Phosphotransferase-mediated Transport of the Osmolyte 2-O-α-Mannosyl-d-glycerate in *Escherichia coli* Occurs by the Product of the *mngA* (*hrsA*) Gene and Is Regulated by the *mngR* (*farR*) Gene Product Acting as Repressor*

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2-O-α-Mannosyl-d-glycerate (MGs) has been recognized as an osmolyte in hyperthermophilic but not mesophilic prokaryotes. We report that MG is taken up and utilized as sole carbon source by *Escherichia coli* K12, strain MC4100. Uptake is mediated by the P-enolpyruvate-dependent phosphotransferase system with the MG-inducible HrsA (now called MngA) protein as its specific EIABC complex. The apparent $K_m$ of MG uptake in induced cells was 10 μM, and the $V_{max}$ was 0.65 nmol/min/10^8 cells. Inverted membrane vesicles harboring plasmid-encoded MngA phosphorylated MG in a P-enolpyruvate-dependent manner. A deletion mutant in *mngA* was devoid of MG transport but is complemented by a plasmid harboring *mngA*. Uptake of MG in MC4100 also caused induction of a regulon specifying the uptake and the metabolism of galactarate and glucarate controlled by the CdaR activator. The ybgG gene (now called *mngB*) encodes a protein with α-mannosidase activity. farR, the gene upstream of *mngA* (now called *mngR*) had previously been characterized as a fatty acyl-responsive regulator; however, deletion of *mngR* resulted in the up-regulation of only two genes, *mngA* and *mngB*. The *mngR* deletion caused constitutive MG transport that became MG-inducible after transformation with plasmid expressed *mngR*. Thus, MngR is the regulator (repressor) of the MG transport/metabolism system. Thus, the *mngR mngA mngB* gene cluster encodes an MG utilizing system.

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2-O-α-Mannosyl-d-glycerate (MG) is a widespread compatible solute of thermophilic or hyperthermophilic bacteria and archaea (1) but not of mesophilic prokaryotes. MG has otherwise only been encountered in the red algae of the order Ceramiales (2) where its function remains unclear. The highly preferential distribution of MG among prokaryotes requiring high temperatures for growth led to the hypothesis that it could play a role in the thermoprotection of cell components in vivo. At least in vitro, MG has been shown to be efficient in the protection of some enzymes against thermal inactivation (3, 4). Recently, we described a method to synthesize radio-labeled MG of high specific activity from radiolabeled mannose by the use of a genetically engineered strain of *Escherichia coli* (5). This prompted us to use this compound to study the potential uptake of MG in bacteria and archaea. Here, we report that carrier-mediated uptake of MG in *E. coli* occurs by the P-enolpyruvate-dependent phosphotransferase system (PTS) with the HrsA (now renamed MngA) protein as a specific EI complex substrate recognition site. So far, *mngA* has not been assigned to the uptake of a particular PTS sugar. However, the gene was recognized during a study of temperature regulation of the outer membrane porin OmpC (6).

Another line of investigation that contributed to the present report is the regulation of fatty acid metabolism gene expression in *E. coli*. The FadR regulator has a dual function in repressing the enzymes of fatty acid β-oxidation and activating the transcription of the *fabA* and *fabB* genes of fatty acid biosynthesis (7, 8). FabR is a repressor that acts downstream of FadR in the transcriptional regulation of the *fabA* and *fabB* genes (9). The existence of a third fatty acid-responsive transcription regulator was proposed by Quail et al. (10), who described a transcription factor adjacent to the citric acid gene cluster that was released from its DNA-binding site by either fatty acid or acyl-CoA. This gene was named *farR* (fatty acyl-responsive regulator). Here, we report that FarR (now renamed *MngR*) has no role in fatty acid metabolism but rather functions as a repressor for the adjacent and divergently oriented genes *mngA* and *mngB*.

† The abbreviations used are: MG, 2-O-α-mannosyl-d-glycerate; IPTG, isopropyl-β-D-thiogalactopyranoside; PTS, P-enolpyruvate-dependent phosphotransferase system; MMA, minimal medium A; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; -P, phosphate.

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Transport of 14C-MG—Bacterial strains (Table I) were grown either in NZA medium (10 g of NZ amine (Sheffield Products Inc.) and 5 g of yeast extract/liter) or in minimal medium A (MMA) (11) with 0.4% glycerol as a carbon source in the presence or absence of 0.1% MG. After overnight growth the cultures were washed three times in MMA with/without carbon source and resuspended to an optical density of 0.8 at 420 nm. The initial rate of uptake was determined in a scintillation counter. The initial rate of uptake was extrapolated. The rate of transport in the standard assay (0.1 mgR strain) was determined in a scintillation counter. The initial rate of uptake was referred to in Fig. 1A. 

