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Production of Glutaconic Acid in a Recombinant *Escherichia coli* Strain▽

Ivana Djurdjevic,[1] Oskar Zelder,[2] and Wolfgang Buckel[1,3]*

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität, 35032 Marburg, Germany[1]; BASF SE, Fermentation Products Research, GVF/D-A30, 67056 Ludwigshafen, Germany[2]; and Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany[3]

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The assembly of six genes that encode enzymes from glutamate-fermenting bacteria converted *Escherichia coli* into a glutaconate producer when grown anaerobically on a complex medium. The new anaerobic pathway starts with 2-oxoglutarate from general metabolism and proceeds via (*R*)-2-hydroxyglutarate, (*R*)-2-hydroxyglutaryl-coenzyme A (CoA), and (*E*)-glutaconyl-CoA to yield 2.7 ± 0.2 mM (*E*)-glutaconate in the medium.

Glutaconic acid, or (*E*)-pentene-1,5-dioic acid, is an unsaturated C_5-dicarboxylic acid (Fig. 1) that can be reduced to the saturated glutaric acid (pentane-1,5-dioic acid), from which its name was derived. In the chemical industry, both acids have the potential to be used as monomers for biodegradable polyesters. Glutaconic acid might be additionally applied for the formation of polyamides by condensation with suitable α,ω-diaminoalkanes (12). The ideal material for biological production of glutaconic acid would be glutamic acid obtained by sugar fermentation. The α,β elimination of ammonia from glutamate to glutaconate, however, is chemically not feasible. Nevertheless, some anaerobic bacteria, such as *Acidaminococcus fermentans* and *Clostridium symbiosum*, catalyze this reaction in five steps during the fermentation of glutamate to ammonia, CO_2, acetate, butyrate, and molecular hydrogen (7). This pathway converts glutamate to ammonia and 2-oxoglutarate, followed by an NADH-dependent reduction to (*R*)-2-hydroxyglutarate (14). Glutaconate coenzyme A (CoA)-transferase mediates the activation to (*R*)-2-hydroxyglutaryl-CoA (8), and an oxygen-sensitive [4Fe-4S] cluster-containing radical enzyme catalyzes the dehydration of this α-hydroxythioester to the α,β-unsaturated thioester, (*E*)-glutaconyl-CoA (Fig. 1) (15). To convert *Escherichia coli* into a glutaconate producer, we expressed six genes in this organism encoding 2-hydroxyglutarate dehydrogenase (HgdH) (14), glutaconate CoA-transferase (GctAB) (13), and the activator of the dehydratase (HgdC) from *A. fermentans* (10), as well as 2-hydroxyglutaryl-CoA dehydratase (HgdAB) from *C. symbiosum* (11). The new pathway diverts from central metabolism at 2-oxoglutarate (Fig. 1).

The first step was the construction of the plasmid pID-3, derived from pACYCDuet-1 (Novagene), which comprises three genes coding for 2-hydroxyglutarate dehydrogenase (*hgdH*) and glutaconate CoA-transferase (*gctAB*). A second plasmid, pID-4, derived from the pASK-IBA3plus vector, harbors the genes of the activator of 2-hydroxyglutaryl-CoA dehydratase (*hgdC*) from *A. fermentans* and 2-hydroxyglutaryl-CoA dehydratase (*hgdAB*) from *C. symbiosum*. *E. coli* BL21(DE3) was transformed with the plasmids pID-3 and pID-4, resulting in the new recombinant strain *E. coli* ID-5 (16). The strain was grown anaerobically at 25°C for 3 h until the optical density at 600 nm (OD_{600}) reached 0.2, induced with 1.0 mM isopropylthiogalactoside and 0.5 μM anhydrotetracycline, and grown for a further 3 h to an OD_{600} of 0.6. The Standard I medium (1.5% peptone, 0.3% yeast extract, 100 mM NaCl, 5 mM glucose; Merck, Darmstadt, Germany) contained carbenicillin (100 $\mu g \cdot ml^{-1}$) and chloramphenicol (50 $\mu g \cdot ml^{-1}$). It was supplemented with 50 mM morpholinepropanesulfonic acid (MOPS), 3 mM cysteine hydrochloride, 10 mM Na-glutamate, 0.2 mM riboflavin, and 2 mM Fe(III) citrate, pH 7.4 (supplemented Standard I medium). No growth was observed on medium in which peptone and yeast extract was omitted.

