Mlc of *Thermus thermophilus*: a Glucose-Specific Regulator for a Glucose/Mannose ABC Transporter in the Absence of the Phosphotransferase System

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We report the presence of Mlc in a thermophilic bacterium. Mlc is known as a global regulator of sugar metabolism in gram-negative enteric bacteria that is controlled by sequestration to a glucose-transporting EIIGlc of the phosphotransferase system (PTS). Since thermophilic bacteria do not possess PTS, Mlc in *Thermus thermophilus* must be differently controlled. DNA sequence alignments between Mlc from *T. thermophilus* (MlcTth) and Mlc from *E. coli* (MlcEco) revealed that MlcTth conserved five residues of the glucose-binding motif of glucokinases. Here we show that MlcTth is not a glucokinase but is indeed able to bind glucose (KD = 20 μM), unlike MlcEco. We found that mlc of *T. thermophilus* is the first gene within an operon encoding an ABC transporter for glucose and mannose, including a glucose/mannose-binding protein and two permeases. *malK1*, encoding the cognate ATP-hydrolyzing subunit, is located elsewhere on the chromosome. The system transports glucose at 70°C with a Kₘ of 0.15 μM and a Vₘₐₓ of 4.22 nmol per min per ml at an optical density (OD) of 1. MlcTth negatively regulates itself and the entire glucose/mannose ABC transport system operon but not *malK1*, with glucose acting as an inducer. MalK1 is shared with the ABC transporter for trehalose, maltose, sucrose, and palatinose (TMSP). Mutants lacking *malK1* do not transport either glucose or maltose. The TMSP transporter is also able to transport glucose with a Kₘ of 1.4 μM and a Vₘₐₓ of 7.6 nmol per min per ml at an OD of 1, but it does not transport mannose.

In *Escherichia coli*, glucose induction of several genes and operons involved in sugar transport and metabolism is mediated by the global repressor Mlc, ptsG, encoding enzyme ICB of the glucose-specific PEP-dependent phosphotransferase system (PTS), is the most prominent gene under the control of Mlc (22). Other genes regulated by Mlc include malT, encoding the activator of the maltose regulon (6); manXYZ, encoding three proteins of the mannose PTS (22); and the genes encoding the general components of the PTS (12, 23, 31). The expression of mlc is autoregulated (5) and partially under the control of the σ₇₀-mediated heat shock response (27).

The particularity of Mlc in *E. coli* is that, unlike normal prokaryotic transcriptional regulators, it is not a low-molecular-weight cytoplasmic molecule that inactivates the repressor by preventing its binding to DNA. Instead, the activity of Mlc as a repressor is regulated (i.e., inhibited) by its binding (sequestration) to the dephosphorylated state of the membrane-associated EIIBC domain of the PtsG protein occurring during glucose transport (14, 19, 27, 30).

The recent sequencing of the thermophilic bacterium *Thermus thermophilus* HB27 genome (10) revealed the presence of a gene (TTC0329) encoding a protein with similarity to the Mlc from *E. coli*. Its amino acid sequence contains the two consensus sequences that characterize the ROK family of transcriptional regulators (for repressors, open reading frames, and kinases), as well as the four residues (one histidine and three cysteines) corresponding to the zinc binding site necessary for the repressor function of Mlc in *E. coli* (25).

Strains of the species *T. thermophilus* are commonly isolated from marine hot springs and can grow at temperatures up to 70°C. Thus far, no PEP-dependent PTSs have been encountered in thermophilic bacteria or archaea (7), an observation in line with the concept of a hot origin of life (1, 11) and implying that the PTS only evolved later in mesophiles. It thus seemed intriguing that a homologue of Mlc would be found in such an organism, since the regulation of Mlc in *E. coli* is known to be dependent on the phosphorylated state of PtsG (13, 14, 19, 26, 30).

The present study began with the observation that the Mlc of *T. thermophilus*, when overexpressed in *E. coli*, had the ability to affect the expression of *ptsG* and *malT* of *E. coli* and also had residual ability to bind to the EIIBC domain of the *E. coli* PtsG. We became curious about the actual role of Mlc in *T. thermophilus* and about its mode of regulation, since it had to be different from PTS-dependent regulation in *E. coli*. DNA sequence alignments between the Mlc of *T. thermophilus* (MlcTth) and that of *E. coli* (MlcEco) revealed that MlcTth, unlike MlcEco, conserved five residues of the glucose-binding motif of glucokinases (15). However, we found that MlcTth was not a glucokinase but, unlike MlcEco, was able to bind glucose as well as mannose. We show here that the Mlc in *T. thermophilus* negatively regulates itself and an entire operon encoding a glucose/mannose ABC transport system in a glucose-dependent manner. The operon contains at least four genes encoding MlcTth, a glucose/mannose-binding protein, and two permeases. We show that the ATP-hydrolyzing subunit for this system is MalK1, which is also the ATP-hydrolyzing subunit of...
optical density at 405 nm (OD405). To calculate the specific activity, we used an carbonate, we clarified the suspension by centrifugation before we measured the hydrolysis of α-D-galactopyranoside at a constant temperature of 28°C. After the reaction was stopped with sodium /H11003 extinction coefficient of 4,860/mol /H9262.

The ABC transporter for trehalose, maltose, sucrose, and palatinose (TMSP) described previously (28).

