Production of glutaconic acid in recombinant *Escherichia coli*

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Die im zeitlichen Rahmen dieser Dissertation erzielten Ergebnisse sind in folgenden Publikationen veröffentlicht:

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METHOD FOR THE PRODUCTION OF GLUTACONATE.
International patent application WO/2010/081885

Djurdjevic, I., Zelder, O., Buckel, W. (2010)

Production of glutaconic acid in a recombinant *Escherichia coli strain*.
Appl Environ Microbiol (in submitting)

„Phantasie ist wichtiger als Wissen,

denn Wissen ist begrenzt.“

Albert Einstein

Contents

Glutarsäure (Propan-1,3-dicarbonsäure) und Glutaconsäure (Propen-1,3-dicarbonsäure) dienen als Monomere zur Produktion von Polymeren. Glutarsäure könnte anstelle von Adipinsäure zur Herstellung von Polyestern, wie das biologisch abbaubare Ecoflex, ein Produkt der BASF, verwendet werden. Glutaconsäure könnte mit Diaminen zu Polyamiden kondensiert werden. Zusätzlich ist diese α,β-ungesättigte Säure für radikalische Polymerisationen geeignet. Aus diesen Gründen wollen wir diese interessanten Säuren biologisch produzieren.

Das ideale Ausgangsmaterial für die biotechnologische Produktion von Glutaconsäure wäre Glutaminsäure, die leicht aus Zucker hergestellt werden kann. Allerdings ist die α,β-Deaminierung dieser α-Aminosäure chemisch unmöglich. Im Gegensatz dazu fermentieren die strikt anaeroben Darmbakterien *Acidaminococcus fermentans* und *Clostridium symbiosum* Glutamat über 2-Oxoglutarat, (*R*)-2-Hydroxyglutarat, (*R*)-2-Hydroxyglutaryl-CoA und Glutaconyl-CoA zu Ammonium, CO_2, Acetat, Butyrat und H_2. Hemmnung der Decarboxylierung von Glutaconyl-CoA zu Crotonyl-CoA würde zu Glutaconat führen. Wir erreichten dieses Ziel auf einem anderen Weg, der Umwandlung von *Escherichia coli* in einen Glutaconatproduzenten. Dazu exprimierten wir in diesem *Bakterium* sechs Gene, die folgende Enzyme kodieren: (*R*)-2-Hydroxyglutarat-Dehydrogenase (HgdH), Glutaconat-CoA-Transferase (GctAB) und der extrem sauerstoffempfindliche Aktivator der Dehydratase (HgdC) aus *A. fermentans,* sowie die ebenfalls sauerstoffempfindliche 2-Hydroxyglutaryl-CoA-Dehydratase (HgdAB) aus *C. symbiosum.* So produzierte der rekombinante *E. coli* Stamm fünf Stunden nach Induktion der Genexpression 2.7 ± 0.2 mM Glutaconat. Das Medium enthielt 1.5% Pepton, 0.3% Hefeextrakt, 100 mM NaCl, 5 mM Glucose, 10 mM Glutamat, 3 mM Cystein, 2 mM Eisen(III)citrat, 0.2 mM Riboflavin und Antibiotika. Interessanterweise sank die Glutamatkonzentration anfangs um 30%, stieg aber später wieder auf den ursprünglichen Wert, während die Glucose fast quantitativ zu zwei Äthanol umgesetzt wurde.

Die Reduktion von Glutaconyl-CoA zu Glutaryl-CoA wird von einem Enzym katalysiert, das an der Synthese von Cyclohexancarboxylat und Benzoat in *Syntrophus aciditrophicus* beteiligt ist. Vorläufige Koexpression der Gene in *E. coli*, die für Glutaryl-CoA-Dehydrogenase und das Electron-transferring Flavoprotein (EtfAB) von *S. aciditrophicus* kodieren, bilden ein Enzymsystem, das zusammen mit

einer Hydrogenase die Bifurkation von 2 NAD(P)H zu Glutaconyl-CoA und Ferredoxin ermöglicht. Somit entstanden Glutaryl-CoA und H_2, obgleich mit einer sehr niedrigen Rate.

Glutaric and glutaconic acids serve as monomers for the production of polymers. Glutaric acid (pentanedioic acid) might be used for polyester synthesis, related to the biodegradable Ecoflex available from BASF. Glutaconic acid (pentenedioic acid) could be applied for the formation of polyamides by polymerization with diamines. Furthermore this α,β-unsaturated dicarboxylic acid is suitable for radical polymerization. Therefore we became interested in the biological production of these dicarboxylic acids.

The ideal material for biotechnological production of glutaconic acid would be glutamic acid, obtained by sugar fermentation. The chemical deamination of this α-amino acid to glutaconate is not executable. In contrary to this, strictly anaerobic bacteria, as are *Acidaminococcus fermentans* and *Clostridium symbiosum* can easily ferment glutamate to ammonia, acetate, butyrate, CO_2 and H_2 via 2-oxoglutarate, (*R*)-2-hydroxyglutarate, (*R*)-2-hydroxyglutaryl-CoA, and glutaconyl-CoA. Inhibition of the subsequent decarboxylation to crotonyl-CoA would lead to glutaconate. We achieved this aim on another route, the conversion of *Escherichia coli* into a glutaconate producer by introducing six genes encoding (*R*)-2-hydroxyglutarate dehydrogenase (HgdH), glutaconate CoA-transferase (GctAB), and the extremely oxygen sensitive activator of the dehydratase (HgdC) from *A. fermentans* as well as the also oxygen sensitive (*R*)-2-hydroxyglutaryl-CoA dehydratase (HgdAB) from *C. symbiosum.* Hence, within 5 h after induction of gene expression the recombinant *E. coli* produced 2.7 ± 0.2 mM glutaconate on a medium containing 1.5% peptone, 0.3 % yeast extract, 100 mM NaCl, 5 mM glucose, 3 mM cysteine, 10 mM glutamate, 2 mM ferric citrate, 0.2 mM riboflavin, and antibiotics. Interestingly, initially the concentration of glutamate decreased by 30% but later regained its original level, whereas glucose was almost quantitatively converted to two ethanol.