Analysis by SDS-PAGE of the cell extract (100,000 × *g*, supernatant) and the pellet revealed, in both fractions, thick protein bands that were absent without induction. Spectrophotometric assays of the specific activities of the enzymes gave the following results: HgdH, 150 U mg^{-1} protein (4); GctAB, 0.25 U mg^{-1} (8); and HgdCAB, 0.46 U mg^{-1} (15). In the absence of the supplements glutamate, riboflavin, and Fe(III) citrate in the medium, the activity of HgdCAB was only 0.08 U mg^{-1} whereas those of the two other enzymes remained unchanged. Riboflavin and Fe are required for synthesis of 2-hydroxyglutaryl-CoA dehydratase and its activator, which contain riboflavin-5′-phosphate (FMN) and three [4Fe-4S] clusters as prosthetic groups (10). Hence, the expression of the six genes appeared to be successful.

The production of glutaconate in the medium was determined enzymatically and by high-pressure liquid chromatography (HPLC). The enzymatic method used acetyl-CoA, glutaconate CoA-transferase (8), glutaconyl-CoA decarboxylase (6), and the NADPH-dependent crotonyl-CoA carboxylase/reductase (9). HPLC was performed at 50°C using a C_{18} reverse-phase column (GE Healthcare) in 20 mM sulfuric acid with UV detection at 205 nm. Both assays demonstrated that glutaconate was indeed produced. After growth for 8 to 20 h, the concentration of glutaconate increased from 0.1 mM (Standard I medium) via 0.3 mM (addition of 10 mM glutamate) to 2.7 ± 0.2 mM (supplemented Standard I medium) (Fig. 2).

* Corresponding author. Mailing address: Fachbereich Biologie, Philipps-Universität, 35032 Marburg, Germany. Phone: 49 6421 2822088. Fax: 49 6421 2828979. E-mail: buckel@staff.uni-marburg.de.
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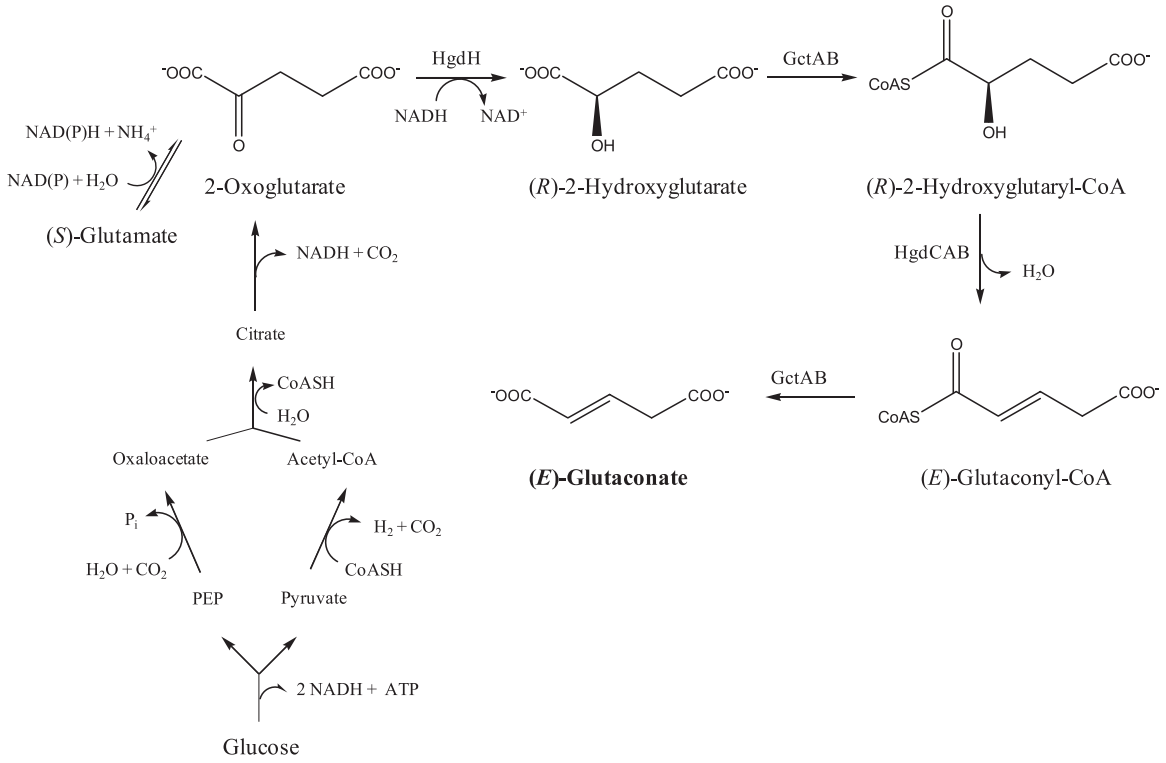

