Synthesis of GDP-Mannose and Mannosylglycerate from Labeled Mannose by Genetically Engineered Escherichia coli without Loss of Specific Isotopic Enrichment

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We report the construction of an Escherichia coli mutant that harbors two compatible plasmids and that is able to synthesize labeled 2-O-α-D-mannosyl-D-glycerate from externally added labeled mannose without the loss of specific isotopic enrichment. The strain carries a deletion in the manA gene, encoding phosphomannomutase isomerase. This deletion prevents the formation of fructose-6-phosphate from mannose-6-phosphate after the uptake of mannose from the medium by mannose-specific enzyme II of the phosphotransferase system (PtsM). The strain also has a deletion of the cps gene cluster that prevents the synthesis of colanic acid, a mannose-containing polymer. Plasmid-encoded phosphomannomutase (cpsG) and mannose-1-phosphate guanylyltransferase (cpsB) ensure the formation of GDP-mannose. A second plasmid harbors msg, a gene from Rhodothermus marinus that encodes mannosylglycerate synthase, which catalyzes the formation of 2-O-α-mannosyl-D-glycerate from GDP-mannose and endogenous glycerate. The rate-limiting step in 2-O-α-D-mannosyl-D-glycerate formation is the transfer of GDP-mannose to glycerate. 2-O-α-D-mannosyl-D-glycerate can be released from cells by treatment with cold-water shock. The final product is formed in a yield exceeding 50% the initial quantity of labeled mannose, including loss during preparation and paper chromatography.

2-O-α-D-Mannosyl-D-glycerate (MG) is one of the most widespread compatible solutes of thermophilic or hyperthermophilic bacteria and archaea (21). This compound has otherwise been encountered only in the red algae of the order Ceramiales (4). The highly preferential distribution of MG among organisms requiring high temperatures for growth led to the hypothesis that it could play a role in the thermostability of cell components in vivo. This speculation still lacks experimental proof, but at least in vitro, MG has been shown to be highly efficient in the protection of enzymes against thermal inactivation (3, 19).

In order to study the metabolic fate of MG and the mechanisms underlying its stabilizing effect on proteins, it is desirable for this compound to be available in a labeled form with a high level of specific isotopic enrichment (14C, 13C); at present, such a compound is not commercially available.

The biosynthesis of MG has been characterized in detail for the thermophilic bacterium Rhodothermus marinus (12) and for the hyperthermophilic archaeon Pyrococcus horikoshii (7). The known pathways involve the transfer of α-mannosyl residues from GDP-mannose to either α-glycerate, forming MG, or δ-3-phosphoglycerate, followed by hydrolysis of mannosyl-3-phosphoglycerate to MG (7, 12). Most organisms in which the synthesis of MG has been observed are not accessible for easy genetic manipulation (21). Thus, the conversion of a simple radioactive sugar for the formation of MG is plagued by its fast metabolism after entering the producing organism. This situation is due to the common separation of catabolic and anabolic pathways seen for sugar-type compatible solutes. The strict separation of trehalose metabolism and its internal synthesis is a typical example of this phenomenon (10). Therefore, we thought of using a genetically altered Escherichia coli strain to synthesize MG from external mannose. E. coli does produce GDP-mannose, mainly for the synthesis of colanic acid via GDP-fucose (2, 9). The cpsB gene, encoding the enzyme for GDP-mannose synthesis (mannose-1-phosphate guanylyltransferase), and the cpsG gene, encoding phosphomannomutase, form an operon that is part of the cps gene cluster necessary for the synthesis of colanic acid (25).

By constructing an E. coli strain that is unable to metabolize mannose and by using plasmid-encoded enzymes, we were able to channel the uptake of mannose preferentially into conversion to GDP-mannose and MG.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are described in Table 1. Strains were grown in minimal medium A (MMA) (15) or in NZA medium (22) supplemented with 0.2% glucose. For confirming strain markers, we used plates containing mannose and arabinose (0.2%) as required and glycerol (0.4%) as a carbon source. Unless stated otherwise, cells were centrifuged at 8,000 × g for 7 min at 10°C. For transport assays, thin-layer chromatography (TLC) analysis, and preparative synthesis, cells were washed twice with MMA in the absence of a carbon source and resuspended in the same medium.