The reduction of glutaconyl-CoA to glutaryl-CoA is catalyzed by an enzyme involved in the synthesis of cyclohexanecarboxylate and benzoate in *Syntrophus aciditrophicus*. Preliminary experiments indicate that coexpression of the genes encoding glutaryl-CoA dehydrogenase and electron-transferring flavoprotein (EtfAB) from *S. aciditrophicus* in *E. coli* yield an enzyme system that together with hydrogenase catalyzes the bifurcation of 2 NAD(P)H to glutaconyl-CoA and ferredoxin. Thus glutaryl-CoA and H_2 were formed though at a very low rate.

Introduction

1.1 Biological production of fine chemicals

From year to year raises the demand for replacement of petroleum-derived chemicals. The biological production of trade chemicals is not a novelty; it has considerable history (Dodds and Gross, 2007). Until 1930, the most important products of that time, such as ethanol, butanol, acetic, citric, lactic acid and other chemicals, were produced from biomass. Afterwards many of these processes were replaced by the cheaper chemical synthesis based on petroleum. At that time, environmental influence and the oil-source limitation were not considered (Willke and Vorlop, 2004).

The list of chemicals produced by biotechnological processes increases every day. Ethanol is so far the largest product by volume and is the most common biofuel. It is mainly produced by sugar fermentation in yeast. It has a lot of advantages over conventional fuels, as are renewable sources and reduced greenhouse gas emissions (Farrell et al., 2006). The disadvantage is that the producing process of biofuels releases CO_2; biofuels give out more nitrogen oxide emissions, implicated in greenhouse gas (N_2O) and acid rain (NO_x), less suitable for use at low temperature, and more expensive than oil based fuel. Butanol, as a better gasoline substitution, can be produced in *Clostridium acetobutylicum* by the ABE process (**a**cetone-**b**utanol-**e**thanol fermentation) (Inui et al., 2008). Also, vitamin C and antibiotics are obtained from biomass. Lactic acid, which has applications in food and detergent industry, is mainly produced by *Lactobacillus* species.

Microbes exclusively produce many compounds in large scale industrial processes. *Corynebacterium glutamicum* was found to be a very efficient fermenter of L-glutamic acid. Lysine, valine, isoleucine, threonine, aspartic acid, and alanine are among other amino acids produced in *Corynebacteria* (Hermann, 2003).

Biotechnological production of vitamin B_2 (riboflavin) successfully replaced chemical productions, due to lower costs and improved eco-efficiency. Various

biotechnological processes have been developed for industrial scale riboflavin biosynthesis using different microorganisms, including fungi (*Ashbya gossypii*), yeast (*Candida famata*) and bacteria (*Bacillus subtilis*) (Stahmann et al., 2000). The chemical company BASF (Ludwigshafen, Germany) has modified *A. gossypii*, which is a natural riboflavin producer. Manipulation of the purine pathway in *A. gossypii* increased riboflavin production tenfold, comparing with the wild type (Jimenez et al., 2005).

1.2 Biopolymers

Biopolymers occur in many organisms. These include polymers out of monomers from renewable resources.

Lactic acid is used as a monomer for producing polylactic acid (PLA), a biodegradable polyester. It is derived from renewable sources, such as corn, starch, or sugarcane. Microbial biosynthesis of polyhydroxybutyrate (PHB) starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA, which is reduced to D-hydroxybutyryl-CoA. This compound is then used as a monomer to polymerize PHB. Poly-3-hydroxybutyrate is the most common type of polyhydroxyalkanoate, but many other polymers of this class are produced by a variety of organisms: these include poly-4-hydroxybutyrate, polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH) and polyhydroxyoctanoate (PHO). Biopol, made from PHB and PHV, is a nontoxic and biodegradable polymer used in medical industry.

Bakelite (polyoxybenzylmethylenglycolanhydride) is one of the first polymers made from synthetic components. Polyamides can be classified as homo- and heteropolyamides, depending on the type of monomer. The amino group and the carboxylic acid group can be on the same monomer (homopolyamide) or the polymer can be constituted of two different bifunctional monomers, one with two amino groups, and the other with two carboxylic acids (heteropolyamide). The most common variants are PA 6-6, also called Nylon 6-6, made of 1,6-diaminohexane and

adipic acid, and PA 6 (Nylon 6), known as Perlon, made of caprolactam. PA 5-10, made from penthamethylene diamine and sebacic acid, was studied by W. Carothers even before Nylon 6-6 and has superior properties, but is more expensive to make.

Cadaverine can be produced by decarboxylation of the amino acid lysine in *C. glutamicum* (Zelder et al., 2009). There is idea to use this 1,5-diaminopentanoate and unsaturated dicarboxylic acid (glutaconic acid) as monomers for production of polyamide, related to Nylon (this work).

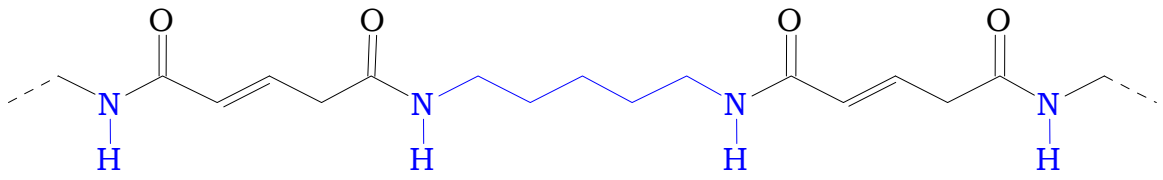


Figure 1. Polyamide made of two different monomers, *trans*-glutaconic acid (black) and 1,5-diaminopentane (blue).

Glutaconic acid is a *trans*-α,β-unsaturated C5-dicarboxylic acid. It exists as a colorless, crystalline compound and is related to the saturated glutaric acid. In the inherited disease glutaric aciduria, glutaric and glutaconic acids accumulate and interfere with brain development (Hoffmann and Zschocke, 1999).

