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Improved Method for the Preparative Synthesis of Labeled Trehalose of High Specific Activity by *Escherichia coli*

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We report an improvement of a published procedure using *Escherichia coli* to synthesize ^{14}C-labeled trehalose from [^{14}C]glucose (B. Brand and W. Boos, Appl. Environ. Microbiol. 55:2414–2415, 1989). Instead of inducing the expression of the trehalose-synthesizing enzymes encoded by the chromosomal genes *otsAB* by high osmolarity, we now induce their expression from a plasmid under normal growth conditions by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). Instead of using a *pgi zwf* double mutant to prevent glucose utilization, we use a *pgi*::Tn*10* insertion only. In addition to being defective in *treA*, which encodes a periplasmic trehalase, the strain is now also defective in *treF*, which encodes a newly discovered cytoplasmic trehalase. This strain is genetically stable; it has no growth defects; and after induction with IPTG, it will transform [^{14}C]glucose to [^{14}C]trehalose in minimal medium without any carbon source under aerobic conditions at a rate of 3 nmol/min/10^9 cells. With the improved method, the overall yield of trehalose from glucose is about 80% and the process takes place without dilution of the specific radioactivity of the glucose residues. The accumulated trehalose is extracted from the bacteria by 70% hot ethanol and can easily be purified radiochemically by chromatographic techniques.

Trehalose is a nonreducing disaccharide composed of two alpha-glycoside-linked glucose moieties. Trehalose is synthesized by many organisms in response to osmotic stress and the stress of dehydration. It acts as a protecting agent for proteins and membranes (12). *Escherichia coli* synthesizes trehalose under osmotic stress (11), and the genes encoding the synthesizing enzymes (7, 8) are under the control of the global stress regulator RpoS (6). *E. coli* can utilize trehalose as its sole source of carbon in two different ways, i.e., under high and low osmolarity (2, 3, 10, 15). These two pathways, as well as trehalose synthesis, are well regulated to avoid futile cycles. For studies of trehalose metabolism and transport, it is useful to have trehalose available in a form labeled with ^{14}C or ^{13}C. Since these compounds are not commercially available, we developed a method to synthesize trehalose from glucose by using *E. coli*. This method was to let glucose enter the cell by means of enzyme II for glucose as glucose-6-phosphate (Glc-6-P) and to prevent its utilization through glycolysis by a block in *pgi* (which encodes phosphoglucose isomerase) and through the pentose phosphate cycle by a mutation in *zwf* (which encodes Glc-6-P dehydrogenase). Glc-6-P is in equilibrium with Glc-1-P via phosphoglucomutase and hence is also in equilibrium with UDP-glucose. The *otsAB*-encoded enzymes form trehalose-6-phosphate (Tre-6-P) from UDP-glucose and Glc-6-P, and Tre-6-P is then hydrolyzed to trehalose. The latter can be isolated by extraction of the cells followed by chromatography (5). A similar scheme for synthesizing labeled trehalose in *Saccharomyces cerevisiae* has been described (16).

Our method with the *E. coli* system had the disadvantage that the strain was genetically unstable, showing a tendency to revert to growth on glucose. Also, the induction of the *ots* genes required growth at high osmolarity, which dramatically reduced the ability of the strain to grow on any carbon source. This curbed the proper induction of the *ots* genes. Therefore, the yield of labeled trehalose was not very high.

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Here we present a new strain with greatly improved properties. Firstly, we abolished the need for high osmolarity by cloning the *ots* genes under the control of the *tac* promoter. By using primers flanking *otsBA*, the operon (8) was isolated from chromosomal DNA by PCR and ligated into plasmid ptrc99A, bringing the expression of the *ots* genes under the control of IPTG (isopropyl-β-D-thiogalactopyranoside) and yielding plasmid pRHo700. Secondly, we introduced into the strain a *pgi*::Tn*10* insertion (1), which abolished the problem of reversion to Glc$^+$. The block in glycolysis by the *pgi*::Tn*10* insertion proved sufficient to prevent glucose utilization, and a second mutation, in *zwf*, appeared not to be necessary. Thirdly, we introduced not only a mutation in *treA*, which encodes the periplasmic trehalase (2), but also a mutation in *treF*, which encodes a novel cytoplasmic trehalase (unpublished data), as well as in the genes encoding trehalose uptake (*treB*) and the utilization of Tre-6-P (*treC*). This increased the yield of cytoplasmically formed trehalose by preventing its hydrolysis to glucose. Thus, the final strain, RHo53, is a derivative of strain DHB3 (4) and carries the following additional mutations: *treBC*, *treA*::Spec, *treF*::Kan, and *pgi*::Tn*10*. The strain was transformed with plasmid pRHo700, which harbors *lacI*^{q} and the *otsBA* genes under *tac* promoter control.

