Characterization of MtfA, a novel regulatory output signal protein of the glucose-phosphotransferase system in *E. coli* K-12

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Abstract

The glucose phosphotransferase system (PTS) in *Escherichia coli* K-12 is a complex sensory and regulatory system. In addition to its central role in glucose uptake, it informs other global regulatory networks about carbohydrate availability and the physiological status of the cell. The expression of the *ptsG* gene encoding the glucose PTS transporter EIICB\[^{Glc}\] is primarily regulated via the repressor Mlc, whose inactivation is glucose dependent. During transport of glucose and dephosphorylation of EIICB\[^{Glc}\], Mlc binds to the B-domain of the transporter resulting in de-repression of several Mlc-regulated genes. In addition, Mlc can also be inactivated by the cytoplasmic protein MtfA in a direct protein-protein interaction.

In this study, we identified the binding site for Mlc in the carboxy-terminal region of MtfA by measuring the effect of mutated MtfAs on *ptsG* expression. In addition, we demonstrated the ability of MtfA to inactivate an Mlc super repressor, which cannot be inactivated by EIICB\[^{Glc}\], by using *in vivo* titration and gel shift assays. Finally, we characterized the proteolytic activity of purified MtfA by monitoring cleavage of amino 4-nitroanilide substrates and showed Mlc’s ability to enhance this activity.

Based on our findings, we propose a model of MtfA as a glucose-regulated peptidase activated by cytoplasmic Mlc. Its activity may be necessary during the growth of cultures as they enter the stationary phase. This proteolytic activity of MtfA modulated by Mlc constitutes a newly identified PTS output signal that responds to changes in environmental conditions.
Introduction

Despite the fact that *Escherichia coli* K-12 has been an important model organism for many decades, the function of more than 20% of all putative open reading frames in its genome remains unknown. Moreover, in many cases, no obvious sequence similarities with previously characterized proteins exist. The *mtfA* gene (formerly named *yeeI*), a monocistronic gene located at 44.1 min in the *E. coli* chromosome, has clearly belonged to this group. Intensive sequence similarity searches revealed that orthologs of MtfA (mnemonic for Mlc-titration-factor A) exist in more than 100 proteobacteria of the α-, β-, and γ- subdivisions. In a previous report (2) we were able to demonstrate that *E.coli* MtfA is a cytoplasmic protein which binds to the carboxy-terminal part of the Mlc repressor protein (mnemonic for Making-large-colonies, gene: *dgsA*) with a very high affinity. Mlc is one of the global regulators of carbohydrate metabolism in *E. coli* and is especially involved in the regulation of the *ptsG* gene expression, which encodes the glucose transporter EIICB<sub>Glc</sub>. The EIICB<sub>Glc</sub> together with the cytoplasmic protein EIIA<sub>Glc</sub>, encoded by the *crr* gene (as part of the *ptsHICrr* operon), forms the glucose-specific phosphoenolpyruvate (PEP) –dependent carbohydrate:phosphotransferase system (glucose-PTS). Both proteins take part in a phosphorylation cascade, which begins with an autophosphorylation reaction of the so called Enzyme I (EI, gene *ptsI*) at the expense of PEP. The phosphate group is subsequently transferred from EI to the phosphohistidine carrier protein HPr (gene *ptsH*), then to the sugar specific Enzymes II and finally to the carbohydrate (for review see (23)). In addition to its transport and carbohydrate phosphorylation functions, the glucose-PTS in enteric bacteria has a central regulatory role in carbon catabolite repression and inducer exclusion (reviewed in (7, 15)). Many recent studies have revealed that the regulation of *ptsG* expression is very complex and takes place both at the transcriptional and post-transcriptional levels (reviewed in (3, 12)). Additionally, there also exists a direct regulation of transport activity that prevents
intracellular hexose phosphate stress (10, 32). In the absence of glucose, *ptsG* expression is repressed by Mlc. In the presence of glucose, dephosphorylated EIICBGlc, which is generated during glucose uptake, binds Mlc and sequesters the repressor away from its DNA-binding sites (13, 16, 28, 34) (review in (3, 21)). Hence, in contrast to other regulatory systems, there is no intracellular low-molecular weight inducer for Mlc. The EIICBGlc/Mlc system is considered to be an important glucose sensor in enteric bacteria (13, 34), since Mlc is also involved in the glucose dependent regulation of the *ptsHicrr* operon (20, 29), the *malT* gene for transcriptional activation of the maltose regulon (6) and the *manXYZ* operon encoding the EII proteins of the mannose-PTS (18).

Although MtfA has been shown to interact with Mlc and to work as an anti-repressor, its true function has not been elucidated yet. We were interested in determining what the cellular role of MtfA was. Thus, in the first part of this study we further characterised the specific interaction between MtfA and Mlc. By generating a collection of MtfA mutants, we identified the Mlc-binding domain within this highly conserved protein. Furthermore, we characterized the interaction between MtfA and the Mlc*H86R* super repressor, which is no longer capable of interacting with EIICBGlc (27). This allowed us to elucidate differences between the inactivation modes of Mlc by membrane-sequestration through EIICBGlc on the one hand, and the cytoplasmic titration by MtfA on the other.

Recently, the crystal structure of the MtfA homologue from *Klebsiella pneumoniae* was characterized (DOI:10.2210/pdb3khi/pdb). Interestingly, the structure revealed similarities to the zinc-dependent metalloprotease Lethal Factor (LF) from *Bacillus anthracis* as well as to other metalloproteases of the gluzincin clan. Hence, the second goal of this study was to characterize the peptidase activity of MtfA and address the influence of Mlc on MtfA’s proteolytic activity. We present a model that postulates MtfA to be a glucose-regulated peptidase, whose activity is regulated by binding to Mlc available in the cytoplasm, which in turn has been released from EIICBGlc during times when no glucose is taken up. Finally, we
speculate about putative biologically relevant MtfA targets, which may have a function in
growth transition under changing environmental conditions.

Materials and Methods

Media and growth conditions. Cells were grown routinely either in Lennox broth without
glucose and calcium ions (LBo), or in 2xTY medium as described in (1). Antibiotics were
used at the following concentrations: tetracycline (Tc), 10 mg/l; ampicillin (Ap), 50 mg/l;
chloramphenicol (Cm) 25 mg/l, respectively.