**MATERIALS AND METHODS**

Transport of 14C-MG—Bacterial strains (Table I) were grown either in NZA medium (10 g of NZ amine (Sheffield Products Inc.) and 5 g of yeast extract/liter) or in minimal medium A (MMA) (11) with 0.4% glycerol as a carbon source in the presence or absence of 0.1% MG. After overnight growth the cultures were washed three times in MMA with/without carbon source and resuspended to an optical density of 0.8 at 420 nm. The initial rate of uptake was determined in a scintillation counter. The initial rate of uptake was extrapolated. The rate of transport in the standard assay (0.1 mgR strain) was determined in a scintillation counter. The initial rate of uptake was referred to in Fig. 1A. 

**TABLE I**

| Strain or plasmid | Known genotype | Origin or reference |
|-------------------|----------------|---------------------|
| Bl21              | E. coli B F−,ompT hsdS (rB mB) gal dcm | Ref. 32            |
| D350              | ΔlacU169 gal4901::cl 857 (Δcro-broA) | Ref. 14            |
| E222              | MC1400 mngB::xam | This study          |
| E223              | MC1400 mngB::xam | This study          |
| J177              | MC1400 edaR::Tn5 | Ref. 23            |
| KM522             | MC1400 malK-lacZ otsA::Tn10 | otsA::Tn10 allele obtained from A. Strain (26) |
| Ljy1              | PDJ1 Δ(mngR)::xam | This study          |
| Ljy2              | PDJ1 Δ(mngR::mngB)::xam | This study          |
| MC4100            | F' araD139 ΔargF-lacU169 deoC1 (ff85301 pTsF25 rbsR relA1 rpsL50) | Ref. 33            |
| MC4100 guaP-lacZ  | MC1400 guaP-lacZ; needs tryptophan for growth | Ref. 23            |
| MC4100 gard-lacZ  | MC1400 gard-lacZ; needs tryptophan for growth | Ref. 23            |
| MC4100 gardP-lacZ | MC1400 gardP-lacZ; needs tryptophan for growth | Ref. 23            |
| Mg1655            | Sequenced E. coli wild type strain | Ref. 34            |
| Ms0112            | MC1400 ΔpufB::Tn5 | This study          |
| Ms0112            | MC1400 Δerr::Tn5 | This study          |
| Ms0112            | MC1400 Δerr::Tn5 | This study          |
| P1D1              | F' metB1 relA1 apoT1 gya216 recD::Tn10 λ- λR | Ref. 9             |
| Rd25              | MC1400 ΔmngA::kan | This study          |
| Rd26              | MC1400 ΔmngR::xam; allele from LYJ1 | This study          |
| X1L-Blue          | Δ(mnga)183 Δ(mgcB::hisdSMR-nrr::175 endA1 supE44 thi-1 recA1 gya216) relA1-lacF (pproAB-lacI) (ZM15 Tn10(TcrD) amy CamR) | Stratagene         |

**Plasmids and phage**

- pAC391: pTre99b mngA
- pAC392: pTre99b mngR
- pET17b: mngR
- pET18b harboring mngR
- pTrec99b: Amp' lacI
- pUC18: Amp'
- ANK1324: Harboring mini cam cassette and transposase

**Southern blots**

Southern blots were performed on genomic DNA isolated from both strain PDJ1 and strain LYJ1 (ΔmngR) by the phenol/chloroform/isoamyl alcohol extraction method (12). Two different restriction enzymes, BglII and PvuII, were used to digest 5 μg of genomic DNA. The digested genomic DNA was separated using a 0.8% agarose gel. The probe was the 320-bp NruI-PvuII fragment from wild type cells (Fig. 1A). The bands were detected with a Molecular Dynamics Storm 860 imager, and their intensity was determined using the ImageQuant 5.1 program. The results from Southern blot analysis with the 320-bp NruI-PvuII fragment corroborated the PCR results, confirming that the chloramphenicol-resistant gene for mngR was inserted in the correct location without compromising the neighboring genes. The probe hybridized with a 2.9-kb fragment of wild type genomic DNA digested with BglII (Fig. 1C, lane 1), whereas the mutant had a 3.2-kb fragment (Fig. 1C, lane 2). The probe also recognized bands of the correct size in the PvuII digests (Fig. 1C). In the case of the ΔmngR strain, the fragments were larger by 650 bp because of the loss of the PvuII digestion site upon insertion of the chloramphenicol gene. The mngR deletion in LYJ1 was transduced into MC4100 by P1vir-mediated transduction selecting for chloramphenicol resistance yielding strain RD26.