**MATERIALS AND METHODS**

**Strains, plasmids, and chemicals.** *T. thermophilus* strain HB27 (DSM7039) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Other strains and plasmids used in the present study are listed in Table 1. All chemicals were reagent grade and were obtained from commercial sources.

**Standard DNA analyses.** Chromosomal DNA from *T. thermophilus* HB27 was extracted by using the QIAampDNA blood minikit (QIAGEN). Plasmids were extracted from *E. coli* strains with the Mini-Plasmid kit (QIAGEN). Digestions by endonucleases (New England Biolabs), ligations (T4 DNA ligase; New England Biolabs), and PCR were performed by standard procedures (18, 24). Proofreading DNA polymerase (Pwo [PqLab] or Phusion [Finnzymes]) was used for all PCR applications. Correct cloning was confirmed by sequencing analysis (GATC Biotech, Konstanz, Germany).

**β-Galactosidase assays.** β-Galactosidase activity was determined according to the method of Miller (19) with alterations. We omitted β-mercaptoethanol from the Z buffer. Hydrolysis of ortho- nitrophenyl-β-galactoside (ONPGal) was done at a constant temperature of 28°C. After the reaction was stopped with sodium carbonate, we clarified the suspension by centrifugation before we measured the optical density at 405 nm (OD405). To calculate the specific activity, we used an extinction coefficient of 4,860×10⁻³ mmol⁻¹ cm⁻¹ for o-nitrophenol. The specific activity (U/mg protein) was given in μmol of ONPGal hydrolyzed per min per mg of protein at 28°C. A specific activity of 1 corresponds to about 1,000 Miller units.

**Cloning of Mlc from *T. thermophilus*, overexpression, and purification of the recombinant protein.** Two versions of *mlc* were produced: an N-terminal His₆-tagged version and a wild-type version. Primers were designed based on the retrieved sequence of gene TTC0329 in *T. thermophilus*. The N-terminal His₆-tagged protein was constructed by producing a PCR product using the genomic DNA of *T. thermophilus* (strain HB27) and the primers 5'-CGG GAG CCC AAG ACC ATA CAC AGC CCC AAC ACC ATA CAC TCC-3' (forward) and 5'-GAG AGG AGT GAG CAC CTG GAG ATC TTG TCC-3' (reverse). After gel purification of the PCR fragment (QIAGEN gel purification kit) and digestion with the restriction enzymes BamHI and HindIII (restriction sites underlined), the fragment was ligated into plasmid pGDR11 (a pQE31 derivative harboring the lacP gene (29), yielding plasmid pFC4 (N-terminally His₆-tagged *Mlc*). Plasmid pFC4 was subsequently used as a template for the construction of the wild-type *Mlc* using the primers 5'-CAT GGC ATG TGC GTA AGG GCC AGC ACC TCC-3' (forward) and 5’-GGA AGATCTA TTA AGC CCC AAC AGC ATA CCG TCC-3' (reverse). These primers were designed so that a methionine start codon was introduced at the N terminal of the construct, while an additional stop codon (TAA) was introduced at the C terminal. After purification of the PCR product and digestion with the NcoI and BglII restriction enzymes (underlined), the PCR fragment was ligated into plasmid pCS19 (a pQE60 derivative harboring the lacP gene (29), yielding plasmid pEM1 (wild-type *Mlc*). This plasmid was transformed into *E. coli* SF120 (2)-competent cells, which were grown in 1 liter of Luria-Bertani (LB) broth containing 100 μg of ampicillin/ml at 37°C. When the OD₆₀₀ reached 0.6, the cells were induced with 0.2 mM IPTG (iso- propyl-β-D-thiogalactopyranoside) and grown for an additional 5 h. The cells were centrifuged for 20 min at 5,000 rpm in a Sorvall SS34 rotor (as in all of the following centrifugation steps) and washed once with 0.9% NaCl. The resulting pellets were stored at −80°C until further use. The frozen cells were resuspended in 15 ml of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole [pH 8]) and French pressed four times at 16,000 lb/in². The crude cell extract was centrifuged at 12,000 rpm for 20 min, the supernatant was incubated at 70°C for 15 min, and the denatured proteins were removed by centrifugation (15,000 rpm for 20 min). The His₆-tagged construct of *Mlc* was further purified by using a Ni-NTA Superflow column. The column was first washed with 2 volumes (40 ml) of lysis buffer containing 20 mM imidazole, and the His tag protein was then eluted by using a linear gradient from 20 to 500 mM imidazole. The protein was dialyzed in equilibration buffer (25 mM Tris, 150 mM NaCl).