Bacterial strains and plasmids. All strains used were E. coli K-12 derivatives. Table 1 list
the genotypes and sources of the relevant bacterial strains. Informations on the plasmids and
oligonucleotides are given in the supplementary materials. P1-transduction was performed as
described previously (2).

Isolation of chromosomal and plasmid DNA, restriction analysis, PCR and DNA-
sequencing. All manipulations of chromosomal or recombinant DNA were carried out using
standard procedures as described previously (1). Plasmid DNA was prepared using QIAprep®
Spin Miniprep Kit (Qiagen, Hilden, Germany). Restriction enzymes were purchased from
New England Biolabs (Schwalbach, Germany) or Fermentas (St. Leon-Rot, Germany) and
used according to supplier recommendations. Oligonucleotides for PCR were purchased from
Thermo Fisher Scientific (Ulm, Germany). DNA sequencing was commissioned to Scientific
Research and Development (Bad Homburg, Germany). Alternatively, DNA was sequenced
with the ABI3100Avant (Applied Biosystems, Foster City CA, USA) at the Center for
Medical Genetics in Osnabrück. Polymerase chain reactions (PCR) were performed as
described by (24) using TaKaRa DNA polymerase from Lonza (Köln, Germany).
**Construction of pTM30mlchis.** To purify Mlc, we used PCR to amplify the corresponding
*dgsA* gene using the oligonucleotides MlcBamHI and MlcHindIII (see supplementary
ingformations) and genomic DNA of *E. coli* K-12. Thus, BamHI and a HindIII restriction sites
were inserted into the PCR product. Afterwards the PCR product was cloned in the pTM30
expression vector. An amino-terminal “his-tag” was added to the protein using his-tag
encoding, complementary oligonucleotides MlcHis+ and MlcHis. 10µg of each
oligonucleotide was dissolved in 10µl of sterile water. After the addition of 2µl of annealing
buffer (GE Healthcare, München, Germany) the mixture was heated to 95°C, and incubated at
RT in a 1l beaker with 95°C water until the water temperature reached 40°C. We then opened
the vector pTM30mlc with BamHI and ligated the previously annealed oligonucleotides with
this vector to generate the vector pTM30mlchis. Additionally, for the MtfA protease activity
assay we amplified the *dgsA* gene using the oligonucleotides Mlchis-tev and MlcHindIII (see
supplementary informations) and ligated it into the pTM30 vector to generate the plasmid
pTM30mlchis-tev. This plasmid encoded an amino-terminal his-tagged *dgsA* gene as
pTM30mlchis, and a TEV-protease cleavage site between the tag and protein. We treated the
purified protein with TEV-protease (AcTEV Protease™, Invitrogen, Darmstadt, Germany)
according to the manufactures’ instructions. This resulted in an untagged Mlc protein with an
additional glycine in front of the native amino acid sequence.

**Site-directed mutagenesis.** We generated defined mutations in the *mtfA* gene using the
pTMByeel-S plasmid (2) and the Quick Change® II Site-Directed Mutagenesis Kit according
to standard protocol (Stratagene; Amsterdam, The Netherlands). In addition, we introduced
mutations into the *dgsA* gene with the same kit and using the pTM30mlchis plasmid as a
template. The oligonucleotides used are listed in the supplementary informations.
Construction of strains JSW1 and JSW2. To characterize the interaction between MtfA and Mlc, we inserted a single copy of the *dgsA* wild-type gene or the *dgsA* * derivative encoding the Mlc*H86R super repressor mutation (in combination with a *cat* gene for a chloramphenicol resistance selection marker) into the chromosome of JEK5 at the *pheU* site via site-directed recombination. In doing so, we applied the RecA-independent recombination mechanism of the conjugative transposon CTnsr94 ((11); exact procedure described elsewhere). PCR was used to confirm correct integration of the desired gene copy.

Western blot analyses and β-galactosidase assays. Bacterial cells harbouring pTMByeeI-S as described in (2) grown in LBo medium were harvested at an optical density at 650 nm (OD$_{650}$) of 1 by centrifugation and resuspended in 100µl sterile water and 100µl of SDS-PAGE loading buffer (125 mM Tris-HCl (pH 6.8), 2 % sodium dodecyl sulphate (SDS), 10 % glycerol, 5 % β-mercaptoethanol, 0.01 % bromophenol blue). If not described otherwise, samples were heated at 95°C for 10min. 15 µl of total cellular proteins were separated by electrophoresis on 0.1 % SDS-containing 15 % polyacrylamide gels and transferred to a Nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). For the detection of MtfA protein derivatives, we used a penta-his antibody (Qiagen, Hilden, Germany) except in the case of the two hybrid studies, where we used a polyclonal anti-MtfA antibody. Detection of antibody-binding was performed using infrared-labeled second antibodies (LI-COR Biosciences, Bad Homburg, Germany). Visualization and quantification were done using an Odyssey infrared imager (LI-COR Biosciences). All β-galactosidase assays were performed as previously described (2).

Bacterial two-hybrid studies. Bacterial two-hybrid studies were performed as described (8). The genes *mtfA*, *mtfAΔ1-83* and *dgsA* were amplified by PCR. PCR products were cloned into the pGEM®-T vector (Promega, Mannheim, Germany) and subsequently transferred into the
vectors pMS604 or pDL804 (8) using a combination of the restriction enzymes BstEII /XhoI
or XhoI/BglII, or in the case of pMM4, PstI /XhoI, respectively. Oligonucleotides were used
in PCR as follows: pMM2: BTH7 and BTH8, pDM2: BTH1 and BTH3, pMM3: MlcPX+/-,
pDM3: MlcXB+/- and for pMM4: Ymlcpms+/-
All oligonucleotide sequences are listed in the supplementary informations.

Protein purification. For purification of MtfA-His₅, cells harbouring pTMByeeI-S or
derivatives were grown in LBo with ampicillin and 500µM IPTG. When cultures reached an
OD₆₅₀ = 3, cells were harvested by centrifugation. Cells were subsequently resuspended in
buffer A (300mM NaCl, 2mM β-mercaptoethanol, 100mM KPi and 10% glycerol pH 8.0) and
disrupted by sonication using a Branson Sonifier W-250D (Danbury, Conn., USA). Cell
debris was removed by low speed centrifugation (14000rpm). We then performed affinity
chromatography using the ÄKTA-FPLC and “His-Trap™ FF” columns (GE Healthcare).
Elution was performed with the same buffer as above with additional 300mM Imidazol.
Subsequently, we obtained the samples and dialysed them against 20mM Tris-buffer, pH 8.0.
Finally, we stored them at 4°C or supplemented them with 50% glycerol and stored them at –
20°C.