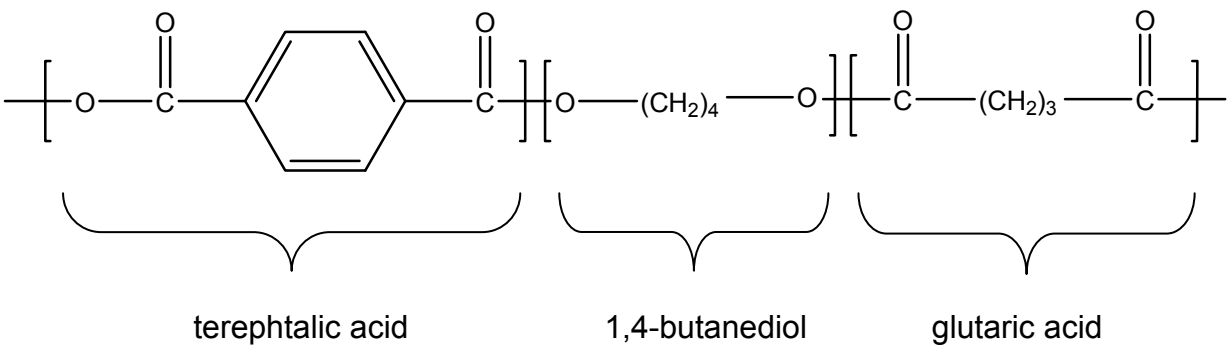


Figure 2. Polyester related to Ecoflex

The saturated C5 dicarboxylic acid (glutaric acid) cannot be involved in the polyamide synthesis, because of cyclization during polymerization and therefore is only used for production of polyesters, related to Ecoflex (instead of one carbon longer adipic acid).

The German chemical company BASF makes Ecoflex for food packaging applications. This fully biodegradable aliphatic-aromatic copolyester is made of 1,4-butanediol, adipic acid and some terephthalic acid (Witt et al., 2001). It was proposed the synthesis of glutarate by oxidative degradation of lysine in *Pseudomonas aeruginosa* (Yamanishi et al., 2007).

Succinic acid is an intermediate of the Krebs cycle and one of the fermentation end-products of the anaerobic metabolism in many microorganisms. It can be obtained from sucrose, glucose or glycerol. Polyamide PA 4-4, based on succinic acid and 1,4-diaminobutane, has been manufactured until now only on a laboratory scale (Bechthold et al., 2008). PA 4-6, known under the trade name Stanyl®, is an aliphatic polyamide formed from 1,4-diaminobutane and adipic acid. Stanyl® has better properties, such as a higher melting temperature, a higher cristallinity, faster crystallization, and better chemical resistance than PA 6 or 6-6. This polyamide has been used in food, pharmaceutical and chemical industries (Song and Lee, 2006).

Some biopolymers are biodegradable; they are broken down to CO_2 and water by microorganisms. Some of these biodegradable biopolymers are compostable and they are marked with a 'compostable' symbol, under European Standard EN 13432 (2000). Packaging marked with this symbol can be put into industrial composting processes and will break down within 6 months (or less). An example of a compostable polymer is a PLA (polylactic acid) film less than 20 μm thick.

The fact that biopolymers are made only from natural materials guarantees that they are not toxic to the environment and decomposing will not relieve any chemicals in the soil or in the water.

But not all the things are favorable to bioplastic. The degradation of the bioplastic is limited by a series of factors, which are impossible to achieve in nature. For example, most biodegradable cups are made from PLA. To degrade PLA, it must hydrolyse for

which heat and moisture are required. So, if we throw that PLA cup in the waste, where it will not be exposed to the heat and moisture, it will be there for decades, much like an ordinary plastic cup.

It is also possible that bacteria have the ability to degrade plastics. There are two types of nylon eating bacteria, *Flavobacteria* and *Pseudomonas*. These bacteria hydrolyse nylon 6, catalyzed by nylonase, to 6-aminohexanoate, which serves as carbon and energy source. (Prijambada et al., 1995).

1.3 Glutamate fermentation by clostridia

Bacteria from the orders Clostridiales, Fusobacteriales and few other anaerobes can use amino acids as energy substrates (Barker, 1961; Boone et al., 2001; Jackins and Barker, 1951). These organisms are able to ferment amino acids to ammonia, carbon dioxide, acetate, short chain fatty acids and molecular hydrogen. Their natural habitats are soil, sewage sludge, marine and freshwater sediments, and the gastrointestinal tract of animals. In these anoxic environments, the bacteria participate in the anaerobic food chain, where polymers such as proteins are finally degraded to methane, CH_4, and carbon dioxide, CO_2. Anaerobic degradation of carbohydrates and proteins starts with the hydrolysis of these macromolecules by the action of exogenous enzymes to sugars, small peptides and single amino acids. The consecutive fermentative step produces CO_2, acetate, short chain fatty acids, H_2, and ammonia.

Acetogenic organisms use carbon dioxide and hydrogen for the synthesis of acetate, whereas syntrophic bacteria oxidise the short chain fatty acids to acetate, CO_2, and H_2. The latter process is thermodynamically possible only if methanogenic archaea keep the partial pressure of H_2 at very low values, by reducing CO_2 to CH_4.

Clostridia ferment glutamate via three different pathways; via (*R*)-2-hydroxyglutarate, (2*S*,3*S*)-3-methylaspartate and 4-aminobutyrate. The major two pathways are the methylaspartate and hydroxyglutarate pathways, named after their specific intermediates. These two pathways lead to ammonia, carbon dioxide, acetate,

butyrate and hydrogen as in the following equation (Buckel and Barker, 1974; Härtel and Buckel, 1996).

equation 1

$$5\ \text{Glutamate}^- + 6\ H_2O + 2\ H^+ \rightarrow 5\ NH_4^+ + 5\ CO_2 + 6\ \text{Acetate}^- + 2\ \text{Butyrate}^- + H_2$$

$$\Delta G^{\circ\prime} = -63.4\ \text{kJ/mol Glutamate}$$

The third pathway of glutamate fermentation has 4-aminobutyrate as intermediate. The pathway requires two organisms, one to decarboxylate glutamate to 4-aminobutyrate and the other to ferment this product to acetate and butyrate.