The concentrations of antibiotics in solid and liquid media were as follows: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; tetracycline, 5 μg/ml; kanamycin, 100 μg/ml.

When needed, amino acids were added to the minimal medium at the following concentrations: histidine, 0.15 g/liter; isoleucine, 0.21 g/liter; leucine, 0.46 g/liter; and valine, 0.32 g/liter. The vitamins pyridoxine and B12 were used at 2.5 and 1 mg/liter, respectively.

Genetic construction of the producer strain. Plasmid transductions were performed as described by Miller (15); P1φ was from a laboratory stock. In order to use arabinose-induced promoters, it was necessary to introduce an araABC-leu
obtained strain MS2601. A lysate of MS2601 allowed us to transfer the ptsG gene. MS4601 can be used to express arabinose-inducible genes. It has lost any sensitivity to mannose and by slow growth on glucose. Additionally, the presence of 0.2% glucose or on glucose minimal medium plates, the strain is sensitive to mannose. To remove the ptsG gene, we obtained strain MS3601. The ptsG gene is harboring a kefE mutation of UE26 into MS1601, we introduced a plasmid carrying the ptsG gene into strain SG20273, and the supernatant was recovered and lyophilized. The remainder of the sample was resuspended in 2 ml of ice-cold water. After incubation on ice for 10 min, the supernatant was harvested, resuspended in MMA to a density at 600 nm (OD600) of 0.5. After induction with 1 mM IPTG and further growth for 6 h, cells were harvested and resuspended in MMA (without a carbon source) to a final OD600 of 1.0. To 6 ml of this cell suspension, [14C]mannose was added to a final concentration of 0.078 µM (0.14 µCi). After different time intervals, 1-ml samples were withdrawn and centrifuged, and the pellet was resuspended in 75 ml of ice-cold water, and incubated for 20 min in an ice bath. Cells were removed by centrifugation, and the supernatant solution was lyophilized, dissolved in D2O, and analyzed by 1H nuclear magnetic resonance (NMR) spectrometry. NMR spectra were acquired with a Bruker AMX300 spectrometer. The presence of MG was estimated as previously described (23).

Preparation of plasmids and transformations were carried out according to standard protocols (20).

**Construction of plasmids.** Two primers (5′-GTCCTAGAATGCGCGGTTC GAAAACCT-3′ and 5′-GCCTGGCAGTCTCTGCAGCAAGCTC-3′) containing the additional XbaI and PstI recognition sequences (underlined) were used to amplify the genes ppsG (encoding phosphomannomutase) and ppsB (encoding mannose-1-phosphate guanylyltransferase) from genomic DNA of E. coli MC4100. The PCR product was digested with the corresponding restriction enzymes and ligated into pTEC99B. The resulting plasmid was named pMS104 and carries ppsG-psnB under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. To construct a vector carrying the mgs gene, we obtained the expression system for R. marinus, and compatible for use in combination with pMS104, we exchanged the antibiotic resistance of pMG37. The sequence coding for chloramphenicol resistance, which was used for the exchange, was obtained from digestion of pACYC184. In this way, we obtained plasmid pMS101. The producer strain was maintained free of plasmids. Only freshly transformed strains were used for MG production.

**[14C]MG and [14C]GDP-mannose production from [14C]mannose in whole cells.** For evaluating the products formed in MS4601 cells, washed cultures of cells with and without plasmids were exposed to external mannose (see Fig. 2). Transformed cells were grown in NZA medium with 0.2% glucose to an optical density at 600 nm (OD600) of 0.5. After induction with 1 mM IPTG and further growth for 6 h, cells were harvested and resuspended in MMA (without a carbon source) to a final OD600 of 1.0. To 6 ml of this cell suspension, [14C]mannose was added to a final concentration of 0.078 µM (0.14 µCi). After different time intervals, 1-ml samples were withdrawn and centrifuged, and the pellet was resuspended in 75 ml of ice-cold water, and incubated for 20 min in an ice bath. Cells were removed by centrifugation, and the supernatant solution was lyophilized, dissolved in D2O, and analyzed by 1H nuclear magnetic resonance (NMR) spectrometry. NMR spectra were acquired with a Bruker AMX300 spectrometer. The presence of MG was confirmed by spiking the NMR sample with the authentic compound. MG was quantified as previously described (23).