**TABLE 1. Strains and plasmids used in this study**

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<thead>
<tr>
<th>Strain or plasmid</th>
<th>Known genotype*</th>
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<td><strong>Strains</strong> E. coli</td>
<td>SF120 ptr32::cm degP4::kan ΔompT</td>
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<td><strong>T. thermophilus</strong> DSM7029</td>
<td>Wild-type strain (HB27)</td>
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<td>HB27 malK1::kan</td>
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<tr>
<td>JN1 ΔmalF::bleo</td>
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<td>Jutta Nesper</td>
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<td>CL4 mlc::kan ΔmalF::bleo</td>
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<td><strong>Plasmids</strong> pFC4</td>
<td>pGDR11 lacP; N-terminally His₆-tagged <em>Mlc</em> (gene TTC0329); Amp’</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
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<td>pCL2</td>
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<td>pTTC0328</td>
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<td>This study</td>
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<td>pREP4</td>
<td>pACYC derivative containing the p15A replicon; Kan’</td>
<td>QIAGEN</td>
</tr>
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</table>
ampicillin/ml and 25 µg of kanamycin/ml. The resulting plasmid, pTTC0328, was grown in 2 liters of NZA medium (0.5% yeast extract, 0.75% NaCl, 1% N-Z-Amine A [Sigma Aldrich, Munich, Germany]) containing 100 µg of ampicillin/ml and 25 µg of kanamycin/ml at 37°C. When the OD600 reached 0.6, IPTG was added to a final concentration of 100 µM. Cells were grown for an additional 3 h and then harvested by centrifugation. The pellet was resuspended in 6 ml of lysis buffer (20 mM Tris–300 mM NaCl). Cells were disrupted by passing them four times at 12,000 lb/in² through a French pressure cell, followed by centrifugation at 17,000 × g for 90 min at 4°C. The supernatant was loaded onto a Ni affinity column (HiTrap chelating HP 1-ml column) equili-
ibrated with lysis buffer. Bound protein was eluted with a linear gradient of 0 to 500 mM imidazole within 20 column volumes. In order to remove imidazole, the protein was extensively dialyzed against 50 mM Tris–300 mM NaCl (pH 7.5).

Cloning and purification of the binding protein of the TMS transporter. Gene TTC0328, encoding the TMS binding protein, was PCR amplified without its signal peptide-encoding sequence, using the genomic DNA of T. thermophilus (strain HB27) as a template and the primers 5'-CCGGATCCCA GTCCGGGCCCCGTGATC-3' (MBPTth forward) and 5'-CCCGGAAGCTTGG GTGTTACGGGAGCA-3' (MBPTth reverse). The PCR product was digested with BamHI and HindIII (sites are underlined) and ligated into vector pQE30 (QIAGEN). Competent E. coli M15 (QIAGEN) harboring the pREP4 (QIAGEN) the lacP repressor-carrying plasmids were transformed with the ligation mixture and plated on 1.5% LB agar plates supplemented with 100 µg of ampicillin/ml and 25 µg of kanamycin/ml. The resulting plasmid, pCL10, carries gene TTC0328 under the control of the T5 promoter lacP operator system. The N-terminal leader peptide sequence (27 amino acids) of the gene is exchanged by a His tag-encoding sequence. E. coli M15 harboring plasmids pREP4 and pCL10 was grown in 2 liters of NZA medium containing 100 µg of ampicillin/ml and 25 µg of kanamycin/ml at 37°C. When the OD600 reached 0.6, IPTG was added to a final concentration of 100 µM. Cells were grown for an additional 3 h and then harvested by centrifugation. The pellet was resuspended in 6 ml of lysis buffer (50 mM Tris [pH 7.5]) containing 300 mM NaCl and 6 M guanidine hydrochloride. Cells were disrupted by passing them four times at 12,000 lb/in² through a French pressure cell. Afterward, the suspension was incubated at 70°C for 30 min and centrifuged at 17,000 × g for 90 min at 4°C. The supernatant was loaded onto a Ni-affinity column (HiTrap chelating HP 1-ml column) equilibrated with lysis buffer. Bound protein was eluted with a linear gradient of 0 to 500 mM imidazole within 20 column volumes in a refolding buffer (50 mM Tris [pH 7.5] containing 300 mM NaCl). In order to remove imidazole, the protein was extensively dialyzed against 50 mM Tris–300 mM NaCl (pH 7.5).

Construction of the mlc::kan mutant in T. thermophilus. The thermostable kanamycin resistance cassette from plasmid pMK18 (6) was amplified by PCR using the primers 5'-TCGGGCCGCCCCGTGATC-3' (MBPTth reverse) and 5'-CCCGGAAGCTTGG GTGTTACGGGAGCA-3' (MBPTth forward) for the T. thermophilus mlc (TTC0211) were amplified by hot start PCR using the primers 5'-AGG CCC GCC ACC ACC ATT TGC AA-3' (TTCTh forward), 5'-TCG CCC TCC TCG CCC TGA GGC AG-3' (TTCTh reverse), and the respective primers as described above. The TLC plate was dipped into methanol containing 5% H2SO4, and after the plate was dried, the sugar-containing spots were visualized by heating at 170°C for 5 min.

Electrophoretic mobility shift assay (EMSA). Promoter regions of mlc (TTC0329), malE1 (TTC1627), and malK1 (TTC0211) were amplified by high start PCRs using genomic DNA of T. thermophilus and the respective primers as follows: ThlMicPro_for (5'-TCC AAGG AGG GCC TGG ACC TTG GCC-3') and ThlMicPro_rev (5'-CTG ATG TGC CCG GCC ACC ATC AA GAA-3') for the mlc promoter, ThlMalE1Pro_for (5'-AGG CCC GCC ACC ACC ATT TGC AA-3') and ThlMalE1Pro_rev (5'-TCG CCC TCC TCG CCC TGA GGC AG-3') for the malE1 promoter, and ThlMalK1Pro_for (5'-AGG CCC GCC ACC ACC ATT TGC AA-3') and ThlMalK1Pro_rev (5'-TCG CCC TCC TCG CCC TGA GGC AG-3') for the malK1 promoter. The resulting plasmid containing about 7 nCi of labeled DNA (ca. 44 fmol) and 250 ng of poly(dI-dC)-poly(dU-dC) competitor DNA (Roche, Germany) per 10 µl was loaded onto a 20% native polyacrylamide gel electrophoresis gel, and run at room temperature under a constant voltage of 200 V.