**Construction of Strain LYJ2**

Construction of Strain LYJ2 was made using the same approach as used for the mngR knockout construct (Fig. 1A). The 846-bp fragment downstream of mngB was amplified by PCR with primers P5 (5'-CGCGGATCCATGCTGATGCTG-3') and P6 (5'-CGCGGATCCATGCTGATGCTG-3'), the BamHI site is underlined. The

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PCR product was ligated to PCR 2.1 vector to generate PCRmngB and sequenced to be right in the Hartwell Center with ABI Prism 3700 DNA analyzer. This plasmid PCRmngB and plasmid pUCmngRC (mngR deletion construct) were double-digested by SacI and BamHI, and the released 846-bp fragment from PCRmngB was cloned into the SacI-BamHI site of pUCmngRC to yield a triple knockout construct (pUC-triC) that replaced mngR, mngA, and mngB with the chloramphenicol resistance gene. This plasmid was digested with AatII and HindIII. The 3.0-kb fragment containing both PCR fragments, the chloramphenicol gene replacing the three genes and a 350-bp fragment of pUC19, was purified from a 0.8% agarose gel. The linear DNA (25 ng) was transformed into strain PDJ1 (recD::Tn10) by electroporation, and chloramphenicol-resistant transformants were isolated. Two pairs of primers, pair P3 and PL and pair P4 and PR, were used to amplify the genomic region of the isolated strain and sent to sequence to confirm that mngR, mngA, and mngB were successfully deleted.

Construction of Strain RD25 Δ(mngA)—We used the technique of Datsenko and Wanner (13). Plasmid pKD13 harboring a kanamycin-
resistant cassette was used as template for creating a PCR product encompassing the kanamycin cassette equipped with the DNA sequence 3’ and 5’ adjacent to the mngA gene. Strain DY330 (14) was transformed with this linear and purified DNA fragment selecting recombinants that were resistant against kanamycin. The successful deletion of mngA was verified by PCR using one primer initiating within the kanamycin cassette and the other from the neighboring mngB gene (Fig. 1A). The deletion was transduced by P1vir transduction (selection for kanamycin resistance) into MC4100, yielding strain RD25.

**Minni-transposon Mutagenesis—**MC4100 (gudP-lacZ) cells were grown at 37 °C overnight in 10 ml of trypton broth (11) containing 0.2% maltose and 10 mM MgSO4. They were washed and resuspended in 1 ml of trypton broth (11) and 20 μl of cells were transferred and plated with phase microscopy (harboring a chloramphenicol insertion together with the gene for the transposase) for 15 min at room temperature and for a further 15 min at 37 °C (15). JKNI324 can only be multiplied in an amber suppressor strain (XLI from Stragateng was used). The multiplicity of infection was kept between 0.1 and 1 phage/cell. The cells were washed twice in 5 ml of LB containing 50 mM sodium citrate and were allowed to grow for 1 h at 37 °C. 100-μl samples were plated on LB containing sodium acetate (20 mM), chloramphenicol (15 μg/ml), XGal (40 μg/ml), and MG (0.5 mM) to grow overnight at 39 °C. Approximately 10,000 colonies were screened for light blue color. After one time purification of 40 such colonies, they were tested for the ability to take up [14C]MG. Two were found; one (2) was selected for purification and the other (3) for sequencing (Fig. 1C). Approximately 10,000 colonies were tested. One was selected for the appropriate chloramphenicol resistance and thus grown on LB agar containing 20 μg/ml chloramphenicol. Two colonies were grown from both plates at the next step.

**Sequence Fragmenting—**Chromosomal DNA (1–2 μg) of the mutants was digested by Sau3A (2 units) (New England Biolabs), and DNA fragments were separated on a 1% agarose gel (SeaKem). DNA fragments between 1000 and 2000 base pairs were cut out of the DNA gel and extracted (Qiagen). The DNA concentration was determined by UV spectrophotometry. The fragments (diluted to a concentration of 0.3 μg/ml in ligation buffer) were circularized overnight by T4 ligase (1 unit) (New England Biolabs) at 16 °C. DNA was then treated by an equal volume of phenol/chloroform. The upper phase was removed, DNA was precipitated with 20% sodium acetate and 2-fold volume of 100% ethanol and collected by centrifugation. After washing the DNA with 70% ethanol, PCR was performed with 0.2 μg of circularized DNA in the presence of 0.2 mM dNTPs and 5 pmol of each primer, ah1 (5’-GATTTCCTACAAAATCATTAGGGAATC-3’) and ah2 (5’-CATTAAGTTAAGGTGGATACACATCT-3’) (16, 17). These primers are complementary to the 3’ and 5’ ends of the insert and permit direct elongation into the adjacent chromosomal DNA. We used an initial denaturation step at 95 °C for 4 min followed by 35 cycles comprising denaturation at 95 °C for 30 s, primer annealing at 56 °C for 1.5 min, and extension by Taq polymerase (Eppendorf) at 72 °C for 1 min. The resulting PCR product was electrophoresed through a 0.8% agarose gel, eluted out, purified (Qiagen), and sequenced using the primers ah1 and ah2 (GATC Biotech AG) (Fig. 1A).