equation 2

$$\text{Glutamate}^- + H^+ \rightarrow CO_2 + \text{4-aminobutyrate}$$

$$\Delta G^{\circ\prime} \text{ ca. } -26.4\ \text{kJ/ mol Glutamate}$$

equation 3

$$2\ \text{x 4-Aminobutyrate} + 2\ H_2O \rightarrow 2\ NH_4^+ + 2\ \text{acetate}^- + \text{butyrate}^- + H^+$$

$$\Delta G^{\circ\prime} \text{ ca. } -37\ \text{kJ/ mol 4-aminobutyrat}$$

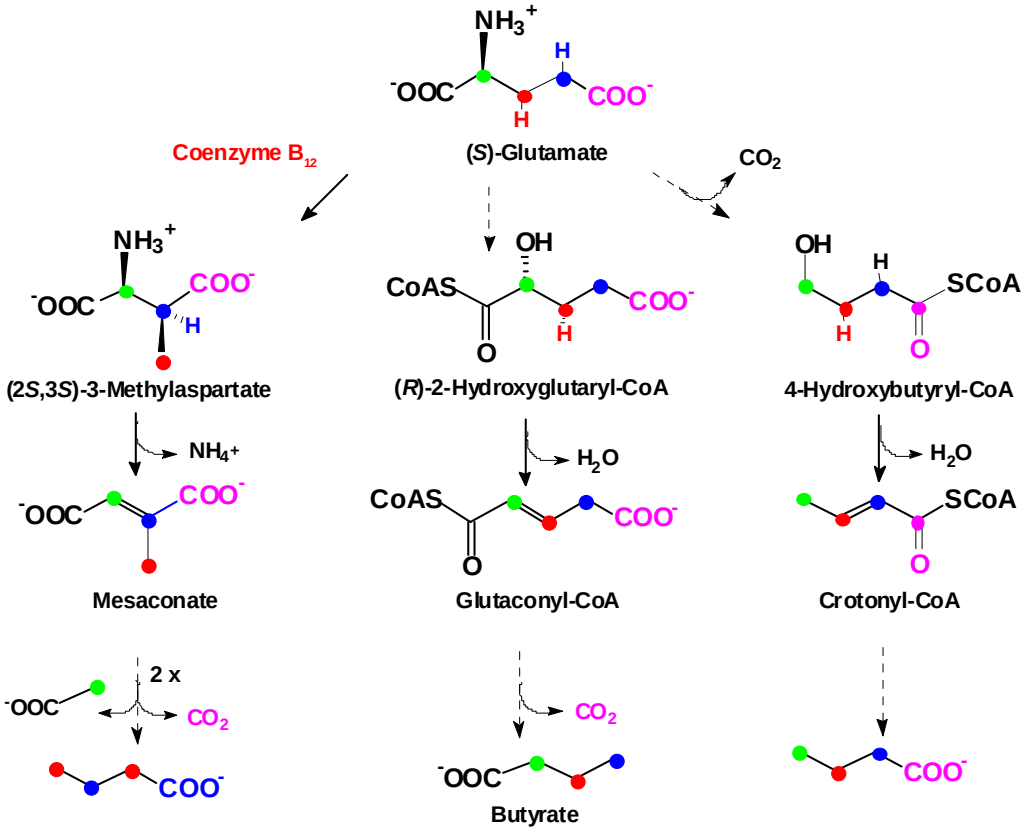


Figure 3. Three different clostridial fermentations leading from (*S*)-glutamate to butyrate as elucidated by the use of isotopically labeled glutamate and characterization of enzymes.

1.3.1 2-Hydroxyglutarate pathway

This pathway has been found in *Acidaminococcus fermentans* (Acidaminococcaceae), *Clostridium sporosphaeroides*, *Clostridium symbiosum* (Clostridiaceae), *Fusobacterium nucleatum* (Fusobacteriaceae) and *Peptostreptococcus asaccharolyticus* (Peptostreptococcaceae) (Buckel, 1980a; Buckel and Barker, 1974). These organisms live in anoxic niches within humans and animals. Glutamate is fermented via (*R*)-2-hydroxyglutaryl-CoA, glutaconyl-CoA and crotonyl-CoA. The latter disproportionates to acetate, butyrate and H_2 (figure 4, equation 1), the same products as those formed by *Clostridium tetanomorphum* via 3-methylaspartate. Coenzyme B_{12} is absent in the hydroxyglutarate pathway, unlike the methylaspartate pathway where this cofactor plays an important role in the first step.

The hydroxyglutarate pathway starts with the oxidative NAD^+-dependent deamination of glutamate to 2-oxoglutarate, which is catalyzed by glutamate dehydrogenase. This step is followed by 2-hydroxyglutarate dehydrogenase and glutaconate-CoA transferase. The key intermediate is (*R*)-2-hydroxyglutaryl-CoA, which is dehydrated to glutaconyl-CoA, followed by decarboxylation to crotonyl-CoA (Buckel and Semmler, 1983). The hydroxyglutarate pathway contains an unusual biochemical transformation, the reversible dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA (Buckel, 1980b; Hans et al., 2000; Hans et al., 1999; Schweiger et al., 1987). This transformation is carried out by the (*R*)-2-hydroxyglutaryl-CoA dehydratase, in the presence of its activator. During this dehydration the 3*Si*-proton has to be removed from the non-activated β-position (pK_a = 40), whereas the hydroxyl anion is released from the α-position. The activation of this proton is achieved by addition of one high-energy electron to the thioester carbonyl, forming a ketyl radical that eliminates the hydroxyl group (figure 5). It has been shown that the pK_a of the 3*Si*-proton of the resulting enoxy radical intermediate is lowered by 26 units (Smith et al., 2003). Deprotonation of this radical leads to the product related allylic ketyl radical that was identified by EPR spectroscopy in case of (*R*)-2-hydroxyisocaproyl-CoA dehydratase from *Clostridium difficile* (Fu et al., 2004). The radical generator is the ATP dependent activator enzyme, which is initially reduced by ferredoxin or dithionite (Kim et al., 2008).