We grew the strain on 1% tryptone broth (TB) (13) or on 1% TB plus 0.4% glycerol or in minimal medium A (MMA) (13) plus 0.4% glycerol at 37°C under aeration. After an optical density at 578 nm (OD_{578}) of 0.5 to 0.7 was reached, 0.1 mM IPTG was added and growth continued for one more hour. The cells were harvested, resuspended in MMA at an OD_{578} of 10, and kept on ice. To measure the ability to take up glucose, the cultures were diluted to an OD_{578} of 0.1 in MMA, MMA plus 0.4% glycerol, 1% TB, or 1% TB plus 0.4% glycerol and warmed for 2 min under aeration. One culture that had been grown on TB and glycerol received no carbon source. To 3 ml of the culture, 0.1 μCi of [^{14}C]glucose was added, unlabeled glucose was added to a final total glucose concentration of 0.1 mM, and the rate of uptake was determined. All cultures, independently of the carbon source, showed about the same rate of uptake, between 2.7 and 3.5 nmol/min/10^9 cells. For the

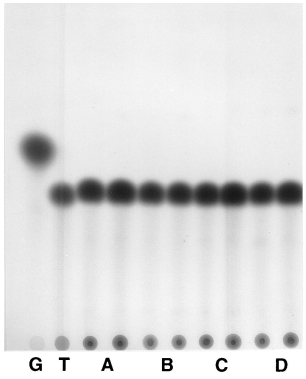

FIG. 1. Ability of strain RHo53/pRHo700 to produce trehalose is independent of the carbon source, and trehalose production takes place even in the absence of a carbon source. Cells were grown in TB (A), in TB plus glycerol (B and D), or in MMA plus glycerol (C). They were incubated in TB (A), in TB plus glycerol (B), in MMA plus glycerol (C), or in MMA only (D) with 0.1 μCi of [^{14}C]glucose for 4 min (first lane for each medium) and 8 min (second lane for each medium). Cellular extracts were obtained, chromatographed on TLC plates, and autoradiographed. Controls: G, [^{14}C]glucose; T, [^{14}C]trehalose.

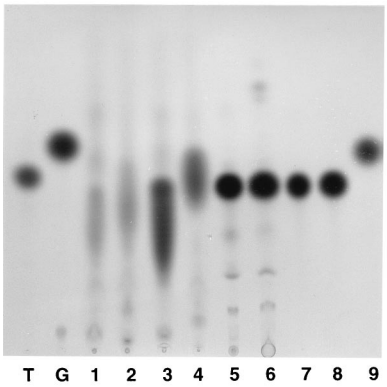

FIG. 2. Analysis of the products of the preparative experiment by TLC. The medium supernatant was treated with trehalase and then placed in a mixed-bed ion exchanger, and the deionized solution was concentrated by evaporation. Lane 1, untreated sample; lane 2, trehalase-treated sample; lane 3, untreated sample plus authentic trehalose; lane 4, trehalase-treated sample plus authentic glucose. Cells were extracted twice with 70% hot ethanol. Lane 5, first extraction; lane 6, second extraction. Both extracts were purified by paper chromatography and eluted. Lane 7, sample of the purified first extraction; lane 8, sample of the purified second extraction; lane 9, trehalase-treated sample of the eluate. Controls: T, [^{14}C]trehalose; G, [^{14}C]glucose.

measurement of the formation of internal trehalose from external glucose, the stock cultures were diluted to an OD_{578} of 1.0 in MMA, MMA plus 0.4% glycerol, 1% TB, or 1% TB plus 0.4% glycerol and warmed to 37°C for 2 min under aeration. One culture that had been grown on TB and glycerol received no carbon source. To 0.2 ml of the culture, [^{14}C]glucose (2 μm final concentration, containing 0.1 μCi of label) was added, and 100-μl samples were taken at 4 and 8 min. The cells were harvested by centrifugation, and the supernatants of both samples were counted. At both time points they contained about 10% of the starting radioactivity. Apparently, this material was no longer taken up and did not consist of glucose. The pellet was resuspended in 5 μl of 10% trichloroacetic acid and incubated for 10 min on ice. The entire sample was transferred to a thin-layer chromatography (TLC) plate and analyzed by chromatography followed by autoradiography (Fig. 1). Internal radioactivity was contained to about 90% in a compound that migrated as trehalose and to about 10% in a material that did not migrate at all. The latter could have been glycogen (14), membrane-derived oligosaccharides (9), or other glucose-containing macromolecules. It was not further analyzed. The results shown in Fig. 1 indicated that within the cell a compound that exhibited the chromatographic behavior of trehalose was formed from external glucose. This material was entirely hydrolyzed by purified trehalase (see Fig. 2), proving its identity with trehalose.