Transport assays in T. thermophilus. Precultures were grown at 70°C in minimal medium A (19) with tryptophan as the sole nitrogen source and threonine as the sole carbon source. The precultures were diluted 1:100 in fresh medium supplemented with 0.4% glucose, maltose, or both. Cells were then grown at 70°C in minimal medium A with Casamino Acids (1%), glucose (0.4%), maltose (0.4%), or a combination of Casamino Acids with glucose or maltose (same proportions as described above).
After 6 h at 70°C the cultures were harvested by centrifugation (5,000 × g, 20°C, 5 min), washed three times with minimum medium without carbon source, and resuspended in minimum medium. To measure the transport of glucose or maltose, a cell suspension with an OD600 of 0.03 (wild type) or 0.1 (mlc mutant) was used. To 3 ml of the cell suspension, prewarmed for 2 min at 70°C, 14C-labeled sugars were added to a final concentration for glucose of 112 nM (311 Ci/mol) and for maltose of 48 nM (680 Ci/mol). Cells were further incubated at 70°C. At each time point (15, 30, 45, and 60 s), 0.5 ml of the cell suspension was filtered through Millipore filters (pore size, 0.45 μm) with a rapid filtration apparatus and washed once with 5 ml of minimal medium at room temperature. The filters were counted in a toluene-based scintillation fluid by using a scintillation counter (LS 1801). Linear correlations of the number of counts versus time were obtained. The rate of transport in *T. thermophilus* is expressed as nanomoles per minute per milliliter of cell culture at an OD 600 of 1. To determine the *Km* and *Vmax* of glucose transport in the wild type and mlc::kan strains, as well as in the ΔmalF::bleo mutant, cells were grown in MMA with CAA as carbon source and 0.4% glucose (wild type and ΔmalF::bleo mutant) and 0.4% maltose (mlc::kan).

**RESULTS**

Mlc of *E. coli* has a homologue in the thermophilic bacterium *T. thermophilus*. BLAST analysis of the sequence of Mlc of *E. coli* (i.e., MlcEco) against the full genome of the thermophilic bacterium *T. thermophilus* HB27 (10) revealed the presence of a homologue of MlcEco in *T. thermophilus*. Gene TTC0329 encodes a protein with 17% amino acid identities with MlcEco over its entire length. It contains the two consensus sequences that characterize the ROK family of transcriptional regulators (gray shading) are shown: the amino acids involved in the zinc-binding site according to Schiefner et al. (25) are used. To 3 ml of the cell suspension, prewarmed for 2 min at 70°C, 14C-labeled sugars were added to a final concentration for glucose of 112 nM (311 Ci/mol) and for maltose of 48 nM (680 Ci/mol). Cells were further incubated at 70°C. At each time point (15, 30, 45, and 60 s), 0.5 ml of the cell suspension was filtered through Millipore filters (pore size, 0.45 μm) with a rapid filtration apparatus and washed once with 5 ml of minimal medium at room temperature. The filters were counted in a toluene-based scintillation fluid by using a scintillation counter (LS 1801). Linear correlations of the number of counts versus time were obtained. The rate of transport in *T. thermophilus* is expressed as nanomoles per minute per milliliter of cell culture at an OD 600 of 1. To determine the *Km* and *Vmax* of glucose transport in the wild type and mlc::kan strains, as well as in the ΔmalF::bleo mutant, cells were grown in MMA with CAA as carbon source and 0.4% glucose (wild type and ΔmalF::bleo mutant) and 0.4% maltose (mlc::kan).

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in an IPTG-inducible E. coli vector, yielding pFC4, and transformed into strains of E. coli containing a ptsG-lacZ translational fusion (22). To our surprise, MlcTth influenced the expression of this major known Mlc target gene. As shown in Fig. 2, MlcTth reduced the activity of ptsG, but the effect was much less than that observed for MlcEco (noticeable only when MlcTth is overexpressed). These assays were conducted with strain JM-G2, which contains the ptsG-lacZ translational fusion but lacks mlc and is ptsG+. Although the expression of ptsG was lifted by glucose in the case of MlcEco (only when not overexpressed), glucose noticeably increased the repression of ptsG by MlcTth when overexpressed (Fig. 2). In contrast to the data reported previously (22), we did not observe a significant repression of ptsG-lacZ by glucose in a mutant lacking Mlc, even though ptsG is known to be weakly dependent on cyclic AMP and CAP.

As a control, we also tested the effect of MlcTth on a tss-lacZ fusion that in E. coli is not controlled by Mlc. No effect was observed (data not shown).

We also found that MlcTth had residual ability to bind to the EIIBC domain of E. coli PtsG, but this binding was not released by glucose transport as is the case with MlcEco (data not shown). However, since T. thermophilus does not possess PtsG, the role of MlcTth in its real host had to be different from that of MlcEco in E. coli. That is the next question we sought to address here.

