35) and for MprA of *M. tuberculosis* (15). The 8-3-8 organization of the MtrA binding site is plausible since the centers of the two direct repeats are separated by 11 bp corresponding to one turn of the DNA. Structural data for OmpR-type response regulators indicate that the phosphorylation of the conserved aspartate residue leads to the active state in which the receiver domains dimerize with a 2-fold rotational symmetry, whereas the effector domains dimerize in tandem, compatible with their binding to direct repeat DNA motifs (3). In the case of MtrA, the structure of a phosphorylated form is not yet available, but the crystal structure of unphosphorylated MtrA from *M. tuberculosis*, which has 72% sequence identity to MtrA from *C. glutamicum*, has been solved (10). MtrA of *M. tuberculosis* and its receiver domain comprised of amino acids 1 to 125 (MtrA_N) are monomers in solution. Phosphorylation of MtrA_N leads to dimerization, indicating that the same could be true for the full-length protein (10). Gel filtration of purified histidine-tagged MtrA from *C. glutamicum* in 1× BS buffer revealed an apparent molecular mass of 33.5 kDa, which is closer to that of the predicted mass of a monomer (27.1 kDa) than to that of a dimer (54.2 kDa) (Fig. 6). As discussed previously for PhoR (35), the larger apparent mass of MtrA can be explained by the assumption that a protein composed of two domains that are connected by a flexible linker migrates more slowly than a globular protein of similar size. Therefore, unphosphorylated MtrA from *C. glutamicum* might also be a monomer in solution. Nevertheless, we could observe binding of MtrA to its target promoters although the protein was presumably unphosphorylated after its purification from *E. coli*. Apparently, MtrA is also able to bind DNA in its inactive form.

As MtrA acts as both a transcriptional repressor and a transcriptional activator, the question arose as to how these different functions are mediated. Repression of genes is often accomplished by binding of the transcriptional regulator close to the transcriptional start site, thereby inhibiting RNA polymerase binding to the promoter. Transcriptional activators usually bind upstream of the RNA polymerase binding site (23). The transcriptional start sites of the target genes *nlpC*, *mepA*, *rpf2*, *betP*, *proP*, *csbD*, and cg1517 revealed that in the case of the repressed genes, the MtrA binding sites overlap with the −10 region, whereas in the case of the activated genes the MtrA binding site is located upstream of the −35 regions (see Fig. S3 in the supplemental material). This suggests that the differential influence of MtrA binding on gene expression is caused by the position of the MtrA binding site with respect to the transcriptional start sites.

Although most response regulators are active in their phosphorylated state, there are also exceptions in which the

response regulator is active in its unphosphorylated state and inactivated by phosphorylation (21). Our transcriptome comparison of $\Delta mtrA$ strains carrying expression plasmids for either wild-type MtrA or MtrA(D53N) clearly indicates that MtrA is active in its phosphorylated form, as the strain expressing MtrA(D53N) showed a gene expression profile similar to that of the $\Delta mtrA$ mutant. This view is supported by the result that MtrA phosphorylated *in vitro* with phosphoramidate shows a much higher DNA-binding affinity than unphosphorylated MtrA (Fig. 7). Besides the binding affinity, phosphorylation of MtrA alters the migration behavior of the bound DNA fragment. Dimerization of MtrA upon phosphorylation is presumably responsible for this effect.

The results reported here considerably expand our knowledge of the MtrAB two-component system. However, to understand its physiological function, further studies dedicated to the role of the MtrA target genes and to the stimuli sensed by MtrB are required.

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