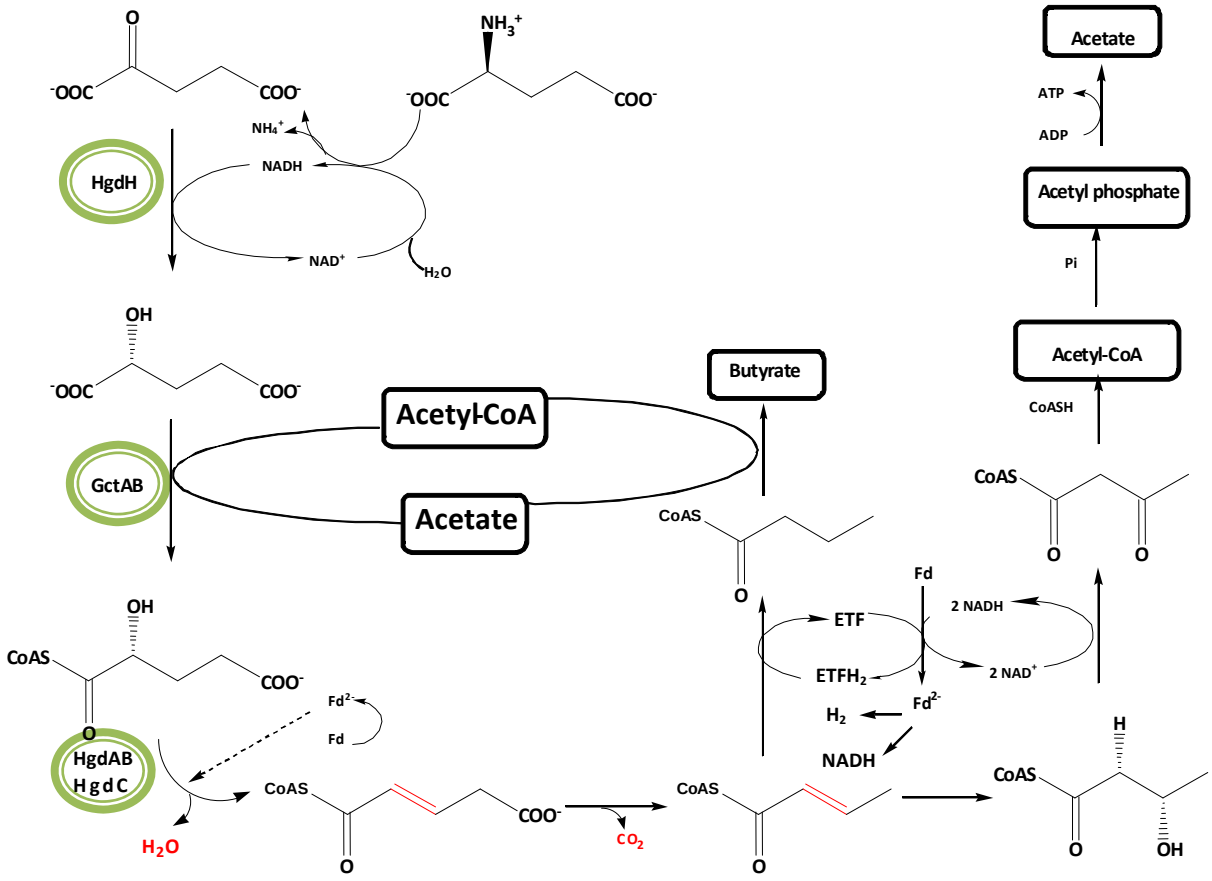


Figure 4. Proposed pathway of glutamate fermentation via 2-hydroxyglutarate. HgdH (2-hydroxyglutarate dehydrogenase), GctAB (glutaconate CoA-transferase), HgdAB (2-hydroxyglutaryl-CoA dehydratase) and HgdC (activator of the dehydratase) were used in this work.

Variations of this remarkable enzyme system are found in *C. symbiosum*, *A. fermentans* and *F. nucleatum*. Component D from *C. symbiosum* contains two [4Fe-4S]$^{2+}$ clusters instead of the one found in component D of *A. fermentans*, although both components D share 70 % sequence identity.

In the 2-hydroxyglutarate pathway (figure 5, equation. 1), extra energy is conserved via $\Delta\mu Na^+$ generated by decarboxylation of glutaconyl-CoA (Buckel, 2001; Buckel and Semmler, 1983). Glutaconyl-CoA decarboxylase is integrated in the membrane, contains biotin and uses the free energy of decarboxylation to pump Na^+ to the outside.