**Construction of pACS32 Harboring mngA under lac Promoter Control—**Plasmid pACS31 harboring mngA under an IPTG-inducible promoter was cloned using pTK18 (6) as template for PCR. mngA was amplified with two flanking primers containing a BamHI cleavage site at the 5’ end (underlined) and a HindIII cleavage site (underlined) at the 3’ end. The first primer was 5’-AAAAAGATATATGTAGTATTTATGTCGGGGC-3’, and the second primer was 5’-AAAAACGTATTTAGGATTTATGCCACCAATCTT-3’. The PCR product was digested with BamHI and HindIII and ligated into pTrc99B (18) that had been opened with the same two restriction endonucleases.

**Construction of pACS29 Harboring mngR under lac Promoter Control—**mngR was amplified by PCR using chromosomal DNA of MC4100 as template and flanking primers containing a BamHI cleavage site and buffer B (10 mM Tris-HCl, pH 7.6, 10–100 mM P-enolpyruvate, 50 mM NaCl and broken in a French pressure cell at 16,000 p.s.i. The extract was shortly centrifuged to remove unbroken cells. The resulting supernatant was ultracentrifugated (90,000 × g for 60 min at 4 °C) to separate the membranes from the cytoplasm. The membranes and crude extract were dialyzed separately overnight at 4 °C against 10 mM Tris-HCl, pH 7.6.

The phosphorylation assay contained 12 μl of an equal mixture of buffer A (10 mM Tris-HCl, pH 7.6, 25 mM dithiothreitol, 125 mM NaF) and buffer B (10 mM Tris-HCl, pH 7.6, 10–100 mM P-enolpyruvate, 50 mM NaCl, 15 μl of crude extract (30 μg protein), and 3 μl of membrane suspension (3–4 μg of protein). The reaction was started by the addition of 5 μl of [14C]Mg (0.025 μCi; 296 mCi/mmol) (5). 10-μl samples were removed at different time intervals and spotted on a TLC (silica) plate. Chromatograms were developed with a solvent system composed of n-propanol/25% ammonia (1:1, v/v). TLC plates were autoradiographed for 16 h at −80 °C.

The control assays were done with membranes from uninduced MC4100 not harboring mngA. When the dependence of P-enolpyruvate was measured, unlabeled MG (100 μM) was added to the complete assay mixture 10 min before the addition of [14C]Mg.
To ensure that the product formed was MG-P, in one assay before spotting the samples onto the TLC plate, the proteins were precipitated by adding 5 μl of 12% trichloroacetic acid to the 10-μl samples. The mixture was kept on ice for 15 min and centrifuged for 2 min at 20,000 x g. The acidic supernatant was neutralized by adding concentrated KOH. After reaching neutrality, 1 unit of alkaline phosphatase was added, and the samples were incubated for 30 min at 37 °C. After the treatment, the samples were spotted on the TLC plate, and the chromatograms were developed using the same solvent system.

Growth Curves—Strain KMS22 is defective in the synthesis of internal trehalose and is therefore highly sensitive to salt. It was grown in MMA and 0.4% glycerol as carbon source. It was diluted 1:100 into fresh medium, one with and one without 0.5 mM MG. After 6 h the cultures were split, and 500 m M salt was added to one portion. The optical density at 600 nm was followed.

**RESULTS**

**Transport of MG in E. coli K12 Strain MC4100**—We tested several bacteria for their ability to use MG as the sole source of carbon using agar plates as well as liquid cultures with MMA as growth medium (Table II). We found that E. coli BL21 and E. coli K12, strain MC4100 were able to grow, whereas Salmonella enterica serovar Typhimurium LT2 and many others were not.

MC4100 was grown in liquid culture overnight in MMA with glycerol as a carbon source and 0.1% MG as potential inducer of MG transport activity. After washing, MC4100 that had been grown in the presence of MG showed time-dependent uptake of radioactively labeled MG at a concentration of 0.1 μM (Fig. 2A). This uptake in the presence of increasing concentrations of unlabeled MG, we found that the transport rate was half-maximal at 10 μM MG, and the maximal rate of transport (Vmax) was 0.65 nmol of MG/min/10^9 cells taken up (equivalent to 150 μg of protein) at room temperature (Fig. 2B). Transport activity was fully inducible by MG in the medium by as little as 0.1 mM, but it was not inducible by 300 mM sodium chloride (Table III; see also Table VI). In contrast, the presence of 300 mM salt during growth reduced the induction by MG by ~50%. Thus, uptake of MG is not a response to salt stress as would be expected if MG was used as an osmolyte, similar to, for instance, the uptake of glycine betaine in E. coli (20). E. coli BL21 showed similar transport activity after growth in the presence of MG (see Table VI), at least at 0.1 mM substrate concentration.