Preparative synthesis of [14C]MG. A culture of MS4601 transformed with pMS104 and pMS101 was grown in NZA medium with 0.2% glucose to an OD600 of 0.5. After induction with 1 mM IPTG, the cells were grown for an additional 6 h, harvested, washed twice, and resuspended in 10 ml of MMA to a final OD600 of 2.0. [14C]Mannose was added to a final concentration of 1 µCi, the cells were incubated for 15 min at 42°C and centrifuged, and the pellet was quickly resuspended in 2 ml of ice-cold water. After incubation on ice for 10 min, the supernatant was recovered and lyophilized. The residual of the sample was dissolves in a minimal amount of water and applied to Whatman 3MM chromatography paper. The chromatogram was developed with n-propanol and 25% ammonia (1:1, vol/vol) for 40 h, dried, and autoradiographed. The paper strip identified by autoradiography as containing labeled MG was chromatographed with water as the solvent; the eluate dripped into a glass vessel. The total radioactivity was contained in 5 drops, which were kept frozen.

The final product was checked by TLC along with authentic unlabeled MG.

**Transport assays.** MS4601, MS4601/pMS104, and MS4601/pMS104/pMS101 cells were grown in NZA medium with 0.2% glucose; 1 mM IPTG was added for induction. Cells were harvested (8,000 × g, 7 min, 10°C), washed twice with MMA, and resuspended in MMA without a carbon source. To measure the transport of mannose, we used a cell suspension with an OD600 of 1.5 to 2.0. To 6 ml of culture, prewarmed for 2 min at 40°C, 0.3 µM [14C]mannose was added.

**TABLE 1. Strains and plasmids used**

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**Construction of plasmids.** Two primers (5′-GTCCTAGAATGCGCGGTTC GAAAACCT-3′ and 5′-GCCTGGCAGTCTCTGCAGCAAGCTC-3′) containing the additional XbaI and PstI recognition sequences (underlined) were used to amplify the genes ppsG (encoding phosphomannomutase) and ppsB (encoding mannose-1-phosphate guanylyltransferase) from genomic DNA of E. coli MC4100. The PCR product was digested with the corresponding restriction enzymes and ligated into pTEC99B. The resulting plasmid was named pMS104 and carries ppsG-psnB under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. To construct a vector carrying the mgs gene, we obtained the expression system for R. marinus, and compatible for use in combination with pMS104, we exchanged the antibiotic resistance of pMG37. The sequence coding for chloramphenicol resistance, which was used for the exchange, was obtained from digestion of pACYC184. In this way, we obtained plasmid pMS101. The producer strain was maintained free of plasmids. Only freshly transformed strains were used for MG production.
For higher concentrations of mannose, cold substrate was premixed with radioactive substrate (we always used the same concentration of labeled material). At each time point, 1 ml of culture was filtered, and the rate of uptake of mannose was determined by filtration. [14C]Mannose was purchased from Amersham Pharmacia. It had a specific radioactivity of 296 mCi/mmol.

RESULTS

Construction of a strain that will produce MG with a high specific radioactivity from external [14C]mannose. In the strain which we constructed, mannose metabolism is directed toward the production of GDP-mannose (Fig. 1). By using an initial strain with a deletion in the cps genes, we were able to block the usage of GDP-mannose mainly for colanic acid synthesis (9). In addition, we deleted manA, encoding phospho-mannose isomerase, preventing the metabolism of mannose by glycolysis. The final strain accumulated [14C]mannose-6-phosphate upon exposure to external [14C]mannose via enzyme II, specific for mannose, of the phosphotransferase system (Fig. 2). The strain also lacks the araABC genes, allowing the expression of genes cloned under the control of the arabinose

FIG. 1. Strategy for construction of producer strain MS4601. Note that mannose cannot be metabolized and is converted to MG without the input of unlabeled cellular precursors. PtsG and PtsM are transport systems for glucose and mannose, respectively. P, phosphate.