For the purification of Mlc, cells harbouring pTM30mlchis were grown in 2xTY or LBo with
ampicillin until an OD₆₅₀= 0.5, induced with 500µM IPTG and harvested at an OD₆₅₀= 2.
Harvesting and disruption were performed as described previously, using a 50mM NaPi pH
8.0, 300mM NaCl and 20mM imidazole buffer. For Mlc purification, it was also suitable to
use affinity chromatography as described for MtfA. Elution was performed with a buffer
containing 50mM NaPi pH 8.0, 300mM NaCl, 500mM imidazol, 200µM ZnCl₂. Afterwards
the sample was dialysed against Hepes-buffer (100mM Hepes pH 9.0, 25mM Na-glutamate,
1mM dithiothreitol) and stored at 4°C.
**Electrophoretic mobility shift assays.** For the electrophoretic mobility shift assays (EMSA) the fluorescence labelled *ptsG*-operator fragment according to (19) encoded by the complementary oligonucleotides *ptsG*01 (5´ labelled with DY782) and *ptsG*02 (5´ labelled with DY682) was annealed as described for the Mlc “his-tag”. 10µg BSA, 0.5µg Herring sperm-DNA as heterologous DNA, 100nM ZnCl₂ and 500nM MgCl₂ were added to 1ng of the operator-fragment (4nM final concentration in the assay). The stated amounts of Mlc and MtfA were incubated for 10min at RT and afterwards added to the mixture, which was then brought to a final volume of 20µl using Hepes buffer containing 1µg/µl BSA. Samples were incubated for an additional 10min at RT and loaded on a 5% acrylamid-bisacrylamide gel. We performed electrophoresis at a constant voltage of 150V and detection using the “Odyssey Infrared Imaging System” (LI-COR Biosciences).

**Protease activity assays.** The Protease Assay Kit from Calbiochem (Merck, Darmstadt, Germany) was used to determine endoprotease activity of purified MtfA according to the manufactures’ instructions. We determined the amino peptidase activities of MtfA by measuring the hydrolysis of L-alanine 4-nitroanilide, L-arginine 4-nitroanilide, L-proline 4-nitroanilide, L-valine 4-nitroanilide or L-alanine-L-alanine-L-alanine-4-nitronalide (Sigma, Taufkirchen, Germany) and performed an endpoint measurement of the chromophoric end product 4-nitroanilide using spectral photometric determination of the absorbance at 390 nm (Shimadzu UV mini 1240 photometer, molar extinction coefficient 11,5x10⁶ cm² mol⁻¹). The assay was carried out in semi micro-cuvettes (Sarstedt, Nümbrecht, Germany). We added 100 µg of purified protein to 500 µl of 1 mM L-amino 4-nitroanilide. The samples were then filled to a final volume of 1 ml with Tris-buffer pH 8.0 and incubated at 37°C for 4 hours. The absorbance was measured against a blank sample without protein (500 µl L-alanine 4-nitroanilide with 500 µl Tris-buffer) at 390nm. In case of hippuryl-L-phenylalanine and hippuryl-L-arginine (Sigma) absorbance was determined at 254 nm. We conducted a
subsequent endpoint measurement, determined the extinction difference $\Delta E$ and calculated the specific activity in U/mg protein of each preparation. All specific activities of purified proteins were compared to a fresh sample of the wild type protein. If indicated, we added the inhibitors AEBSF (Applichem, Darmstadt, Germany), EDTA (Roth, Karlsruhe, Germany) or phenantroline (Sigma) in denoted concentrations to the assay.

Results

Multiple alignments to identify highly conserved amino acids. MtfA is a highly conserved protein that is not only restricted to the group of enterobacteria, but can also be found in the subdivisions of $\alpha$, $\beta$, and other $\gamma$-proteobacteria as well as in cyanobacteria. In order to further characterize the interaction between MtfA and Mlc, we created a multiple alignment of various MtfA proteins to identify highly conserved amino acid residues which might be of functional importance (Fig.1). In this alignment, MtfA homologs from ten different bacteria and covering a broad spectrum of identity were used (39 to 89% identical amino acid residues). All of these bacteria harbor an Mlc-homolog protein. Twenty-five amino acid residues were identified as absolutely invariant, and another 32 residues were highly conserved. One important conserved motif is composed of the sequence $^{149}\text{HExxH}^{153}$ which characterizes zinc-dependent metalloproteases. Interestingly, many invariant amino acid residues were clustered at the carboxy-terminal end of MtfA, indicating another important region of the protein. Based on this multiple alignment, a collection of highly conserved amino acid residues were separately substituted by site-directed mutagenesis (marked in grey).

Substitution of conserved amino acids and determination of protein stability. To further analyse the interaction between MtfA and the repressor protein Mlc, we used site-directed mutagenesis to define mutants of MtfA. In order to identify specific effects of single amino
acid residues, we created substitutions with chemically related residues. Western blot analysis 
was used to test protein stability of all MtfA derivatives. In almost all cases a signal was 
detected at the expected size of 32 kDa. Only the two derivatives with the substitutions I148E 
(in contrast to I148Y) and A215D (in contrast to A215S) exhibited massive protein decay 
(data not shown). Thus, compared to wild type, the majority of MtfA derivatives provided an 
equally stable protein, which could then be further characterized.