MlcTth, unlike MlcEco, binds glucose and mannose and shows no glucokinase activity. Since the sequence of gene TTC0329 retained the five residues necessary for glucose binding in glucokinases and given the high structural similarity between Mlc and kinases (25), it seemed likely that the corresponding protein (MlcTth) was in fact a glucokinase. We therefore investigated its glucose-binding and glucokinase activities.

MlcTth was purified to homogeneity (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) both as an N-terminally His-tagged version and in its native form. The His-tagged version was used for the binding and enzymatic assays. MlcTth indeed showed glucose-binding activity. At a 0.8 μM initial substrate concentration and a 2.5 μM protein concentration, it showed its highest binding activity at 70°C (294 nM bound glucose), whereas its activity at 37°C was 30% lower (208 nM bound glucose). The protein worked best in the presence of MgSO₄ in the binding buffer (294 nM bound glucose versus 207 nM bound glucose in a binding buffer without MgSO₄). In contrast, ZnCl₂ reduced its activity (145 nM bound glucose). We therefore kept MgSO₄ in the buffer for our standard assays. The apparent Kᵦ value for glucose binding was determined to be 20 μM, and the 2.5 μM protein solution bound maximally a 2.1 μM concentration of substrate (Fig. 3A). It is surprising that MlcTth would bind glucose with a much higher affinity than the E. coli glucokinase, which shows a Kᵦ for glucose of 0.78 mM (17).

Other sugars were also tested for their possible binding to MlcTth. Among them, mannose, the 2-epimer of glucose, was bound by MlcTth with a Kᵦ value of 134 μM and in equimolar proportion (ca. 5 μM Mlc bound maximally 4.76 μM mannose; Fig. 3B). In contrast, we did not observe any binding between MlcTth and the following sugars: maltose, maltotriose, sucrose, fructose, lactose, galactose, trehalose, α-methyl-glucopyranoside, or glucose-6-phosphate.

Given its high affinity for glucose and its structural homology to E. coli glucokinase, the glucokinase activity of MlcTth was investigated by using TLC for product identification and compared to the activity of E. coli glucokinase. No glucokinase activity was found for MlcTth (data not shown). Neither divalent ions such as Mg²⁺ or Zn²⁺ nor different phosphoryl donors (ATP, ADP, GTP, and CTP) would initiate glucose phosphorylation.

MlcTth is a repressor of its own gene. A closer examination of the genomic surroundings of mlc in T. thermophilus revealed that, unlike the gene encoding MlcEco, which is on its own in E. coli, the gene encoding MlcTth belonged to an operon encoding...
of the protein. One possibility is that, but without inactivating the protein. One possibility is that, 

Figure 5A shows that MlcTth was able to shift DNA upstream activated by glucose. We used EMSAs to test this proposal.

act MlcTth band shifting, since mannose is also bound, albeit weakly, by MlcTth. However, mannose did not counteract DNA shifting by MlcTth. It is not clear why mannose was bound by MlcTth, without inactivating the protein. One possibility is that, by competing with glucose binding, mannose would interfere with glucose-dependent induction.

Identification of the mlc operon encoding a glucose/mannose-specific ABC transporter. To demonstrate that the genes distal to mlc encode an ABC transporter, we cloned the gene encoding the binding protein downstream of mlc, purified the protein to homogeneity, and tested its binding of glucose, mannose, maltose, sucrose, α-methyl glycopyranoside, glucose-6-phosphate, ribose, and fructose. The purified binding protein (N terminally His tagged and purified from pTTC0328) bound glucose and mannose with respective apparent $K_D$ values of 0.67 and 47 μM and an apparent stoichiometry of 1:2 (substrate-polypeptide), whereas no binding activity was detected for any of the other sugars tested (Fig. 6). At present it is unclear why the glucose/mannose-binding protein only showed an approximate stoichiometry of 1:2. It was not possible to test whether all protein molecules were active.

Recently, we became aware of the work H. W. Hellinga and coworkers at Duke University Medical Center, who crystallized a glucose/galactose-binding protein from Thermus thermophilus (4). Therefore, we tested whether the glucose/mannose-binding protein described here was identical to the protein crystallized by Cuneo et al. (4). Indeed, we found that the glucose/mannose-binding protein bound also galactose with the same affinity as glucose, indicating identity of the two binding proteins.

BLAST analysis of the adjacent two open reading frames, i.e., the annotated permeases, revealed homology to membrane components of the ABC transporters. Thus, the operon seemed to encode a standard ABC transporter for the uptake of glucose and mannose in T. thermophilus.

Transport and regulation of glucose and maltose. Glucose transport assays with wild-type T. thermophilus (HB27) demonstrated glucose- and maltose-inducible transport of glucose and maltose (Table 2) when measured at fixed substrate concentrations of 320 nM glucose and 48 nM maltose, respectively. The kinetic analysis of glucose uptake in glucose-grown wild-type cells is shown in Fig. 7A. A kan insertion in mlc of T. thermophilus was isolated (strain CL3) that most likely is polar on the distal genes encoding the glucose/mannose ABC transporter. Transport activity of glucose at a 0.32 μM substrate concentration in this mutant was lower than in the wild type. It was weakly induced by glucose but induced threefold by maltose (Table 2). Considering the polar effect of the kan insertion on the glucose transport genes, we suspected that the remaining glucose transport in the mutant was mediated by another system, most likely the TMSP transporter, as shown below. Kinetic analysis of glucose transport in the mlc::kan mutant grown in the presence of maltose gave a $K_m$ of 1.4 μM and a $V_{\text{max}}$ of 7.6 nmol/min per ml of cells at an OD of 1 (Fig. 7C). Also, as shown in Table 3, mannose no longer inhibited glucose uptake.