FIG. 2. Formation of 14C-labeled products from external [14C]mannose. (A) MS4601. (B) MS4601/pMS104. (C) MS4601/pMS104/pMS101. In each experiment, 6 ml of cells (OD600, 2) was incubated with 0.14 μCi of [14C]mannose, equivalent to 0.078 nmol. The supernatants of TCA-treated cells were analyzed by TLC and autoradiographed. Lanes contain samples from the following time points: 1, 10 s; 2, 1 min; 3, 5 min; 4, 15 min; and 5, 30 min. The positions of the known products are indicated. When GDP-mannose (GDP-man) was present (panels B and C), an additional product was formed and ran ahead of MG; it was tentatively assigned as fucose.
promoter. A galU mutation (resulting in a defect in the synthesis of UDP-glucose) will prevent the synthesis of the internal osmoprotectant trehalose (8).

Genes necessary for the expression of enzymes involved in the synthesis of mannose-1-phosphate and GDP-mannose as well as the enzyme MG synthase were introduced by using two plasmids (pMS101 and pMS104).

Production of mannose-6-phosphate, GDP-mannose, and MG. Cells of strain MS4601 without plasmids, harboring plasmid pMS104, or harboring plasmids pMS104 and pMS101 were exposed to labeled mannose, and the internally formed products were analyzed by TLC (Fig. 2). In the absence of any plasmid, one major spot, representing mannose-6-phosphate, was seen (Fig. 2A). In the presence of plasmid pMS104 (Fig. 2B), the additional production of GDP-mannose was observed. Since mannose-6-phosphate and mannose-1-phosphate run at the same positions in the solvent system used, the ratio of these two compounds could not be determined. In the presence of both plasmids (Fig. 2C), an additional compound representing MG was detected. A weakly labeled spot running above MG was also observed and tentatively assigned as fucose. The strain synthesizing GDP-mannose but not MG synthesized small amounts of a product running below mannose-1-phosphate or mannose-6-phosphate. This material was present in smaller amounts when MG synthase was present. Therefore, we believe it to be a mannose product transferred to an unknown acceptor. Occasionally, we observed a spot running at the same position as free mannose. This spot was more prominent in strains lacking MG synthesis, indicating that GDP-mannose in the absence of a suitable acceptor might be slowly degraded internally to mannose.

Estimation of the rates of synthesis of MG and GDP-mannose. Cells containing both plasmids were exposed to 0.078 μM [14C]mannose in the presence of increasing amounts (up to 10 μM) of unlabeled mannose. The time-dependent formation of labeled products was analyzed by TLC (Fig. 3). Under the chosen conditions, external mannose was taken up by the cells in all experiments within 2 min. At a high specific radioactivity and a low external concentration of mannose (0.078 μM), the synthesis of MG was immediate. Considering the internal cell volume of the culture, the initial internal concentration of the precursors under these conditions was estimated to be about 80 μM. With decreasing specific activity and increasing amounts of external mannose, the formation of labeled MG seemingly declined, while free pools of mannose-6-phosphate or mannose-1-phosphate and GDP-mannose increased. We used an experiment in which cells were exposed to 10 μM mannose (Fig. 3F) to estimate the in vivo rate of production of MG. Under these conditions, the internal concentration of GDP-mannose was on the order of 10 mM. The rate of MG formation was calculated by measuring the radioactivities of the different spots with phosphorimaging, taking into account the dilution of the radioactivity. We found that the synthesis of MG (0.08 nmol/min/10⁹ cells) was slow in comparison to the formation of GDP-mannose (0.25 nmol/min/10⁹ cells). This has to be compared to the rate of mannose uptake at 10 μM mannose, which is 2.9 nmol/min/10⁹ cells (see below).