Titration of Mlc by MtfA and its derivatives in vivo. The intensity of the interaction 
between Mlc and MtfA wild type or its derivatives was tested in vivo using a quantitative 
measure of the β-galactosidase activity of a single copy ptsGop-lacZ fusion (34). The test 
system is shown in figure 2. We introduced plasmids encoding MtfA or its derivatives into the 
indicated strain background (e.g. dgsA+, deletions of ptsG and lacZ), induced mtfA expression 
and measured the strength of Mlc titration by determining β-galactosidase activities. The β-
galactosidase activity of completely induced MtfA wild type was always used as a control and 
set to 100%, basal expression levels were determined using the empty expression vector 
pTM30 (shown by grey columns in Fig. 3). 25 of the 59 MtfA derivatives tested exhibited no 
or only minor reduction in Mlc titration (at least 80% wild type activity, white columns in 
Fig.3). However, 34 different MtfA mutants exhibited a significantly reduced Mlc titration (as 
indicated by black columns). Within the amino-terminal part of the MtfA, only the two 
substitutions L40E and A70D led to decreased interaction with Mlc. In contrast, a 
replacement of these two residues by other neutral amino acids (L40A or L40T and A70S, 
respectively) had almost no effect, indicating that negative charges at these positions either 
interfere with the protein-protein interaction or with the overall conformation of the protein. 
Within the carboxy-terminal part of MtfA, many different amino acid substitutions led to 
substantial changes in the interactions with Mlc. In particular, the two regions, from position 
132 to 149 and position 214 to 243, seemed to be essential for the binding of Mlc. Due to
PCR-artifacts; we found eight derivatives with an additional mutation next to the substitution of interest. In cases where strong effects were present, we reintroduced the conserved amino acid residue to study the impacts of each single substitution. In the case of the double mutant D156N/D161E, the latter mutation suppressed the phenotype of the first. In the double mutant Y205S/A207D, the decreasing interaction with Mlc appeared to be due to a cumulative effect, since each single mutation exhibited higher β-galactosidase activity. In the case of the double mutant V216L/L217I the decrease in interaction seems to be caused by the latter change, because the single V216L mutation had almost no effect. However, changing V216 to an aspartate residue again reduced the activity to less than 50%, indicating that both residues may be of importance. For all other double mutants, the second substitution did not change the result of the single substitution of the conserved amino acid.

The carboxy-terminus of MtfA binds Mlc in a bacterial-two-hybrid assay. Given the results from the in vivo titration assays, the carboxy-terminal part of MtfA appears to be responsible for the binding of Mlc. To verify this hypothesis, we used a bacterial-two-hybrid assay (8). This assay employed a chromosomal lacZ gene under the control of a hybrid lexA operator-promoter region in the E. coli test strain SU202. One half of this artificial operator is recognized by the helix-turn-helix DNA-binding domain of the LexA wild type repressor, the other half by a LexA* mutant. Both DNA-binding domains are encoded by compatible plasmids and can be coupled to different proteins of interest. Expression of these hybrid genes can be induced by IPTG. Only in the case of crosstalk between the two hybrid proteins, the two different DNA-binding domains of LexA and LexA* come together to repress lacZ expression as was seen in the two control plasmids pMS604 (encoding a LexA-Fos-hybrid protein) and pDL804 (encoding a LexA*-Jun-hybrid protein). As shown in figure 4, when fused to the LexA-DNA-binding domain, both full length MtfA and MtfAΔ1-83, which is missing the amino-terminal part of the protein, are able to interact with Mlc fused to LexA*. 
These results clearly demonstrate that the carboxy-terminal part of MtfA is sufficient for Mlc binding. Furthermore, as an internal control, we fused Mlc to both different DNA-binding domains of the LexA repressor. Expression of these two hybrid proteins resulted in a low β-galactosidase activity after induction, confirming the previously published dimerization (tetramerization) of Mlc (26). Cells with only one of the two LexA-Mlc hybrid proteins always showed a very high β-galactosidase activity, indicating a high specificity of the different LexA DNA-binding domains for their cognate operator sites (data not shown).

**Gel shift assays confirm Mlc titration by MtfA.** Our in vivo results indicated that the interaction between MtfA and Mlc prevents binding of the repressor to the ptsG-operator. In order to further characterize this effect, we employed electrophoretic mobility shift assays (EMSA) with purified Mlc and MtfA proteins. Addition of Mlc to a ptsGop fragment led to the formation of a repressor-operator complex and thus a mobility shift (Fig. 5a). In contrast, addition of MtfA to the labeled operator-fragment had no effect, indicating that MtfA has no DNA-binding properties. Incubation of Mlc and MtfA prior to incubation with the DNA reduced the amount of the repressor-operator complex. Increasing amounts of MtfA led to an increasing fraction of free ptsGop-DNA. A super complex of DNA, Mlc and MtfA was never observed.

**MtfA binds to the super repressor Mlc*H86R in vivo and in vitro.** In order to distinguish between the two currently known inactivating mechanisms for Mlc, we used an Mlc super repressor. This Mlc* derivative carries an amino acid substitution of histidine 86 to arginine and is unable to bind the unphosphorylated B domain of the EIICBGlc (27). Thus, in contrast to the wild type, the super repressor cannot be inactivated by EIICBGlc. The in vivo interaction between the super repressor Mlc*H86R and MtfA was investigated using a similar system as presented in figure 2. In the case of the Mlc wild type in strain LZ110, overexpression of
MtfA caused a high β-galactosidase activity of the single copy ptsGop-lacZ fusion as expected (Fig. 6). In contrast, the introduction of a dgsA deletion into this strain background (resulting in JEK5) led to a constitutive β-galactosidase activity. Interestingly, overexpression of MtfA caused a significant increase of ptsG expression in the absence of Mlc. Since MtfA does not directly bind to the ptsG operator as shown in figure 5, this might indicate some yet unknown interaction of MtfA with a different regulator of ptsG expression. Subsequently, we introduced into JEK5 a single copy of either a wild type dgsA+ (giving strain JSW1), or the dgsA* allele encoding the Mlc*H86R super repressor (giving strain JSW2). Both repressors strongly reduced the ptsGop-lacZ expression. Remarkably, JSW2 harbouring the super repressor Mlc*H86R exhibited almost no basal β-galactosidase activity. Induction of plasmid-encoded mtfA+ in both strain backgrounds resulted in high expression of the ptsGop-lacZ fusion, meaning that both wild type Mlc and Mlc*H86R can be bound and inactivated by MtfA. However, corresponding to the very low basal expression level in the presence of the super repressor, β-galactosidase activity in JSW2 after induction of mtfA+ were always lower compared to the wild type.

To further characterize the MtfA interaction with Mlc*H86R in vitro we purified the super repressor and used it in an EMSA experiment with the ptsGop-fragment. In accordance to the in vivo titration data and the results from the band shift assays with the wild type repressor, addition of MtfA to the complex of Mlc*H86R with the ptsGop-fragment led to a release of the operator DNA (Fig. 5b). This seemed to indicate that the inactivation mechanisms of Mlc by unphosphorylated EIIBGlc and MtfA differ significantly.