**Uptake of MG Shows the Characteristics of a PTS**—Isogenic derivatives of MC4100 were constructed lacking the general PTS protein EI as well as EIIGlc (21, 22). To reduce effects caused by catabolite repression in these mutants, 1 mM cAMP was added to the growth medium. Although the EI mutant failed to transport MG, the EIIGlc mutant still showed inducible MG transport, albeit at reduced levels (Table III). This indicated that MG transport was mediated via a PTS but was most likely independent of EIIGlc. We observed weak inhibition with the PTS sugars glucose, mannose, and mannitol, less inhibition with trehalose, and no inhibition by fructose. No inhibition was observed with the non-PTS compounds maltose, arabinose, gluconate, glycerate, 1-α-glucosylglycerol, 2-α-glu-cosylglycerate, and 2-α-mannosylglycerol.

The Search for the MG Transporter—Strain MC4100 was grown in MMA with glycerol as a carbon source and in the presence and absence of 0.1% MG for the induction of the MG transporter. Messenger RNA was isolated from logarithmic cultures and used to hybridize a microarray chip charged with E. coli genes obtained from the sequenced strain E. coli K12 MG1655. Because transport activity for MG is induced by more than 10-fold, we initially considered only genes in the microarray analysis that were induced by more than 10-fold. In addition, we only considered those genes that showed significant and reliable chip responses under both growth conditions. Using these criteria, 6 of the 4288 genes present on the chip were induced 12–32-fold in the presence of MG. Together with additional genes that are induced more than 2-fold, they are listed in Table IV. Five of the more than 10-fold induced genes belong to a regulon that is controlled by the cdaR gene product acting as an activator (23). Interestingly, cdaR itself is also induced 3-fold by MG consistent with the proposal that cdaR in the presence of inducer is positively regulated by its own product (23). The CdaR regulon comprises two operons and one gene that are geared for the uptake and metabolism of galactarate and glucarate (Fig. 3A). The regulon is known to be induced by glycerate, galactarate, and glucarate. All of them contain the α-glycero moiety as part of their structure. LacZ reporter gene fusions are available for garD, garP, and gudP, three genes of the CdaR regulon (23). They are weakly but consistently induced by MG (Table V). Considering the partial structural identity of the inducers of CdaR with MG (Fig. 3B), the induction of the CdaR regulon by MG was not surprising. The question arose of whether the glucarate or the galactarate transporter, encoded by garP and gudP, respectively, were identical to the MG transporter. A kanamycin resistance insertion mutation in cdaR (strain JA177) (23) was available leading to the loss of gar and gud gene expression. This mutant was tested for inducible MG uptake, which was found to be unimpaired. Thus, neither gudP nor garP could encode the MG transporter.

hrA, the sixth gene identified in the microarray analysis as a strongly induced gene, was not part of the CdaR regulon (Table IV). It had been previously identified as a gene complementing the defective temperature-regulated expression of ompC in a micF deletion (6). We obtained hrA cloned under its natural promoter from Dr. Utsumi (plasmid pTK18). pTK18 still contained the upstream gene farR but was deleted for the downstream gene ybgG (Fig. 4A). When this plasmid was transformed into the wild type strain, high but still inducible MG transport was observed (Table VI). We also cloned the hrA under an MG-independent but IPTG-inducible promoter (vector pTrc99b) (Fig. 4A). This plasmid, pACS31, exhibited constitutive transport activity as seen below (Table VI).

We used the technique of Datsenko and Wanner (13) in combination with the method of Yu et al. (14) to construct a complete deletion of the mngA gene replacing it with a kanamycin resistance cassette (Fig. 1A). The deletion was transduced by P1vir transduction into MC4100 yielding strain RD25. This strain was tested for its ability to transport MG after growth in the presence of MG. Table VI shows that this mutant lost the ability to transport MG but could be complemented for this activity after transformation with the plasmid.
harboring the IPTG-inducible mngA gene. RD25 was also unable to grow on MG as the only carbon source (Table II). Thus, hrsA encoded the transporter for MG and will now be called mngA.

MngA-containing Membranes Are Able to Phosphorylate MG Depending on P-enolpyruvate and the General PTS Proteins Enzyme I and HPr—Inverted and washed membrane vesicles of strain MC 4100 containing the plasmid harboring the IPTG-inducible mngA gene were incubated with dialyzed cellular extracts of strain MC4100 containing EI, HPr, and EIIa Glc of the PTS. 14C-Labeled MG was transformed by this mixture into another compound as seen by TLC analysis (Fig. 5, lanes 1–3). The membranes of MC4100 without the mngA-harboring plasmid showed the same activity in this test, but only when the strain was induced with MG during growth prior to the isolation of the membranes (not shown). When the assay mixture was treated with alkaline phosphatase, MG was dephosphorylated, identifying the original product as MG-P. Surprisingly, complete dephosphorylation of MG-P by alkaline phosphatase was only achieved after trichloroacetic acid precipitation of the involved macromolecules. Because the phosphorylated product was not altered by trichloroacetic acid, we interpret this phenomenon as protein-mediated sequestration of MG-P from alkaline phosphatase (Fig. 5, lanes 4–6). The dependence of P-enolpyruvate in the MngA-dependent phosphorylation reaction was initially difficult to demonstrate. Apparently, the washed membranes or the dialyzed extracts still contained a limited amount of phosphorylated PTS proteins (EI-P or HPr-P) to partially phosphorylate MG in the absence of added P-enolpyruvate. Only the addition of 100 μM unlabeled MG to the incubation mixture (10 min prior to the addition of 14C-labeled MG) dephosphorylated the endogenous phosphoryl groups and allowed us to demonstrate the subsequent dependence of P-enolpyruvate in the [14C]MG phosphorylation reaction (Fig. 5, B and C). When [14C]MG-P was incubated with 2-a-d-mannosyl-3-phosphorylglycerate phosphatase, an enzyme involved in the alternate synthesis of mannosylglycerate removing the phosphoryl group from the 3-position of glyceral in mannosylglycerate-phosphate (24), MG-P was not altered as demonstrated by TLC analysis (data not shown). This made it unlikely that the phosphoryl group in MG-P was positioned in the glyceral moiety.

