APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1989, p. 2414–2415
0099-2240/89/092414-02$02.00/0

Vol. 55, No. 9

Convenient Preparative Synthesis of [^{14}C]Trehalose from [^{14}C]Glucose by Intact *Escherichia coli* Cells

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Received 15 December 1988/Accepted 5 June 1989

At high osmolarity, *Escherichia coli* synthesizes trehalose intracellularly, irrespective of the nature of the carbon source. Synthesis proceeds via the transfer of UDP-glucose to glucose 6-phosphate, yielding trehalose 6-phosphate, followed by its dephosphorylation to trehalose (H. M. Giaeyer, B. O. Styrvold, I. Kaasen, and A. R. Strøm, J. Bacteriol. 170:2841–2849, 1988). This reaction was exploited to preparatively synthesize [^{14}C]trehalose from exogenous [^{14}C]glucose by using intact bacteria of a mutant (DF214) that could not metabolize glucose. The total yield of radiochemically pure trehalose from glucose was routinely more than 50%.

Trehalose is a nonreducing disaccharide composed of two alpha-glycoside-linked glucose moieties. This sugar plays an important role in many organisms as a storage carbohydrate as well as an osmoprotectant under conditions of severe dehydration and high osmolarity (10). In *Escherichia coli*, trehalose is synthesized in response to high osmolarity (4), but it also is an excellent carbon source (7), both at high and low osmolarities (1; C. Gutíerrez, M. Ardouel, E. Bremer, A. Middendorf, W. Boos, and U. Ehmann, Mol. Gen. Genet., in press).

Here, we present a fast and convenient method of synthesizing [^{14}C]trehalose from [^{14}C]glucose. This method is based on the ability of *E. coli* to synthesize trehalose under conditions of high osmolarity (4) and on the use of mutant DF214, isolated by Dan Fraenkel (11), which no longer grows on glucose because of two mutations: the first in *pgi* prevents the transformation of glucose 6-phosphate to fructose 6-phosphate, and the second in *zwf* prevents the oxidation of glucose 6-phosphate to gluconate 6-phosphate. However, the strain is still able to transport glucose under simultaneous and phosphotransferase system-mediated phosphorylation to glucose 6-phosphate. The latter can be transformed into glucose 1-phosphate and subsequently into UDP-glucose. Thus, exogenous [^{14}C]glucose can be incorporated into glucose-containing polymers without radiochemical dilution from gluconeogenesis. Indeed, this strain has been used to radioactively label membrane-derived oligosaccharides (9). At high osmolarity, the synthesis of membrane-derived oligosaccharides (5) and the synthesis of glycogen (2), which is mediated via the transfer of ADP-glucose to maltodextrins (6), are strongly suppressed. Therefore, synthesis of trehalose should occur preferentially. Nevertheless, to avoid entirely the synthesis of glycogen, a mutation (*glgAC*) was introduced into DF214 with a nearby Tn*10* insertion (3), resulting in strain CB30 [*eda-1 pgi*::Mu Δ(*edd-zwf*) *his rpsL glgAC zhf*::Tn*10*].

Strain CB30 was grown aerobically at 37°C in minimal medium A (MMA) (8) with 0.2% glycerol as the carbon source, supplemented with 0.1 mM histidine and 1 mM gluconate. The strain cannot be grown at high osmolarity, possibly because of its inability to synthesize trehalose in the absence of exogenous glucose. After growth overnight, the cells were suspended in MMA containing various amounts of NaCl to different optical densities (at 578 nm) and incubated for 15 min at 37°C before the addition of 4.5 μM [^{14}C]glucose (270 mCi/mmol). Samples (150 μl) were centrifuged at different time intervals, and 30 μl of 6% trichloroacetic acid was added to the pellet. The clarified soluble extract was treated with mixed-bed ion-exchange resin (Ionenaustauscher V; E. Merck AG) and chromatographed on thin-layer silica plates (Kieselgel 60, without fluorescence indicator; Merck). The solvent was *N*-butanol-ethanol-water (5:3:2, vol/vol/vol). The dried chromatogram was analyzed by autoradiography. This test was used to optimize the conditions for maximal in vivo synthesis of trehalose with respect to the NaCl concentration, time of incubation, and density of the culture.

Figure 1 demonstrates the time-dependent conversion of glucose to trehalose, using cell densities of 0.2 and 2.0 and an NaCl concentration of 350 mM (which appeared to be the optimum for the formation of trehalose). Glucose itself is not seen on the chromatogram, since it was present only in the medium that was removed by centrifugation. Using the lesser amount of cells nicely revealed the time-dependent conversion into trehalose. The first recognizable products were glucose and trehalose phosphates, followed by trehalose. One can estimate from the results shown in Fig. 1 that the rate of trehalose formation under these conditions (glucose concentration in the medium in the vicinity of the K_m of glucose transport) is about 8 nmol/min × 10^9 cells at 37°C. For the enzymatic identification of the compounds separated on the thin-layer chromatography plate, the silica coating was scraped off and the compounds were extracted with water and clarified by centrifugation. Fifty-microliter samples in 10 mM Tris hydrochloride, pH 8, were treated with *E. coli* alkaline phosphatase or *E. coli* trehalase or both. After incubation for 30 min, the reaction mixture was analyzed by thin-layer chromatography followed by autoradiography. In this way, the first products after glucose uptake were identified as glucose and trehalose phosphates (results not shown).