MtfA shows amino-peptidase activity. The MtfA crystal structure from Klebsiella pneumoniae was recently characterized (DOI:10.2210/pdb3khi/pdb). 76% of the amino acids are shared by MtfA of K. pneumoniae and E. coli MtfA. The analysis of the overall MtfA architecture revealed structural similarities to the zinc-dependent metalloprotease Lethal
Factor (LF) of *Bacillus anthracis* or to the Mop protein, which is involved in virulence of *Vibrio cholera* (Qingping Xu, Anna Göhler et al., in preparation). Therefore, we tried to characterize the putative protease activity of *E. coli* MtfA. As the natural target and the substrate specificity of MtfA are currently unknown, we made use of several relatively unspecific and artificial protease activity assays, which monitor the cleavage of peptide substrates fused to 4-nitroanilide. Originally, a 4-nitroanilide substrate was used to characterize the *in vitro* activity of LF from *B. anthracis* (30). Various different L-amino 4-nitroanilide derivatives were chosen to look for amino-peptidase activity as indicated. The greatest activity and specificity was observed for L-alanine fused to 4-nitroanilide as shown in figure 7. Other artificial substrates (arginine, proline or valine fused to 4-nitroanilide, respectively) were cleaved off with significantly lower activities. Moreover, L-glutamic-acid-4-nitroanilide as an acidic amino acid and substrates larger in size like L-alanine-L-alanine-L-alanine-4-nitronalide were not cleaved at all (data not shown). Subsequently, hippuryl-L-phenylalanine and hippuryl-L-arginine were used to test for carboxy-peptidase activity. Again, only very low activities were obtained for these artificial substrates. Furthermore, we tested FTC-casein, a substrate for many endo-peptidases regarding proteolytic conversion by MtfA. In contrast to the artificial amino-peptidase substrates we did not detect any protease activity after incubation of FTC-casein with purified MtfA (data not shown). These results suggest that MtfA is an amino- rather than a carboxy- or endo-peptidase. Since the highest efficiency of MtfA activity was monitored by cleaving off an amino acid from the artificial substrate L-alanine-p-nitroanilide, this substrate was used to further characterize the peptidase activity of MtfA. Subsequently, we tested several protease inhibitors. Addition of the chelators EDTA or phenantroline significantly reduced the peptidase activity of MtfA, whereas the addition of other protease inhibitors (e.g. AEBSF) had much less effect. This finding is consistent with the idea that MtfA is a zinc-dependent protease. Addition of higher amounts of EDTA or phenantroline caused an irreversible denaturation of MtfA, meaning that zinc-ions are...
absolutely required to stabilize the protein structure and keep it in solution. Furthermore, lack of complete inhibition by the weaker chelator EDTA was previously also observed with other HExxH metalloproteases, such as PepN (4).

Amino-peptidase activities of MtfA mutants. In the next step we examined the collection of MtfA mutants for their amino-peptidase activities. All mutants were purified and tested for their ability to cleave off the alanine residue from the L-alanine-p-nitroanilide substrate. Freshly prepared, wild type MtfA protein was always taken as a control and set to 100%. The results for all MtfA derivatives are given in figure 8. Mutants with substitutions at positions H149, E150, H153 (HExxH motif) or E212 displayed clearly reduced protease activities probably due to the fact that the coordination of the zinc-ion, the overall conformation of the protein and thus the catalytic activity were affected. With respect to the histidine residues 149 and 153 and their involvement in zinc-coordination, a substitution for lysine led to a stronger effect than a replacement by glutamine. A triple mutant with replacements (by alanine) of all three functionally important residues of the HExxH motif was insoluble and could not further be analysed (data not shown). Substantial changes in the proteolytic activities were also observed for mutants with substitutions of the two glutamate residues at positions 150 and 212. On the basis of the MtfAKpn crystal structure one would expect these residues to be involved in catalysis. The substitution E150Q influenced the protease activity most significantly. In contrast, as shown in figure 3, the replacement of this residue with aspartic acid or glutamine, respectively, did not affect Mlc binding in vivo. Moreover, as illustrated in figures 5c and 5d, the purified MtfAE150D or MtfAE150Q proteins are still capable of causing a release of the ptsGop -fragment in a band shift assay with purified Mlc in vitro.

These results demonstrate that the two MtfA functions of Mlc-binding and proteolysis are clearly distinct from each other. Other substitutions that led to reduced proteolytic activities, but retained Mlc binding include Y94S and P225S. MtfA derivatives with the substitutions
S142F, D156N, A207D, V216D, or Y243S, respectively, exhibited both reduced enzyme activity and reduced Mlc binding, which might indicate severe changes in structure and function. Furthermore, replacements of the residues isoleucine 148 with glutamic acid and alanine 215 with aspartic acid resulted in protein instability. Thus, the reduced activities of MtfAI148Y and MtfAA215S may also be caused by enhanced protein degradation.

Mlc influences protease activity. The results described above raised the question as to whether Mlc is a substrate for MtfA or whether it has an influence on its protease activity. Therefore, purified MtfA and Mlc were incubated together in a ratio of 1 to 2 (MtfA is known to form dimers, Mlc to form tetramers) and tested for amino-peptidase activity. As illustrated in figure 9, the addition of amino-terminal his-tagged Mlc to the standard assay led to an increased proteolytic activity. The effect was even more severe with untagged Mlc. Addition of equal amounts of BSA caused no significant effect. The mutant protein MtfAN145D, which displayed a reduced interaction with Mlc in vivo (Fig. 3) but almost wild type proteolytic activity (Fig. 8), was significantly less stimulated by his-tagged Mlc. The presence of some stimulation may have been caused by the in vitro test conditions, which required high amounts of purified protein. Regardless, Mlc can clearly be excluded as a biologically relevant substrate.