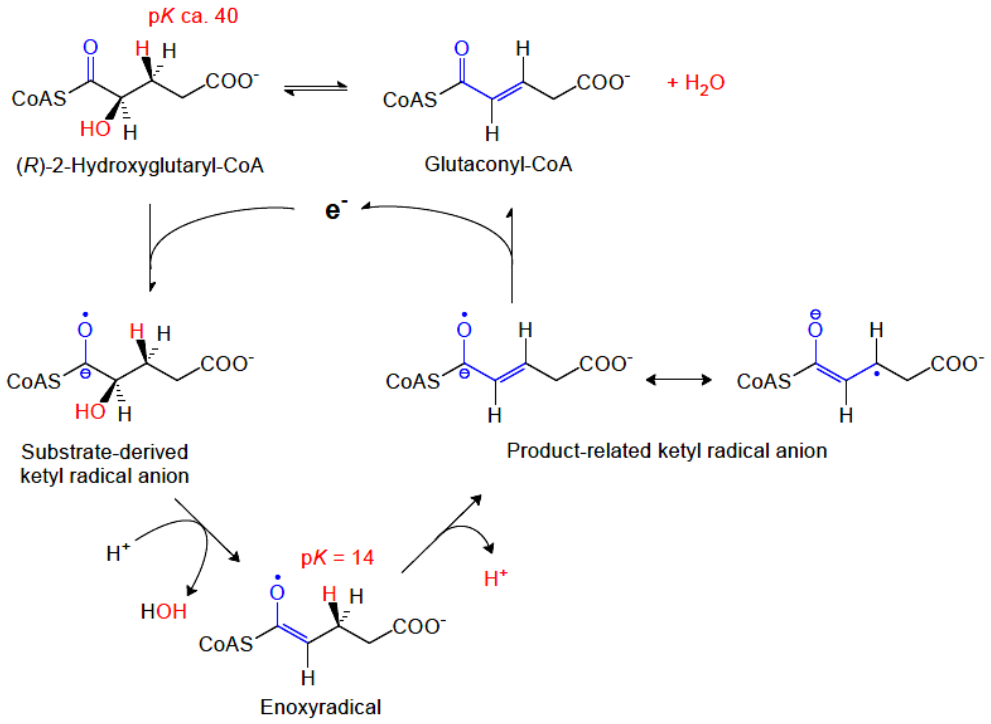
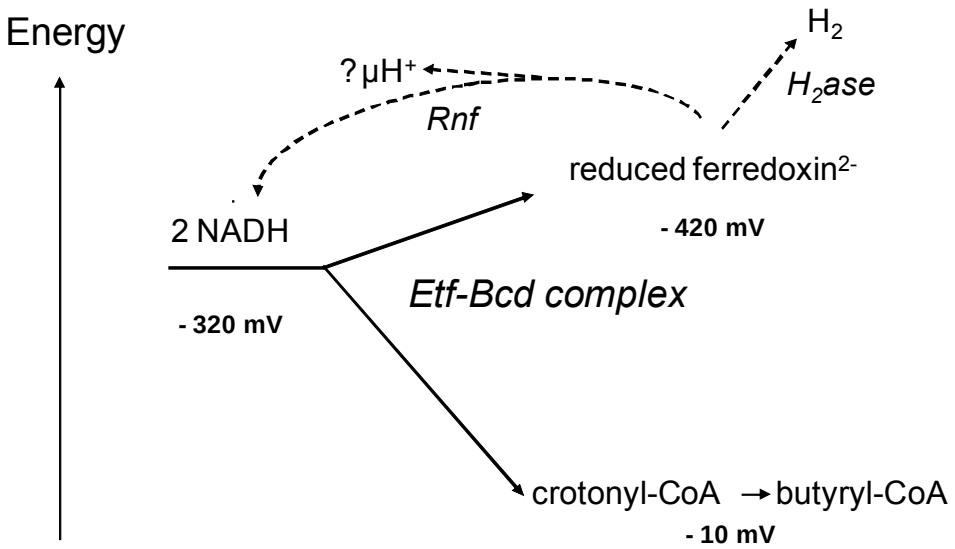


Figure 5. Proposed mechanism for the dehydration of (*R*)-2-hydroxyacyl-CoA to (*E*)-2-enoyl-CoA. As example (*R*)-2-hydroxyglutaryl-CoA was used.

After decarboxylation, the pathway branches in an oxidative and a reductive part. In the reductive part, crotonyl-CoA is reduced to butyryl-CoA (E_0' = - 10 mV) by NADH (E_0' = - 320 mV). This reduction is highly exergonic and irreversible under physiological conditions ($\Delta G^{\circ`}$ = -60 kJ mol^{-1}) and, as proposed by Herrmann et al. (Herrmann et al., 2008) and experimentally verified by Li et al. (Li et al., 2008), is coupled with the exergonic reduction of ferredoxin (E_0' = - 410 mV) with NADH. Two electrons from NADH are bifurcated by the Etf-Bcd complex from anaerobic bacteria, one electron is transferred via butyryl-CoA dehydrogenase to more positive electron acceptor crotonyl-CoA and the other electron is transferred to the more negative acceptor ferredoxin (Fd → Fd^-) (figure 6). The next NADH carries the electrons for complete reduction of crotonyl-CoA to butyryl-CoA and Fd^- to Fd^{2-}. The reduced ferredoxin can be reoxidized either by NAD^+, catalyzed by Rnf with generation of $\Delta\mu H^+$ or $\Delta\mu Na^+$ (Herrmann et al., 2008), or by protons, mediated by a hydrogenase with the formation of molecular hydrogen. In the oxidative part, crotonyl-CoA is

hydrated to 3-hydroxybutyryl-CoA and oxidized to acetoacetyl-CoA, where at the end two acetates are produced.