We then also measured glucose uptake kinetics in a mutant lacking the TMSP transporter (ΔmalF::bleo). As displayed in Fig. 7B, glucose transport in this mutant showed a $K_m$ of 0.15 μM and a $V_{\text{max}}$ of 4.22 nmol/min per ml cell at an OD of 1 corresponding to a fourfold-higher affinity than that of the cognate glucose/mannose-binding protein for glucose (0.67 μM). The transport kinetics were in accord with Michaelis-Menten kinetics. When $[^{14}C]$glucose transport at 0.1 μM was measured in the presence of 100 μM unlabeled mannose, glucose transport was abolished (data not shown).

We also transferred the malF::bleo mutation from strain JN1 into the T. thermophilus strain CL3 harboring the mlc::kan insertion, yielding strain CL4 harboring both mutations. As expected, we could no longer detect transport of maltose in...
this double mutant. The transport of glucose in the double mutant was still measurable but amounted to only 3.7% of that in the wild type.

We conclude that uptake of glucose in the \textit{malF}::\textit{bleo} mutant represents the activity of the glucose/mannose ABC transporter only. Thus, transport of glucose, as shown in Fig. 7, is mediated by two transporters systems (Fig. 7A), exclusively by the glucose/mannose system (Fig. 7B), and exclusively by the TMSP system (Fig. 7C).

Surprisingly, the presence of glucose in the growth medium also induced the uptake of maltose. This indicated that MlcTth may also be involved in the regulation of the TMSP ABC transporter. However, the \textit{mlc}::\textit{kan} mutant, even though reduced in maltose transport, was still maltose inducible. Thus, MlcTth may stimulate the expression of the TMSP system, but it cannot be its major regulator. MlcTth did shift DNA containing the promoter region of \textit{malE}, the first gene of the TMSP ABC transport gene cluster, a finding consistent with its stimulatory action, but glucose did not interfere (Fig. 8). When both glucose and maltose were present in the medium, transport of either sugar was reproducibly reduced (Table 2). We interpret this finding by MalK1 becoming limiting for transport through either system when both transporters were induced.

As shown below, MalK1 serves the glucose and the TMSP transporters as an ATP-hydrolyzing subunit.

In parallel, we cloned and purified the binding protein from the TMSP transporter to homogeneity (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and assessed its binding affinity for glucose and maltose. Although the protein bound maltose very well with a standard Michaelis-Menten pattern and a \(K_d\) of 0.1 \(\mu\)M (Fig. 9, upper curve) as

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**FIG. 4.** Mlc glucose/mannose transport operon and TMSP ABC transport operon organization in \textit{T. thermophilus}. The TTC numbers refer to the gene numbering in the genome sequencing of \textit{T. thermophilus} \textit{HB27} \((10)\). We propose the names GlcE, GlcF, and GlcG for the glucose/mannose-binding protein and the two membrane components of the glucose/mannose ABC transporter.

**FIG. 5.** EMSA between Mlc\textsubscript{Tth} and the \textit{T. thermophilus} \textit{mlc} promoter. (A) Lanes 1 to 10 represent reactions using 8 ng of labeled DNA amplified from the promoter region of \textit{mlc} (TTC0329) with the following concentrations of wild-type Mlc\textsubscript{Tth}: 0, 0.21, 0.28, 0.56, 0.67, 0.84, 1.11, 1.26, 1.67, and 3.32 \(\mu\)M. (B) Each reaction was done with the same quantity of labeled DNA (8 ng).

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\(6567\)
reported previously (30), the affinity for glucose did not show Michaelis-Menten characteristics but appeared negatively co-operative (Fig. 9, lower curve). We could not reach saturation at a reasonably high concentration of glucose (ca. 100 μM). In order to remove any possible hidden unlabeled ligand, both binding tests were done with the same protein preparation (and the same protein concentration of 5 μM) that had been denatured with 6 M guanidinium chloride, dialyzed, and renatured in dilute buffer. Although this treatment resulted in substantial loss of binding activity (at saturation only 1.6 μM maltose was bound), it did not affect the maltose-binding characteristics (Fig. 9, upper curve). However, it did not alter the peculiar binding pattern for glucose.

**TABLE 2. Transport of glucose and maltose in T. thermophilus**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth conditions</th>
<th>Initial rate of glucose transport (μM)</th>
<th>Initial rate of maltose transport (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>at 0.32 μM [14C]glucose</td>
<td>at 0.048 μM [14C]maltose</td>
</tr>
<tr>
<td>HB27 (wild type)</td>
<td>Glucose</td>
<td>10.23</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>7.01</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>Glucose plus maltose</td>
<td>6.26</td>
<td>0.99</td>
</tr>
<tr>
<td>CL3 (mlc::Kan)</td>
<td>Glucose</td>
<td>0.81</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>1.84</td>
<td>0.91</td>
</tr>
</tbody>
</table>

* Growth conditions were MMA with Casamino Acids (1%) with no addition (−) or with glucose (0.2%) or maltose (0.2%) or both added in the logarithmic phase 6 h prior to harvest.