Discussion

In Escherichia coli K-12, one of the best studied model organisms, the function of many putative open reading frames remains unknown. These had included mtfA (formerly yeeI), despite the fact that identification of orthologs of the E. coli mtfA gene in more than 100 proteobacteria of the α-, β-, and γ- subdivisions have implied an important function of this protein. Using surface plasmon resonance assays we were able to demonstrate in a previous paper that MtfA binds with high affinity to the glucose-repressor Mlc (2). Moreover, we could
demonstrate that MtfA binds specifically to the carboxy-terminal segment of Mlc, which is usually involved in protein tetramerization. In this manner, MtfA amongst other factors seems to play an important role in the regulation of the glucose-PTS. In turn, the glucose-PTS has an important function in the regulation of carbohydrate metabolism in *E. coli*. Mlc, for example, not only regulates *ptsG* gene expression, but is also a signal mediator for glucose induction of the *ptsH/crr* operon (20, 29, 34), the *malT* gene for the transcriptional activator of the maltose regulon (6), the *manXYZ* operon for the mannose-PTS (18) and enzymes involved in glycolysis (5), respectively. Interestingly, DNA-binding activity of Mlc in *E. coli* is not regulated by a small molecular inducer. Instead, Mlc is inactivated by binding to the dephosphorylated EIIB\(^{Glc}\) domain of the EIICB\(^{Glc}\) during glucose transport (13, 16, 28, 34). The localization of EIIB\(^{Glc}\) at the membrane appears to be essential for this kind of inactivation mechanism (27), since overproduction of the liberated soluble EIIB\(^{Glc}\) domain does not lead to Mlc inactivation (13, 34), though unphosphorylated EIIB\(^{Glc}\) alone binds to Mlc as shown in surface plasmon resonance assays (16). In contrast, membrane-bound fusion proteins consisting of the EIIB\(^{Glc}\) domain in combination with either the lactose permease LacY (27) or the bacteriophage M13 membrane coat protein Gp8 (26) are perfectly able to sequester Mlc. Thus, binding of EIIB\(^{Glc}\) to Mlc might either not cause any conformational change of the repressor, or there might be no similar conformational change as caused by MtfA binding. Instead by anchoring the Mlc tetramer to the membrane via EIICB\(^{Glc}\), the structural flexibility of Mlc monomers within the complex becomes restricted, which seems to be responsible for the loss of its DNA binding activity (17). Crystal structure analysis of *E. coli* Mlc (25) revealed the existence of three functional domains: the amino-terminal part containing the helix-turn-helix DNA-binding motif (D-domain), an oligomerization domain from residues 195-380 (O-domain) and an inner EIIB\(^{Glc}\) binding domain (E-domain). There appears to be only weak interactions between the three domains and in particular the O-domain is separated from the residual protein by a highly flexible hinge. However, structural
rearrangements of the O-domain remotely affect the conformation of the DNA-binding
domain by an unknown mechanism (17). In contrast to EIIBGlc, inactivation of Mlc by MtfA
occurs in the cytosol and is not membrane-dependent (2). Tanaka et al. (2004) isolated an Mlc
super repressor, which carries a H86R amino acid substitution. According to the domain
model, Mlc*H86R cannot be inactivated by EIICB\textsuperscript{Glc}. In this paper we could demonstrate that
MtfA is still able to bind and inactivate Mlc*H86R both \textit{in vivo} and \textit{in vitro}. This clearly
supports the notion that MtfA-Mlc interaction \textit{de facto} occurs at the C-domain of Mlc, which
may in contrast to the EIIB\textsuperscript{Glc}-Mlc interaction, result in either severe changes in the
conformation of the D-domain or prevention of repressor oligomerization. Both mechanisms
would affect the DNA-binding activity of Mlc. Both the presence of a ternary complex \textit{in vivo}
and that of cooperative binding effects remain uncertain and thus are areas in need of further
elucidation.

Using systematic site directed mutagenesis of conserved amino acid residues in MtfA and
subsequent \textit{in vivo} activity assays, we identified a Mlc interaction domain within the carboxy-
terminal part of MtfA. The results of \textit{in vivo} phenotyping of the interaction between MtfA and
its derivatives with Mlc led to the conclusion that amino acids from residue 132 onwards
appear to be necessary for this interaction. Further experiments employing a bacterial two-
hybrid assay have also supported this idea. However, two amino acid substitutions (L40E and
A70D) in the amino-terminus also caused a significant reduction in Mlc titration, whereas
conserved exchanges at these two positions had no effect. This might indicate that there are
two classes of amino acid substitution that affect Mlc binding: Substitutions of the first class
disturb the overall conformation of MtfA, whereas substitutions of the second class directly
affect the MtfA-Mlc interaction.

Recently, the crystal structure of MtfA from \textit{Klebsiella pneumonia} (MtfAE\textsubscript{Kpn}) was
characterized at the Joint Center for Structural Genomics (DOI:10.2210/pdb3khi/pdb). A
projection of the MtfAE\textsubscript{Ec} sequence onto the MtfAE\textsubscript{Kpn} structure revealed that at least eight of the
identified amino acid residues in the carboxy-terminus are exposed on the surface of MtfA.

Furthermore, these residues are clearly clustered at one side of the protein (Fig. 10). Thus, it is highly likely that these residues are directly involved in Mlc binding. In contrast, other residues like L40 or A70 are almost or completely buried inside the protein.

The MtfA<sub>Kpn</sub> structure revealed overall similarities to the zinc-dependent metalloprotease Lethal Factor (LF) from Bacillus anthracis as well as other metalloproteases in the gluzincin clan. Furthermore, we previously confirmed the presence of proteolytic activity of the MtfA<sub>Kpn</sub> protein and the existence of the artificial substrate L-alanine 4-nitroanilide (Qingping Xu, Anna Göhler et al., manuscript in preparation). In this study, we were able to demonstrate a similar activity for the E.coli protein. With an assortment of MtfA derivatives, our results support the notion of MtfA as a metal dependent protease. Single substitutions of amino acids involved in zinc-coordination or catalysis of the substrate show, as deduced from the structure, significantly reduced <i>in vitro</i> activities. An MtfA mutant with a triple substitution at positions 149, 150 and 153 (zinc-coordination-residues) for alanine, could not be investigated <i>in vitro</i>, suggesting a structural instability of the residual protein without the metal ion. Further analysis to identify a metal-bound and metal-unbound state by electrospray ionization mass spectrometry failed due to irreversible unfolding of MtfA at low pH conditions. However, once again this result points to a metal-dependent peptidase activity of MtfA.