FarR (MngR) Is the Repressor for mngA—To test whether or not mngA is controlled by farR, the gene product of the upstream gene, we constructed a deletion in farR (Fig. 1A). Total RNA was isolated from exponentially growing cells, both wild type and ΔfarR, using MasterPure RNA purification kit (Epigen Technologies). The samples were subjected to analysis using the Affymetrix E. coli gene chip array. Our analysis of four sets of global gene expression profiles comparing mRNA levels in strain LYJ1 (ΔfarR) with the wild type were made, and the expressions of only two genes were significantly up-regulated by the absence of farR. The expression level of mngA and ybgG changed 9- and 23-fold, respectively. Therefore FarR must be the repressor for mngA and is now called MngR.

The mngR deletion of LYJ1 was transduced into MC4100 (yielding strain RD26). RD26 grew well on MG as the only

| Strain | Relevant genotype | MG (0.5 mM) in the growth medium (+/−) | Rate of uptake (pmol/min/10⁹ cells) at 5.1 μM |
|--------|------------------|----------------------------------------|---------------------------------------------|
| MC4100 | wild type        | −                                       | 0.22                                        |
|        | +                | +                                       | 6.12                                        |
| MS0112 | MC4100 ΔptsIH    | −                                       | <0.10                                       |
|        | +                | +                                       | <0.10                                       |
| MS2112 | MC4100 crr       | −                                       | <0.10                                       |
|        | +                | +                                       | 1.84                                        |

TABLE III
Transport of MG in EI and EIIA mutants and in the presence of different sugars

Fig. 2. Transport of [14C]MG by MG-induced cells of MC4100. Cells had been grown in MMA and glycerol as carbon source in the presence of 0.5 mM MG. Uptake of [14C]MG was measured in MMA in the absence of a carbon source. A, time-dependent accumulation of [14C]MG at an initial external [14C]MG concentration of 0.1 μM. The data are given in pmol of MG/10⁹ cells taken up. B, rate of [14C]MG uptake at different external [14C]MG concentrations. The data are given in nmol of MG/min/10⁹ cells taken up. From these data, a Kₘ of 10 μM and a Vₘₐₓ of 0.65 nmol/min/10⁹ cells was estimated.
carbon source without the lag phase observed with MC4100. As shown in Table VI, RD26 exhibited high constitutive transport activity for MG that again became MG-inducible after transformation with the mngR-harboring plasmid pACS32. This demonstrated that MngR acts as a transcriptional repressor for mngA.

DNase I footprint analysis showed that MngR protected two AT-rich sites upstream of mngR (10). The two genes just downstream of mngR in the E. coli chromosome genetic map are mngA and mngB. The binding sites of MngR are located in the same intergenic region (Fig. 4B). One of these sites is the mngR promoter, and the other is located in the putative promoter region of the mngA-mngB operon, suggesting how MngR can regulate the expression level of mngA and of itself.

The Function of the YbgG (MngB) Protein—Before conducting the microarray analysis as described above that lead to the identification of the MG transporter, we had attempted to isolate chloramphenicol insertions mutations in the elusive MG transporter gene. The observation that MG induced the CdaR-dependent gadP-lacZ fusion was used to screen a collection of random chloramphenicol resistant insertions for their loss of gadP-lacZ inducibility by MG (light blue colonies on X-gal plates containing MG). The rationale of this experiment was that transport of MG was needed to induce the gadP-lacZ fusion, and mutations in the elusive gene for the MG transporter would result in a white or light blue colony phenotype. Of approximately 10,000 independent chloramphenicol-resistant insertions, 44 were found to be light blue on X-gal plates. Of these, two mutants (yielding ET222 and ET223 after the transduction of the mutation into MC4100) were -3 to 4-fold reduced in MG-induced MG uptake in comparison with the parent strain (data not shown). However, the sequence of the gene in which one of these insertions had occurred (ET223) was not the mngA but was found to be identical with the ybgG, located immediately downstream to mngA (Fig. 1A). PCR analysis with ET222 showed that the second cam insertion was also within the ybgG gene. As seen in Table IV, the microarray analysis showed that mngR, mngA, and ybgG were induced by MG 2.2-, 16.4-, and 5.7-fold, respectively. The three genes form a cluster with mngA and ybgG in one putative transcriptional unit, and mngR oriented divergently (Fig. 4A).