* The rate of transport is given in nanomoles of substrate taken up per minute per milliliter of culture at an OD600 of 1.
TABLE 3. Inhibition of glucose transport by mannose and maltose

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth condition</th>
<th>Inhibitor concn (μM)</th>
<th>% Remaining transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB27 (wild type)</td>
<td>Glucose</td>
<td>10 (mannose)</td>
<td>24</td>
</tr>
<tr>
<td>CL3 (mlc::Kan)</td>
<td>Maltose</td>
<td>10 (mannose)</td>
<td>90</td>
</tr>
<tr>
<td>CL3 (mlc::Kan)</td>
<td>Maltose</td>
<td>10 (maltose)</td>
<td>3.2</td>
</tr>
<tr>
<td>JN1 (∆malF::Bleo)</td>
<td>Glucose</td>
<td>10 (maltose)</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>50 (maltose)</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>100 (maltose)</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

*That is, compared to glucose transport in the wild-type strain HB27 without inhibitor.

Thus, a hidden unlabeled ligand cannot be the reason for this peculiar binding behavior toward glucose. Inhibition studies of glucose uptake showed that mannose was not transported via the TMSP ABC transporter. As seen in Table 3, mannose inhibited ca. 80% of the glucose transport in the wild-type strain possessing both transporters. In the mlc::kan mutant, mannose only inhibited glucose transport by 10%. In contrast, 100 μM mannose completely inhibited transport of 0.32 μM [14C]glucose in the ∆malF::bleo mutant. Thus, in contrast to glucose, mannose is only transported by the glucose/mannose transporter but not by the TMSP transporter.

MalK1 is the shared ATP-hydrolyzing subunit for both the glucose and the TMSP transporters. We had previously identified MalK1 as the ATP-hydrolyzing subunit of the TMSP ABC transporter. A kan insertion mutant in malK1 had lost the ability to grow on maltose or trehalose (28). We now tested the transport of glucose and maltose in the malK1::kan mutant and found that both activities had been lost completely. Thus, MalK1 is shared by both the glucose and the TMSP transporters.

Band shift analysis showed that MlcTth did not shift the malK1 operator (not shown), meaning that the regulation of malK1 is MlcTth independent.

Quaternary structure of MlcTth. MlcTth cloned as an N-terminally His-tagged version was purified, and its molecular weight was determined in the presence or absence of glucose by molecular sieve chromatography. In both cases, an identical molecular weight of 145,000 at ambient temperature was estimated, indicating a tetrameric quaternary structure (not shown). However, native MlcTth purified by ion-exchange chromatography behaved differently. It showed a strong tendency to multimerize and to precipitate from solutions, preventing a meaningful characterization of its quaternary structure.

**DISCUSSION**

We report here for the first time the presence and function of Mlc in a thermophilic bacterium. Mlc has been widely studied in _E. coli_ and it is well known that its activity as a global repressor for sugar uptake is inhibited by binding to the dephosphorylated state of the membrane-associated EIIB^Glc^ domain of the PtsG protein occurring during the transport of glucose (13, 14, 19, 26, 30). What makes the presence of Mlc in a thermophilic bacterium so special is that thermophilic bacteria do not possess PTS transport systems. Therefore, the inactivation of Mlc in _T. thermophilus_ cannot occur via sequestration to a PTS transporter. Nevertheless, MlcTth did affect the expression of _ptsG_ in _E. coli_, revealing its relatedness to Mlc_Eco. The effect on _ptsG_ expression was weaker and in the same direction as that of Mlc_Eco, even though glucose increased the repression instead of releasing it.

We found that MlcTth is a transcriptional regulator for the glucose/mannose ABC transporter in _T. thermophilus_ and that it is controlled by glucose binding, which affected (reduced) its operator binding. The best evidence for the function as a glucose-specific transcriptional regulator was its ability to shift a DNA fragment containing the upstream regulatory region of the operon harboring the glucose ABC transporter. The presence of glucose counteracts the shift, identifying it as an inducer.

An insertion mutation in _mlc_ did not lead to constitutivity of glucose transport, as expected for a repressor, but resulted in strongly reduced transport activity. This can be explained by the polar effect that the _kan_ insertion in _mlc_ exerts on the downstream glucose transport genes.

Glucose transport remaining in the _mlc_ mutant was due to the action of the TMSP ABC transporter. This could be demonstrated by near-complete inhibition of glucose transport by maltose in the _mlc_ mutant and by the fact that the isolated TMSP-binding protein could also recognize glucose.

The TMSP ABC transporter has been characterized as a constitutive system of high activity (28). Here, we demonstrate that, with Casamino Acids as the major carbon source and maltose or glucose as an additive in the medium, the transport activity of maltose was slightly induced by glucose and three-fold induced by maltose. The expression of maltose transport activity is clearly lower in the _mlc_ mutant than in the wild type, indicating that MlcTth is involved in the regulation of the TMSP ABC transporter genes as well. MlcTth shifted a DNA fragment containing the promoter/operator sequence of the

**FIG. 8. EMSA between MlcTth and the malE1 promoter region.** Lanes 1 to 5 represent reaction mixtures with 12 ng of labeled DNA amplified from the promoter region of _malE1_ and increasing concentrations of wild-type MlcTth. Lanes 1 to 4, 0, 0.84, 1.67, and 3.32 μM, respectively. Lane 5 contained 1.67 μM MlcTth (identical to lane 3) but was done in the presence of 200 μM glucose.
TMSP ABC transporter genes. However, in contrast to its action on the mlc promoter/operator sequence, glucose does not prevent shifting. Thus, if anything, MlcTth has to act as an auxiliary activator of the TMSP system rather than as a repressor, as observed with the glucose/mannose ABC transporter genes.