These findings now raise questions about the biological relevance of the MtfA-Mlc interaction. Mlc is clearly not a target protein. On the contrary, addition of Mlc stimulates MtfA amino-peptidase activity. Preliminary analysis of the Mtf<sub>Kpn</sub> structure revealed the existence of a self-inhibitory complex (Qingping Xu, Anna Göhler et al., in preparation). It is tempting to speculate that Mlc binding causes a conformational change in MtfA, which may override self-inhibition. Transcriptome analysis by Lemuth (14) under glucose limitation revealed a significant up-regulation of <i>mtfA</i> expression under growth conditions during the...
transition from the exponential to the stationary phase. According to the Mlc regulation model, running out of glucose would lead to a rephosphorylation of EIICB\textsuperscript{Glc} and thus to a release of Mlc into the cytoplasm. Both effects would boost the overall MtfA protease activity at this growth transition point. This leads to a model according to which the glucose sensor Mlc/EIICB\textsuperscript{Glc} not only controls gene expression by Mlc, but that at least in \textit{E. coli} it also regulates the proteolytic activity of MtfA in a glucose-dependent manner. This reflects a novel regulatory output signal by the glucose-PTS.

Questions still remain regarding which proteins are natural targets for MtfA. For example, the structurally related endopeptidase LF of \textit{B. subtilis} cleaves off the amino-terminal peptides of mitogen-activated protein kinase kinases (MAPKKs) during host infections (9, 31). It is tempting to speculate that MtfA also may cleave off short peptides from its natural target proteins rather than working just as an amino-peptidase. In a series of unspecific crosslinking experiments to identify further interaction partners of MtfA, several putative target proteins were identified (our unpublished results). Among others, the \(\gamma\)-subunit of the ATP synthase was identified 4 times by 5 screening approaches under different conditions. Variations in the number of ATP synthase complexes might be necessary for growth adaptation under changing environmental conditions, making the \(\gamma\)-subunit a promising target candidate of MtfA.

In conclusion, by elucidating that MtfA is a glucose-regulated peptidase, we have identified a novel regulatory output mechanism of the glucose-PTS, which may be involved in the adaptation of \textit{E. coli} cells during the transition from the glucose-phase to the next consecutive growth phase. Since the physiologically relevant target is not known yet, further investigation is necessary.

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References


TABLE 1. *Escherichia coli* strains used in this study

<table>
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<th>Strains</th>
<th>Relevant Genotype or Phenotype</th>
<th>Source or Reference</th>
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<td>JM109</td>
<td><em>thi-1</em> Δ*(lac-proA,B)* <em>recA1</em> <em>hsdR1</em> / <em>F</em>′<em>traD36 proA</em>′ <em>B</em>′ <em>lacF</em>′<em>Z</em> M15</td>
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<td>LZ110</td>
<td><em>LJ110 ΔlacU169 zah-735::Tn10tet</em></td>
<td>(34)</td>
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<td>LZ150</td>
<td><em>LZ110 Δ(ptsG::cat)</em></td>
<td>(34)</td>
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<td>this study</td>
</tr>
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<td><em>JEK5 pheU::dgsA</em>′ <em>cat</em>′</td>
<td>this study</td>
</tr>
<tr>
<td>JSW2</td>
<td>*JEK5 pheU:: dgsA <em>(Mlc</em>′*H86R) <em>cat</em>′</td>
<td>this study</td>
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<td>XL1-Blue</td>
<td><em>recA1 endA1 gyrA96 thi-1</em> <em>hsdR17 supE44 relA1</em> <em>lac /F</em>′<em>proAB</em>′ <em>lacF</em>′<em>Z</em> M15 <em>Tn10Te</em>′</td>
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Legends

Figure 1. Multiple Alignment of MtFA\textsubscript{Eco} and ortholog proteins from other bacteria.

The amino acid sequence of MtFA\textsubscript{Eco} was aligned with the presented homologues using the ClustalW program. Abbreviations are as follows: Eco: \textit{Eschericha coli} gi|90111364; \textit{Escherichia fergusonii} gi|22034302; Kpn: \textit{Klebsiella pneumoniae} gi|152970975; Sty: \textit{Salmonella enterica} serovar Typhimurium gi|16420534; Ype: \textit{Yersinia pestis} gi|22126457; Cvi: \textit{Chromobacterium violaceum} ATCC gi|34104211; Psy: \textit{Pseudomonas syringae} pv \textit{tomato} DC3000 gi|28851187; Xca: \textit{Xanthomonas campestris} pv. \textit{Campestris} strain ATCC 33913 gi|21115196; Rso: \textit{Ralstonia solanacearum} GMI 1000 gi|17428198; Npu: \textit{Nostoc punctiforme} PCC 73102 gi|23129869. Identical residues are marked by a *, highly conserved residues by a :, conserved residues by a . Numbers of identical residues in binary comparisons between \textit{E. coli} MtFA and the respective homologues of all other bacteria are given in the box.

Substituted amino acids are marked in grey.

Figure 2. Test system to quantify the MtFA interaction with Mlc \textit{in vivo}.

This figure illustrates the test system for phenotyping MtFA mutants. The strain background LZ150F\textsuperscript{-}::\Phi(ptsGop-lacZ) provides a chromosomally encoded \textit{dgsA}\textsuperscript{+} gene (mlc\textsuperscript{+}), chromosomal deletions of the ptsG and the lacZ genes, and a single copy \textit{ptsGop-lacZ} fusion on an F\textsuperscript{-}-plasmid. MtFA or its derivatives were introduced via the IPTG inducible expression plasmid pTMByeel-S or derivatives thereof, respectively. Since Mlc cannot bind to the EIIB\textsubscript{Glc} domain in this strain, it can only be inactivated by MtFA. Thus, inactivation of Mlc by MtFA is reflected by high \textit{\beta}-galactosidase activities.

Figure 3. \textit{\beta}-galactosidase activities for the quantification of the interactions between MtFA wild type or derivatives thereof with Mlc \textit{in vivo}.

The results of phenotyping MtFA and defined mutants with regard to Mlc titration are shown. The test system presented in figure 2 was used. Cells harboring the pTMByeel-S vector or
derivatives thereof were grown in LBo, mtfA expression was induced with IPTG, and cells were harvested for β-galactosidase activity assays. The activity of the wild type control (pTMByeeI-S), which was usually around 850 nmol per mg protein and min, was set to 100% in each set of experiments. The empty expression vector pTM30 served as a negative control (represented by grey bars). Mutants, which exhibited β-galactosidase activities of more than 80% of the wild type level, were drawn in white columns. In contrast, MtfA mutants with less than 80% activity, which reflected a severe reduction of Mlc binding, are presented by black columns. MtfA mutants K/Q/K/Q and A/A/A denote substitutions H149K/E150Q/H153K/E212Q and H149A/E150A/H153A, respectively. Averages of at least three independent tests with standard deviations are given.