FIG. 1. Proposed pathway of glutaconate production. The genes coding for the following enzymes have been introduced into *E. coli*: HgdH, GctAB, and HgdCAB.

From measurements of the extract from cells harvested after 20 h, the cytoplasmatic glutaconate concentration was calculated as 16 mM by assuming an internal volume of 2.4 ml g^{-1} dry cells (1, 5). Hence, the export of glutaconate could be one of the rate-limiting steps. As shown in Fig. 2, glucose (5 mM) (2) was almost stoichiometrically converted to ethanol (8.7 ± 0.2 mM) (3), indicating that glutaconate was mainly produced from another carbon source, either peptone, yeast extract, or citrate. Surprisingly, this source was not glutamate, because the concentration of this essential amino acid (18) initially decreased but recovered to the original value of 13 mM when the concentration of glutaconate reached its maximum. A similar behavior of the glutamate concentration was also observed with the "wild-type" *E. coli* strain, indicating that the concentration of the most abundant compound in the cell (1) is tightly regulated.

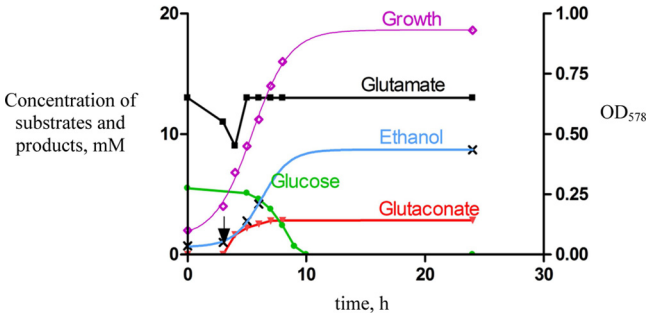

FIG. 2. Growth of the recombinant *E. coli* strain ID-5 in the supplemented Standard I medium. The arrow indicates the time of induction with isopropylthiogalactoside and anhydrotetracycline. Growth was measured as the OD at 578 nm.

In summary, the data demonstrate that the biological production of glutaconate is possible (I. Jurdjevic, O. Zelder, and W. Buckel, 22 July 2010, patent application, publication no. WO/2010/081885, German Patent and Trade Mark Office). To reach higher concentrations, however, many parameters need to be optimized, e.g., the glucose concentration, expression of the *gctAB* and *hgdCAB* genes, attenuation of ethanol production, and facilitation of glutaconate export. A further goal is the reduction of glutaconyl-CoA to glutaryl-CoA, possibly by expressing the gene coding for the nondecarboxylating glutaryl-CoA dehydrogenase (17). This could lead to a glutarate-producing strain.

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