When transformed with the MngA-encoding plasmid pASC31, the ybgG mutant strains (ET222 or ET223) showed again high transport in the absence of inducer. However, the presence of MG in the growth medium at concentrations higher than 30 μM prevented growth, indicating the toxicity of the accumulated MG-P in the absence of the ybgG gene product.

Bioinformatic analysis of the YbgG protein sequence showed that it belongs to the α-mannosidase subgroup of the glycohydrolase superfamily (Pfam0101074). Deletion mutants were constructed in which mngR was deleted (strain LYJ1) and in which the entire gene cluster mngR mngA ybgG was deleted (strain LYJ2). These strains, together with the corresponding wild type strain (PDJ1), were tested for α-mannosidase activity (after growth in minimal medium with succinate as carbon source) in an in vivo assay using p-nitrophenyl-α-mannoside with intact cells. The results are shown in Fig. 6. There was high α-mannosidase activity in strain LYJ1 (ΔmngR), whereas only a trace of activity was detected in strain PDJ1 (wild type), and no activity was detected in strain LYJ2 (ΔmngR-αmngB). It will now be called MngB. It has to be kept in mind that the in vivo test does not differentiate between p-nitrophenyl-α-mannoside and p-nitrophenyl-α-mannoside-6-P hydrolysis. The latter could arise internally by the MngA-mediated uptake and phosphorylation of p-nitrophenyl-α-mannoside.

The Phenotype of a mngB::cam Mutation in a mngA plus Genetic Background—Initially, we had concluded that the product of mngB would influence induction of the mngA mngB operon, because the isolation of mngB::cam insertions in the gadP-lacZ reporter strain showed not only reduced β-galactosidase activity but also reduced MG transport after induction with MG. However, after realizing that MG in the medium caused the cessation of growth in a mngB::cam strain harboring an intact MngA protein, it became clear that these strains can only grow on MG-containing plates when the MngA is reduced in its activity by secondary mutations in mngA. In-
When the mngB::cam carrying mutants transformed with the mngA harboring plasmid were induced with sublethal concentration of MG in the medium, the resulting MG transport activity of the outgrown culture varied from experiment to experiment and was usually lower than after growth in the absence of MG, indicating the outgrowth of mngA mutants (data not shown).

It is clear that MngB is not an enzyme that is needed for inducer production of the mngA mngB operon. mngB::cam-harboring strains, even though reduced in the capacity to transport MG, are still induced by MG in the medium (Table VI). A wild type strain is not induced for MG transport by mannose in the medium. Mannose is transported in E. coli by the mannose PTS and appears in the cytoplasm as mannose-6-P (25). This compound is the expected product of MG-P hydrolysis by MngB, but it is apparently not an inducer of the mngA mngB operon. Thus, the MngB enzyme is likely to remove the inducer rather than to produce it.

**Cellular Response to the Transport of MG**—The finding that MngA that previously had been recognized as a heat-responsive element (HrsA) for the expression of the osmo-controlled outer membrane porins OmpC and OmpF transports MG made it likely that inversely, the transport of MG should have an effect on porin expression. We made an effort to find an effect of MG transport either by chromosomally encoded MngA or by plasmid-encoded MngA (pACS31) on the expression of transcriptional lacZ fusions to ompF and ompC. Surprisingly, this analysis did not reveal any connection of MG transport to the transcriptional (NaCl- or temperature-controlled) regulation of ompF or ompC (data not shown). However, when IPTG-inducible and plasmid-encoded MngA (by plasmid pACS31) was present in any strain, even in an mngB plus strain, MG in the medium above 30 μM prevented the growth of the culture.
again indicating the toxicity of the accumulated MG-P. This toxicity by MG was not seen in MC4100 harboring a single chromosomal copy of the mngR mngA mngB gene cluster. In contrast, the growth yield of a glycerol-grown culture was reproducibly 10–15% higher when it was grown on the presence of 0.1% MG. This indicated that the amount of MngA has to be balanced by the equivalent amount of MngB for effective "de-toxification" of MG-P, most likely by hydrolyzing MG-P to mannose-6-P and glycerate. MG toxicity was not recognized in wild type strains transformed with plasmid pTK18 harboring mngA and mngR both under their own promoters, indicating that the expression of mngA in this plasmid was sufficiently curbed by the mngR gene product to handle MG-P hydrolysis.