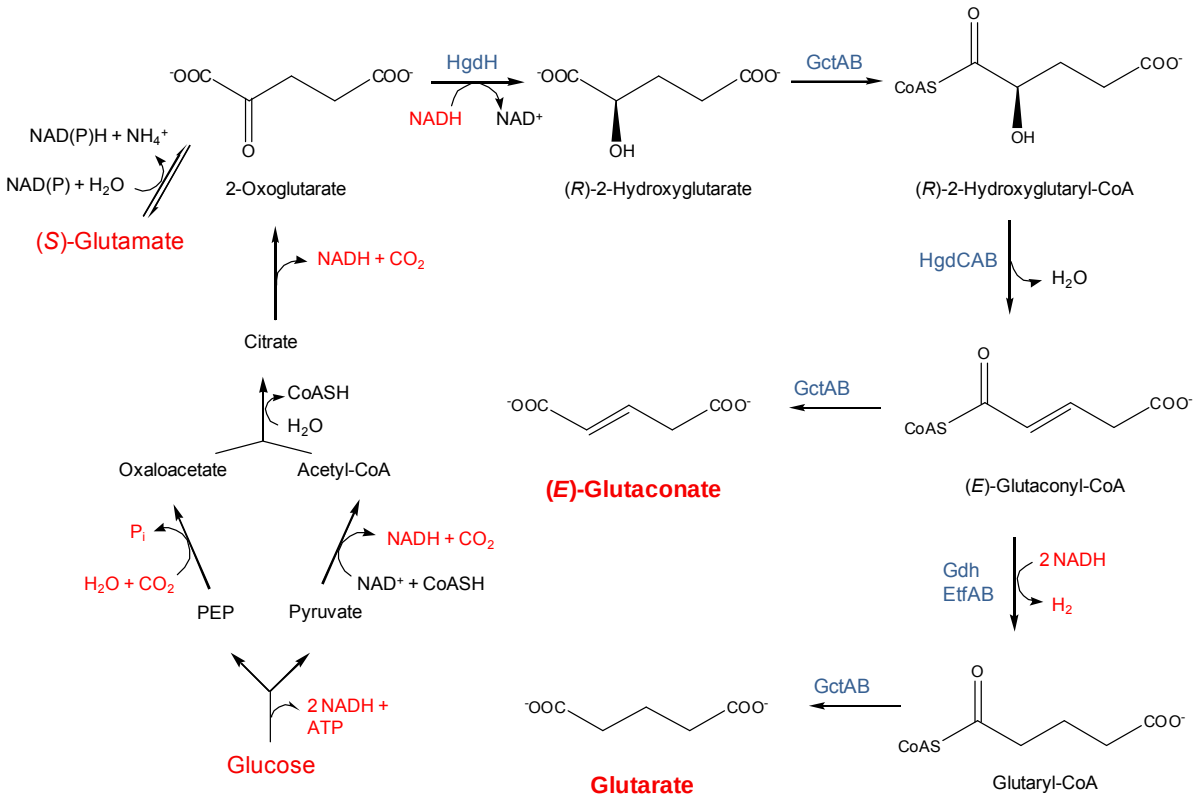


$\Delta E_0' = (-420\ \text{mV}) + (-10\ \text{mV}) - (2 \times -320\ \text{mV}) = +210\ \text{mV}$

$\Delta G^{\circ\prime} = -41\ \text{kJ mol}^{-1}$ butyryl-CoA

Figure 6. Electron bifurcation in BCD/ETF complex. Electrons are transfered to the butyryl-CoA dehydrogenase and ferredoxin. Oxidation of ferredoxin is catalysed by hydrogenase or Rnf complex.

1.4 Proposed pathway for glutaconate and glutarate production



$$\text{Glusose } (C_6H_{12}O_6) \longrightarrow \text{Glutaconate}^{2-}\ (C_5H_4O_4) + 2\ H^+ + CO_2 + H_2 + 4\ H$$

$$\text{Glusose } (C_6H_{12}O_6) \longrightarrow \text{Glutarate}^{2-}\ (C_5H_6O_4) + 2\ H^+ + CO_2 + 2\ H_2$$

Figure 7. Proposed pathway for glutaconate and glutarate production from glucose. HgdH: (*R*)-2-Hydroxyglutarate dehydrogenase, GctAB: Glutaconate CoA transferase, HgdCAB: (*R*)-2-Hydroxyglutaryl-CoA dehydratase (AB) and activator (C), Gdh: Glutaryl-CoA dehydrogenase, EtfAB: Electron-transferring flavoprotein.

$$\text{Pyruvate}^- + \text{CoASH} \rightarrow \text{Acetyl-CoA} + \text{Formate}^- \quad \text{(Pyruvate formate-lyase)}$$

$$\text{Formate}^- + H^+ \rightarrow CO_2 + H_2 \text{ (Formate hydrogen-lyase)}$$

For the conversion of *Escherichia coli* to a glutaconate producer, six genes encoding 2-hydroxyglutaryl-CoA dehydratase (HgdAB, figure 7) from *C. symbiosum,* the activator of the dehydratase (HgdC), 2-hydroxyglutarate dehydrogenase (HgdH) and glutaconate CoA-transferase (GctAB) from *A. fermentans* were introduced. The new pathway can divert at 2-oxoglutarate derived from glucose via the Embden-Meyerhof pathway and the citrate cycle.

For production of glutarate, the genes *gdh* and *etfAB* encoding glutaryl-CoA dehydrogenase and electron transferring flavoprotein, respectively, must be additionally introduced into the *E. coli* (figure 7). Both proteins are present in *S. aciditrophicus*, a strictly anaerobic bacterium with syntrophic metabolism.