In contrast, glucose was seen to weakly induce the TMSP ABC transporter, meaning that either the uptake of glucose in the cytoplasm leads to inducer formation for the TMSP ABC transporter genes or the glucose itself is acting as an inducer. Since all substrates of the TMSP ABC transporter contain glucose, the metabolism of these sugars will form free glucose that may act as an inducer.

The characterization of the MlcTth-controlled ABC glucose transporter in a mutant lacking the TMSP system revealed a high affinity ($K_m = 0.15 \mu M$) and a $V_{max}$ of 4.22 nmol/min per 1 ml of cells at an OD of 1, which is sufficient for the maintenance of growth on glucose as the sole source of carbon. The corresponding glucose-binding protein exhibited a $K_D$ for glucose binding of 0.67 $\mu M$, which is somewhat higher than the transport $K_m$. This may well be due to an in vivo stoichiometric excess of binding protein over the membrane components and the exclusive interaction of only the substrate-loaded binding protein with the latter, which is in contrast to the situation of the maltose transporter in E. coli (16). The glucose-binding protein also recognizes $d$-mannose, the 2-epimer of glucose, with a clearly reduced affinity ($K_D = 47 \mu M$). This and the inhibition of glucose transport in the wild type (but not in the mlc mutant) by mannose showed that the glucose transporter of T. thermophilus also accommodates $d$-mannose. A similar overlap of specificities is also seen in the PTS-dependent glucose and mannose transporter of E. coli (8). The overlap between the glucose and the mannose specificity of the cognate-binding protein is reflected in the specificity of Mlc. The $K_D$ for glucose binding was 20 $\mu M$ and for mannose binding was 134 $\mu M$, both with a stoichiometry of 1:1 (polypeptide-substrate). However, whereas glucose interfered with the binding of MlcTth to its operator, mannose did not. Thus, the inducer of the system is only glucose. This is reminiscent of the situation wherein MlcEco regulates both the glucose-specific PtsG (22) and the mannose/glucose-specific PtsM (ManXYZ) (21), but only transport of glucose via PtsG controls the activity of MlcEco (13, 14, 19, 26, 30).

We have demonstrated that glucose can also be taken up by the TMSP ABC transporter. This became clear when we analyzed the remaining glucose transport in the mlc::kan mutant: it was no longer inhibited by mannose but was completely inhibited by maltose. In addition, a strain carrying a mlc::kan and a malF::bleo mutation could no longer transport glucose (3.7% remaining transport). Glucose transport via the TMSP ABC transporter (as determined in the mlc::kan mutant) is somewhat peculiar. Its transport $K_m$ of 1.4 $\mu M$ reflects a reasonably high affinity, but high concentrations of glucose do not completely inhibit maltose or trehalose transport in the wild type (28). The recognition site of the system, the TMSP-binding protein which binds maltose with high affinity (Fig. 9, upper curve) and Michaelis-Menten characteristics, does bind glucose, but its binding isotherm is not of the Michaelis-Menten type. Rather, it indicated that binding of glucose is negatively cooperative.

We observed that mutants lacking MalK1, which had previously been shown to be devoid of the TMSP ABC transport activity (28), had also lost glucose transport via the glucose/mannose ABC transporter. Thus, this ABC subunit must be shared by both systems. One observation is relevant in this respect. When wild-type cells were grown in the presence of both glucose and maltose, the transport activity for either glucose or maltose was significantly less than when the cells were
grown in the presence of either sugar. Possibly, MalK1 (which is not controlled by ThMlc) is constitutively expressed and becomes limiting for transport when both systems are induced. Are there other ABC transporters that would make use of MalK1? Sequence analysis of sugar ABC transporters in T. thermophilus showed seven systems (including the glucose/manose and TMSP systems) without an ATP-hydrolyzing enzyme encoded within their gene clusters. Moreover, there are only two "isolated" genes encoding "sugar ATPases" including MalK1. Therefore, it is not unlikely that MalK1 may serve yet another ABC transporter as an energizing subunit.

Upon examination of the structure of Mlc_Eco, and glucokinase of E. coli, their close structural relatedness becomes apparent (25). It seems reasonable to conclude that Mlc_Eco has evolved from a glucokinase by the acquisition of the DNA-binding domain. Mlc_th has lost the kinase activity but kept glucose binding to control gene expression. Interestingly, Mlcقد does have residual binding affinity for the PtsG of E. coli even though T. thermophilus has not yet acquired the PTS-type transporters. One might speculate that it was only after the appearance of PTS in mesophilic bacteria that PtsG was optimized for the binding of Mlc, which in turn lost its ability to recognize glucose as repressor-controlling principle, replacing it by sequestration to PtsG.

It will be interesting to compare the structure of Mlcقد, to that of Mlc_Eco, with respect to the evolutionary alteration toward the sequestration mode of repressor regulation.

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