We also performed microarray analysis by RNA hybridization to E. coli K12 genes with MngA as the differentiating factor. The two sets of RNA preparations were isolated from a derivative of MC4100 that was grown in MMA with glycerol as carbon source and in the presence and absence of the mngA harboring plasmid (pACS31). Although the data for genes induced by mngA were too marginal to be significant, we found that six genes were repressed more that 5-fold in the presence of MngA. They are listed in Table VII. The connection of these
genes encoding only unknown hypothetical proteins to regulation of porin expression, if any, is not obvious. Two of these genes (rzpR and yncH) were also seen to be slightly repressed in the microarray analysis using the presence of MG as an inducer for a differentiation criterium (data not shown). In neither of these analyses was the expression of ompF or ompC altered to any significant extent.

**MG Transport Does Not Provide Protection against Salt Stress in E. coli**—Because MG has been recognized as an osmoprotective substance at high temperature and salinity. Even though the apparent $K_m$ of uptake in MC4100 is in the range of classical sugar PTSs, its $V_{max}$ of 0.65 nmol/min/10^9 cells appears rather low. One might also argue that MG is not the natural substrate of the MngA/MngB system. However, neither mannose, 1-β-D-mannopyranosylglycerol, 2-α-D-mannopyranosylglycerate, nor 2-β-D-mannopyranosylglycerol, even at 1 mM concentration, significantly inhibited the uptake of 0.1 mM [14C]MG, rendering the uptake of MG by MngA rather specific. The fact that a PTS is transporting a negatively charged compound is intriguing. If transport of MG is indeed electrogenic, it will increase the membrane potential and might disturb the energy balance of the cell.

**DISCUSSION**

We report the PTS-dependent transport of MG, a compound that is used by hyperthermophilic prokaryotes as a protective osmolyte at high temperature and salinity. Even though the apparent $K_m$ of uptake in MC4100 is in the range of classical sugar PTSs, its $V_{max}$ of 0.65 nmol/min/10^9 cells appears rather low. One might also argue that MG is not the natural substrate of the MngA/MngB system. However, neither mannose, 1-α-D-glucosylglycerol, 2-α-D-glucosylglycerate, nor 2-α-D-mannosylglycerol, even at 1 mM concentration, significantly inhibited the uptake of 0.1 μM [14C]MG, rendering the uptake of MG by MngA rather specific. The fact that a PTS is transporting a negatively charged compound is intriguing. If transport of MG is indeed electrogenic, it will increase the membrane potential and might disturb the energy balance of the cell.

The evidence for MngA being the PTS EII complex recognizing MG is based on the observations that MG induces the expression of mngA, that a deletion in mngA no longer transports MG, and that plasmid-encoded and MG-independent expression of MngA allows transport of MG in cells that had not been induced by MG. Moreover, membranes harboring MngA were able to phosphorylate MG in a P-enolpyruvate and PTS protein-dependent manner. The signature of MngA is clearly that of a PTS. Its sequence reveals the presence of a composite PTS EII complex consisting of covalently linked EIIA, IIB, and IIC domains with correctly conserved His and Cys residues. Thus, MngA has all the capability to function as a PTS transporter. From its sequence, MngA (HrsA and Frx) has been grouped with fructose like PTS transporters (22).

**mngA** is induced in the presence of MG in the medium. Thus, one would conclude that MG-P is the inducer of mngA. One might also argue that the function of MngB is that of a MG-P
vitro experiments showing that MngR was released from its response to fatty acids (10). This conclusion was based on posed to be involved in the regulation of the citric acid cycle in product acting as a repressor. MngR had previously been pro-

It is likely that mesophilic bacteria will transport MG preferably by MngA-like PTS transporters that generate intracellular MG-P. This molecule, like many other phosphorylated sugars, is toxic to the cell (30, 31), and bacteria will use exogenous MG as a carbon source (after hydrolysis to mannose-6-P and glycerolate) but not as a substance taken up in unmodified form to function as an osmolyte. Indeed, the presence of MG in the growth medium does not provide protection against salt stress in E. coli, excluding MG-P as an effective osmolyte. So far, archaea have not been reported to possess PTS, and the transport of MG has not been studied in these organisms. If there is transport of MG in archaea, it would be expected to be of the proton motive force or of the ATP-binding cassette type, leading to the accumulation of unmodified MG. This observation must be related to the finding that MG is used as an osmolyte preferentially by hyperthermophilic archaea but not by mesophilic bacteria.

Analyzing the genome of sequenced enteric bacteria we found that the mngR mngA mngB gene cluster has been exactly deleted in E. coli O157:H7, S. enterica serovar Typhimurium LT2, Vibrio cholerae, Yersinia pestis, and Klebsiella pneumoniae. In these bacteria, the genes adjacent to the mngA mngB cluster in E. coli, namely sucD on one side and cydA on the other side, are perfectly preserved. On the other hand, Shigella flexneri, like E. coli K12, does contain the mngR mngA mngB cluster at the identical position, indicating a common function.

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