For a large-scale preparation of [^{14}C]trehalose, 50 μCi of [^{14}C]glucose at a final concentration of 6 μM was added to 30 ml of CB30, washed, suspended in MMA containing 350 mM NaCl at an optical density of 1.5, and incubated for 30 min at 37°C. After centrifugation, the cells were extracted with 3 ml of 6% trichloroacetic acid, centrifuged, and treated as described above. The clarified and deionized supernatant was chromatographed on Whatman 3MM filter paper, using the same solvent as described above. Trehalose was identified

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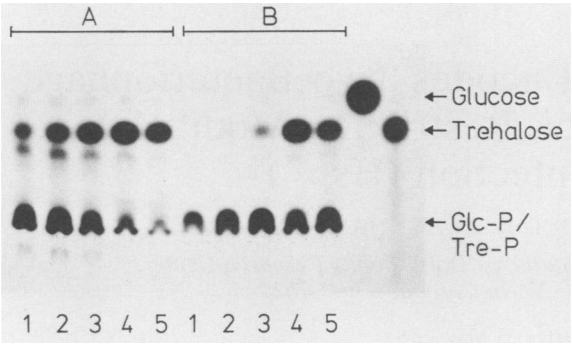

FIG. 1. Thin-layer chromatographic analysis of the time- and bacterial density-dependent conversion of [^{14}C]glucose to [^{14}C]trehalose. Strain CB30 at optical densities (578 nm) of 2 (A) and 0.2 (B) was incubated with 4.5 μM [^{14}C]glucose. Cellular extracts of 150-μl samples were chromatographed (butanol-ethanol-water, 5:3:2) on thin-layer glass plates coated with silica, followed by autoradiography. The following incubation times (in minutes) were used: lane 1, 2; lane 2, 5; lane 3, 10; lane 4, 20; lane 5, 30. The positions of the authentic [^{14}C]glucose and [^{14}C]trehalose (both from Amersham) and of glucose and trehalose phosphates (Glc-P/Tre-P) are indicated.

on the chromatogram by autoradiography. The band was cut out and eluted by descending chromatography, using water as the solvent. The total yield of [^{14}C]trehalose with respect to the starting material of [^{14}C]glucose was 53%. The radioactive material, when incubated with periplasmic trehalase, yielded glucose, as evidenced by thin-layer chromatography and autoradiography (data not shown).

To further identify the isolated compound as trehalose and to compare it with commercial [^{14}C]trehalose, transport assays with strain UE15 (MC4100 *treA*) (Gutierrez et al., in press), induced for the trehalose transport system, and strain KRIM4, a transport-negative derivative of UE15, were done by using [^{14}C]trehalose synthesized by the method described above as well as by using authentic [^{14}C]trehalose obtained from Amersham Corp. Both substrates were added so that the amounts of radioactivity were identical. Commercial [^{14}C]trehalose was less well transported in UE15 than that which we synthesized, while KRIM4 did not take up any radioactivity at all (Fig. 2). The comparison of the two preparations of [^{14}C]trehalose by uptake measurements is valid despite the fact that they exhibit different specific radioactivities (150 versus 540 mCi/mmol). The chemical concentrations of trehalose in the uptake assay (in the absence of unlabeled trehalose) of 1.0 and 0.3 μM were far below the apparent K_m of the trehalose uptake system (10 μM). Thus, the number of counts per minute taken up per minute should be identical when the same amount of radioactivity is present in the medium, irrespective of the difference in chemical concentration. This experiment demonstrates that [^{14}C]trehalose synthesized in the manner described above does not contain unlabeled compounds that interfere with trehalose transport.

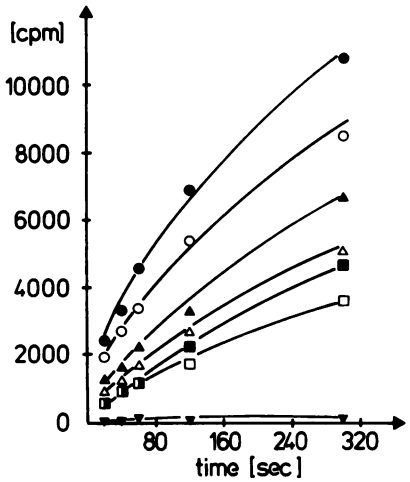

FIG. 2. Transport of [^{14}C]trehalose in strain UE15 (lacking the periplasmic trehalase) and its trehalose transport-negative derivative KRIM4. Cells were grown overnight in MMA–0.2% glycerin–0.2% trehalose, washed three times in MMA, and suspended to an optical density of 1 (578 nm). Samples (500 μl) were filtered through membrane filters (Millipore Corp.; 0.45-μm pore size) after different time intervals. The assay was done with synthesized (closed symbols) and authentic (open symbols) (Amersham) [^{14}C]trehalose, using equal amounts of radioactivity (0.3 and 1 μM chemical concentrations, respectively). Inhibition by unlabeled trehalose was with 0 (circles) and 10 (triangles) μM sugar and 20 μl (squares) of unlabeled sugar, mixed with the radioactive substrate prior to addition in the transport assay. Inverted triangles represent uptake measurements in KRIM4, a mutant negative in trehalose transport.

On the basis of the properties of strain CB30 of not utilizing glucose as a carbon source and not producing glucose-containing polymers via gluconeogenesis (9, 11), it can be concluded that trehalose synthesized from two molecules of glucose will have twice the specific radioactivity of the [^{14}C]glucose used as the substrate.

We thank Dan Fraenkel for strain DF214 and Michael Ehrmann for stimulating discussions.

This work was supported by grants SFB 156 from the Deutsche Forschungsgemeinschaft and by a grant from the Fond der Deutschen Chemischen Industrie.

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