As an alternative method for extracting the labeled compounds, we treated the harvested cells with ice-cold water after exposing them to different external concentrations of [14C] mannose for different times. The corresponding supernatants were lyophilized and analyzed by TLC (Fig. 4). Again, it became apparent that the in vivo synthesis of MG was limited by the conversion of GDP-mannose to MG. The cold-water shock released, without preference for any of the labeled compounds, at least 95% of the accumulated radioactivity, as demonstrated by TLC analysis of TCA-treated pellets (data not shown). As shown in Fig. 4, when a higher initial concentration of MG (20 μM) was used, the percentage of radioactivity declined over time. We believe this to be due to the release of labeled MG prior to the cold-water shock treatment. Indeed, small
amounts of labeled MG (only MG, no other labeled compound) could be detected in the medium supernatant prior to the shock treatment (data not shown).

The synthesis of MG was confirmed by NMR analysis of the supernatant solutions resulting from the cold-water shock treatment of cells exposed to unlabeled mannose. The proton spectra showed that, in addition to MG, a variety of compounds were released into the external medium. The area of the anomeric proton resonance in MG was used for quantification, and an intracellular concentration of 5 mM was determined (data not shown).

Preparative synthesis of [14C]MG. For the preparation of radioactively labeled MG, undiluted [14C]mannose was used, and the final product was separated by paper chromatography of material released from harvested cells by ice-cold water treatment. The yield with respect to the input of mannose was 56%. This value includes the loss of material during the different procedures (cold shock, concentration, chromatography, and elution from paper). According to the metabolic scheme (Fig. 1), no dilution of the specific radioactivity should occur. The isolated product was checked by TLC, and one major spot was detected. The radioactive material behaved like authentic nonlabeled MG (Fig. 5).

A sample of the medium supernatant (after cell harvest by centrifugation) was analyzed by paper chromatography. The only radioactivity present (15% the initial radioactivity supplied as mannose) behaved like MG (data not shown). Thus, internally produced MG does leak out, possibly carrier mediated, presumably after the concentration of internal MG reaches levels in the millimolar range.

**DISCUSSION**

In this publication, we describe the production of labeled MG, an osmolyte that is preferentially found in thermophilic or hyperthermophilic prokaryotes (21). These organisms are useful for isolating MG but do not allow the labeling of this compound, since labeled precursors added to the medium are degraded in the cells and the precursors actually used for MG synthesis are derived from internal metabolites and hence are not labeled. *E. coli* does not produce MG and was therefore chosen for metabolic engineering to synthesize MG from external mannose in the absence of mannose metabolism. Figure 1 explains the simple strategy. The deletion of *manA*, encoding mannose-6-phosphate isomerase, prevents the formation of...
fructose-6-phosphate and thus the metabolism of mannose. It also prevents dilution of the specific labeling of mannose-6-phosphate from internal gluconeogenesis via glucose-6-phosphate. We removed the activity of the \textit{ptsG}-encoded transport of glucose for future selection purposes (see below). In addition, by overcoming \textit{araD}-dependent sensitivity to arabinose (not shown in Fig. 1), we gained the possibility of using the engineered strain with arabinose-inducible promoters in future studies.

![FIG. 5. Confirmation of authenticity of radiolabeled MG. (A) Radiolabeled MG isolated from preparative synthesis and paper chromatography. (B) Paper chromatogram of MG and mannose. Lane 1, radioactive material obtained by the paper chromatography shown in panel B; lane 4, authentic nonlabeled MG; lanes 2 and 3, mixtures of labeled product and nonlabeled authentic MG. Lanes 1 and 2 show samples subjected to TLC and autoradiography; lanes 3 and 4 show samples stained with α-naphthol–sulfuric acid solution and charred at 120°C. Note that lanes 2 and 3 represent the same spot that has been autoradiographed (lane 2) and charred (lane 3). The small spot ahead of MG has not been identified but could be D-fucose.]