Figure 4. Bacterial-two-hybrid assays to test in vivo interaction of MtfA with Mlc. Cells were grown in LBo without (-) and with (+) IPTG, harvested and analyzed for their β-galactosidase activities. Control samples (SU202: test strain without any plasmid as a negative control; pMS (LexA-Fos-hybrid protein)/pDL (LexA*-Jun-hybrid protein) as a positive control) are shown in grey. The results for the analyzed test samples (pMM3 and pDM3: Mlc full-length constructs; pMM2: MtfA full-length construct; pMM4: MtfAΔ1-83 construct) are illustrated by the white bars. All test combinations showed reduced β-galactosidase activities (given in nmol per min and mg total cell protein) under inducing conditions, which indicates an interaction of the fusion proteins. Averages of at least three independent measurements and standard deviations are given.

Figure 5. Electrophoretic mobility shift assays (EMSA) with purified Mlc and MtfA proteins. a. A fluorescence-labeled DNA fragment containing the Mlc-binding sites in front of the ptsG gene (ptsG operators) was incubated with different combinations of purified Mlc and MtfA and subsequently run on a native gel. A control sample without any protein (-) led to an unshifted signal of the DNA. After incubation of the operator fragment with purified Mlc (+), a retarded signal was observed.
Mlc-DNA complex could be detected. Addition of purified MtfA (150 nM in the assay) caused not band shift. Incubation of Mlc (usually 25nM in the assay) with rising amounts of MtfA (usually 25 to 150nM in the assay) as indicated prior to the incubation with ptsGop led to an increasing release of operator fragments.

b. Incubation of the ptsG-operator fragment with purified super repressor Mlc*H86R led to the generation of an Mlc-DNA complex. Similar to the results obtained for the wild type repressor, addition of MtfA to the band shift assay prior to the incubation with ptsGop led to released operator fragments.

c. Incubation of the ptsG-operator fragment with purified wild type Mlc and increasing amounts of MtfA E150D.

d. Incubation of the ptsG-operator fragment with purified wild type Mlc and increasing amounts of MtfA E150Q, further explanations are given in the text.

Figure 6. β-galactosidase assays for the characterization of the in vivo interaction between MtfA wild type and either Mlc wild type or Mlc*H86R super repressor. The test system was similar to the one described in figure 2. LZ110 was used as dgsA+ wild type control (as indicated by grey bars). JEK5 carries a dgsA deletion. JSW1 carries a dgsA deletion and a single wild type copy of dgsA+ at a different position in the chromosome. JSW2 carries also a dgsA deletion and a single copy dgsA* gene encoding the Mlc*H86R super repressor. All strains harbor the mtfA wild type expression plasmid pTMByeeIs. Cells were grown in the absence (-) or presence of IPTG (+) and tested for their β-galactosidase activities, which are given in nmol per min and mg total cell protein. All activities are mean values of at least three independent experiments with standard deviations.

Figure 7. Peptidase assays of purified MtfA with different substrates. The activity measurements were carried out as described in methods. For L-amino 4-nitroanilide substrates, activity was monitored by measuring the change of the optical density
at 390 nm (ΔOD390) triggered by the release of 4-nitroanilide after cleavage. The specific activity [U/mg*min] was calculated. The highest cleavage rate was reached with L-alanine 4-nitroanilide as substrate and set as 100%. The typical activity level under the test conditions for this substrate was around 9 mU per mg protein and min. Lower activities were detected for L-arginine 4-nitroanilide as a basic amino acid or for other none polar amino acid substrates such as L-proline or L-valine fused to 4-nitroanilide. Glutamic-acid 4-nitroanilide as an acidic amino acid or alanine-alanine-alanine 4-nitroanilide (data not shown) could not be cleaved by MtfA. Substrates for caroxy peptidase were also investigated. Neither hippuryl-arginine nor hippuryl-L-phenylalanine was cleaved sufficiently by MtfA. The cleavage of L-alanine 4-nitroanilide was not strongly inhibited by the protease inhibitor AEBSF. In contrast, the chelator reagents EDTA and phenantroline reduced proteolytic activity severely as it is shown in the last three columns. All activities are given in % relative to the cleavage of L-alanine 4-nitroanilide. Data are mean values of three independent experiments with standard deviations.

Figure 8. Protease activity of MtfA wild type and defined MtfA mutants. MtfA and defined mutants (as indicated) were purified and the amino-peptidase activities towards the cleavage of L-alanine 4-nitroanilide were monitored. Each activity was calculated and is diagrammed relatively to the activity of the MtfA wild type protein, which was analyzed in parallel. Wild type activity, which was typically around 9 mU per mg protein and min was set to 100% and is presented by the grey bars. Activities of mutant proteins, which showed more than 80% wt-activity, are displayed by the white columns. In contrast, black columns represent MtfA derivatives, which showed less than 80% wt- proteolytic activity. The MtfA derivative 149/150/153/212 carries the substitutions H149K/E150Q/H153K/E212Q. Standard deviations and average values of at least three independent experiments are indicated.
Figure 9. Protease activities of MtfA wild type and MtfAN145D in the absence or presence of Mlc. The cleavage efficiency of L-alanine 4-nitroanilide by MtfA or MtfAN145D was assayed in the presence or absence of Mlc as indicated. Control samples were carried out incubating MtfA either without any supplements or with accessory bovine serum album (BSA). Addition of purified Mlc with an amino-terminal his-tagged or addition of an untagged Mlc version stimulated MtfA enzyme activity up to 175%. In contrast, the MtfA derivative N145D with reduced Mlc binding capacity was only stimulated to 123% of the wild type activity. Activity values are averages of at least three independent experiments with standard deviations.

Figure 10. Projected MtfA<sub>Ec</sub> protein structure. We used the POLYVIEW-3D web-based tool (http://polyview.cchmc.org/polyview3d.html; (22)) for molecular structure visualization of MtfA<sup>Ec</sup><sup>Kpn</sup>. The positions of amino acid residues 132, 142, 145, 149, 153, 205, 207, 216, 219 are indicated in red color.