For the preparative synthesis of [^{14}C]trehalose, the strain was grown at 37°C in TB to an OD_{578} of 0.5, 0.1 mM IPTG was then added, and growth was allowed to continue for 1 h. The culture was harvested and resuspended in 30 ml of MMA (without a carbon source) to an OD_{578} of 2, and 350 μCi of [^{14}C]glucose (250 μCi/μmol), corresponding to 46 μM glucose, was added. Incubation of the culture under aeration at 37°C for 10 min was followed by centrifugation. The pellet was carefully freed of supernatant and extracted twice with 1 ml of 70% hot ethanol. The extracts were centrifuged, and the supernatant was chromatographed by descending chromatography on Whatman 3MM filter paper with butanol-ethanol-water (5:3:2 [vol/vol/vol]) as the solvent. The pellet was resuspended in 1% sodium dodecyl sulfate. The counting of a sample indicated that the pellet contained about 10% of the original radioactivity. Since 10% of the initial radioactivity remained in the culture supernatant, this means that about 80% of the initial radioactivity was transferred onto the chromatography paper.

The paper strip identified by autoradiography as containing the labeled trehalose was chromatographed with water as the solvent; the eluant dripped into a glass vessel. The total radioactivity was contained in less than 20 drops, which were kept frozen.

Figure 2 shows the TLC analysis of the material produced in the preparative experiment and of the same material after its treatment with trehalase. Figure 2 also shows a sample of the culture supernatant after all glucose had been taken up. The amount of radioactivity left in the supernatant was less than 10% of the initial radioactivity associated with glucose. The supernatant was treated with trehalase, concentrated, treated with ion-exchange material, and chromatographed. Unfortunately, the analysis of this concentrated material was not very clear. By adding authentic glucose or trehalose to the mixture (Fig. 2, lanes 3 and 4), we concluded that the supernatant most likely also contained trehalose originating from internally synthesized material.

The method for producing trehalose from glucose can of course also be applied to the synthesis of [^{13}C]trehalose needed for nuclear magnetic resonance studies, since ^{13}C-labeled glucose is available commercially. The conditions for the uptake of glucose can be varied to some extent. Since the K_m for glucose uptake is rather low (on the order of micromolar concentrations), both the density of the cells and the concentration of the labeled glucose can be varied. The guideline is given by the rate of glucose uptake, which under conditions of V_{max} in our hands and with the strain described above is about 3 nmol/min/10^9 cells. As a general rule, we allow twice the minimal time needed to take up the given amount of glucose before harvesting. In addition, we did not allow the incubation with glucose to exceed 10 min, in order to avoid substantial excretion of internally formed trehalose.

In Fig. 3 the advantage of using the *E. coli* strain described above for the production of labeled trehalose from labeled glucose is clearly demonstrated. The figure shows a TLC analysis of the entire culture (no separation of bacteria and me-

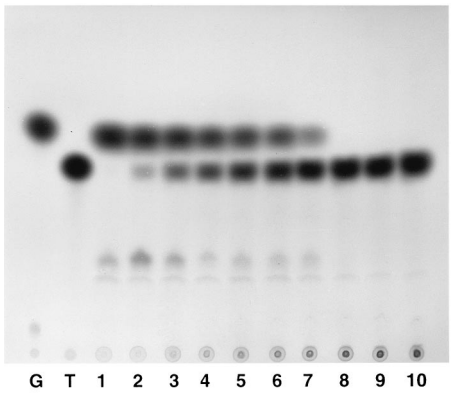

FIG. 3. Kinetics of the formation of trehalose from glucose. Fifty microliters of cells (OD_{578} of 2) were incubated with 0.3 μCi of [^{14}C]glucose, corresponding to 1.1 nmol. Samples (5 μl) were withdrawn and added to 5 μl of 10% trichloroacetic acid, and the mixture was kept on ice. The total mixture (containing medium and cellular extract) was applied to a TLC plate, chromatographed, and autoradiographed. Lanes contain samples from various time points, as follows: 1, 0 s; 2, 10 s; 3, 30 s; 4, 60 s; 5, 90 s; 6, 120 s; 7, 180 s; 8, 300 s; 9, 600 s; 10, 900 s. Controls: G, [^{14}C]glucose; T, [^{14}C]trehalose.

dium) after the addition of labeled glucose to the medium. As can be seen, the transformation of glucose into trehalose is almost complete and the concentration of intermediates, mainly phosphorylated glucose and trehalose, is minimal. This is in contrast to the previously reported method (Fig. 1 in reference 5), in which substantial amounts of phosphorylated intermediates allowed the synthesis of other glucose-containing polymers.

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