![FIG. 6. Transport of [14C]mannose. Washed cultures of strain MS4601 harboring pMS101 and pMS104 were tested in MMA without a carbon source. The assay was done at 42°C, and [14C]mannose was added at increasing concentrations (0.05 to 40 μM). Initial rates of uptake were determined and are plotted against substrate concentration. A \( K_m \) of 5.6 μM and a \( V_{max} \) of 5.8 nmol/min/10^9 cells were determined for the strain containing the two plasmids. Strains containing only pMS101 or no plasmid showed transport activities that were identical within the limit of experimental error (not shown).]
plasmid constructs. *E. coli* does contain the *cpsG* gene, encoding phosphomannose mutase (catalyzing the conversion of mannose-6-phosphate to mannose-1-phosphate), and the *cpsB* gene, encoding GDP-mannose pyrophosphorylase (catalyzing the formation of GDP-mannose from GTP and mannose-1-phosphate). However, these genes are contained in a gene cluster responsible for the formation of colanic acid (2). Since the latter could interfere with the yield of MG, we chose to delete the *cps* cluster but introduced *cpsB* and *cpsG* on an IPTG-inducible plasmid. Finally, the *mgs* gene, encoding MG synthase, was available only from a thermophilic organism (*R. marinus*). The activity of the encoded enzyme was optimal at 85°C but very low at the ambient temperature of *E. coli* (12). Nevertheless, after induction, the enzyme was sufficiently active to detect the synthesis of MG (Fig. 2C and 3). The accumulation of mannose-1-phosphate or mannose-6-phosphate was observed when GDP-mannose pyrophosphohydrase was absent (Fig. 2A and B), and the additional formation of GDP-mannose was observed in its presence (Fig. 2B). The fact that these compounds were metabolically stable proves the quality of the engineered pathway.

The overall synthesis of MG appears to be limited mainly by the transfer of α-mannosyl residues from GDP-mannose to β-glycerate. The external addition of glycerol or glycerate did not increase the yield of MG (data not shown). Thus, the internal pool of glycerate cannot be the limiting factor in MG synthesis. Neither does mannose transport appear to be limiting in the producer strain, since the mannose uptake rate was approximately 70-fold higher than the rate of MG synthesis (5.8 compared to 0.08 nmol/min/10^9 cells). Surprisingly, the rate of formation of GDP-mannose also was not very high despite the fact that GDP-mannose pyrophosphohydrase was plasmid encoded and should have been present in large amounts. This result suggests that the formation of GTP is the next rate-limiting step after MG synthase activity is increased.

Engineered strain MS4601 is sensitive to mannose, most likely due to the accumulation of intermediates. By using this sensitivity as a selection marker, conceivably at increasing salt concentrations, it might be possible to isolate mutations leading to an overall higher rate of MG synthesis and thus increase the concentration of internal MG as an effective protective osmolyte. Glucose used as a carbon source in the presence of mannose would ensure the maintenance of PtsM as a port of entrance for glucose and mannose, avoiding an undesired mode of resistance (loss of mannose uptake). If successful, the strain might also be used for the synthesis of large amounts of unlabeled MG from mannose; in addition, with a small alteration in the strain (deletion of phosphofructokinase and introduction of wild-type mannose-6-phosphate isomerase), glucose might be used as an inexpensive starting material for the synthesis of MG.

Although limited by several shortcomings (presence of two plasmids and low rate of MG synthesis), the producer strain allows the high-yield formation of labeled MG from labeled mannose without the loss of specific radioactivity. Labeled MG can now be used for studying the properties of this novel osmolyte. After exposure to limited amounts of mannose, uptake of mannose into cells, and the formation of internal MG, we did detect small quantities of labeled MG in the medium without other labeled compounds. Thus, this material must have leaked from the cells in a selective process. This observation suggests the possibility of optimizing the production and selective release of MG after cloning of the responsible exit carrier for MG.

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**REFERENCES**