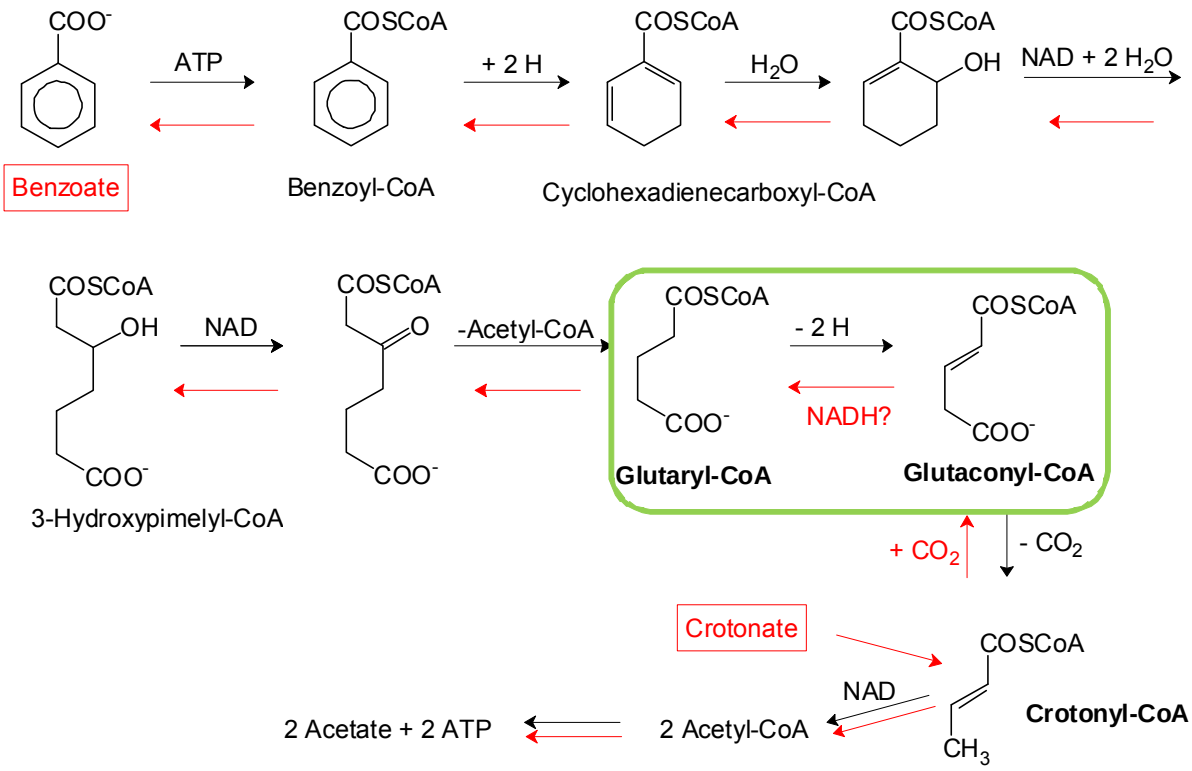


Figure 8. Benzoate oxidation and crotonate fermentation by *S. aciditrophicus SB*

When *S. aciditrophicus* is grown on benzoate, glutaryl-CoA and glutaconyl-CoA are late intermediates, where glutaryl-CoA is oxidized to glutaconyl.CoA. This *β*-oxidation reaction is catalyzed by the non-decarboxylating glutaryl-CoA dehydrogenase (Gdh). Also these two intermediates are present when this organism is grown on crotonate. Probably the same enzymes catalyze the reaction in both directions (figure 8).

1.4.1 (*R*)-2-Hydroxyglutarate dehydrogenase from *A. fermentans*

(*R*)-2-Hydroxyglutarate dehydrogenase (HgdH) belongs to the D-2-hydroxyacid dehydrogenase protein family (Grant, 1989). This enzyme is involved in the second step of the hydroxyglutarate pathway in glutamate fermentation. It catalyzes the reduction of 2-oxoglutarate to (*R*)-2-hydroxyglutarate, coupled with the oxidation of NADH to NAD^+ (figure 9). The 4*Re*-hydrogen at the nicotinamide moiety of NADH is transferred as hydride (Berk et al., 1996), making HgdH an "A-side" specific dehydrogenase (Benner, 1982).

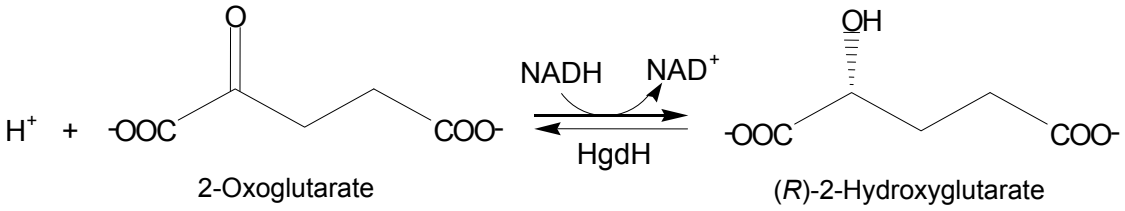


Figure 9. Reduction of 2-oxoglutarate to (*R*)-2-hydroxyglutarate with NADH catalyzed by (*R*)-2-hydroxyglutarate dehydrogenase (HgdH).

(*R*)-2-Hydroxyglutarate dehydrogenase from *A. fermentans* is a homodimer (2 x 36.5 kDa) with the optimum pH 8.0 and a K_m value of 134 µM for 2-oxoglutarate (at 100 µM NADH), the reported specific activity is 4800 U/mg (Bresser, 1997). The equilibrium constant, K_{eq} = 1.47×10^{-12} M at pH 7 lies on the side of (*R*)-2-hydroxyglutarate production (Buckel and Miller, 1987).

As possible alternative substrates, two dicarboxylic acids were tested in our lab, oxalocrotonic acid or 2-oxohexene-dioic acid and 2-oxoadipic acid (Parthasarathy, 2009). The kinetic parameters measured at 0.1 mM of NADH for 2-oxoadipic acid were V_{max} = 800 ± 30 U/mg and K_m = 0.88 ± 0.03 mM, and for 2-oxohex-3-enedioic acid were V_{max} = 390 ± 10 U/mg and K_m = 1.1 ± 0.3 mM.

1.4.2 Glutaconate CoA-transferase from *A. fermentans*

Glutaconate CoA-transferase belongs to the coenzyme A-transferase family I (Heider, 2001). Members of this group of enzymes are found in anaerobic, aerobic bacteria and in the mitochondria of humans and other mammals.

Glutaconate CoA-transferase (GctAB) from the strict anaerobic bacterium *A. fermentans* takes a role in the glutamate fermentation via the hydroxyglutarate pathway (Buckel et al., 1981). The enzyme catalyzes the transfer of the $CoAS^-$ moiety from acetyl-CoA to (*R*)-2-hydroxyglutarate, to yield (*R*)-2-hydroxyglutaryl-CoA (figure 10). Glutarate, (*E*)-glutaconate, acrylate and propionate are also good acceptors. With (*Z*)-glutaconate and C4-dicarboxylic acids no reaction was observed.

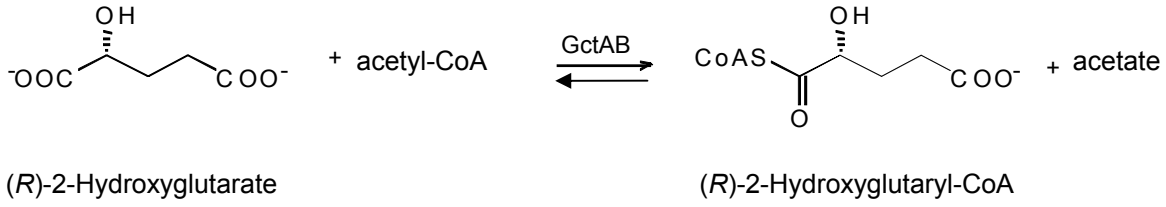


Figure 10. Transfer of the $CoAS^-$ moiety from acetyl-CoA to (*R*)-2-hydroxyglutarate to yield (*R*)-2-hydroxyglutaryl-CoA, catalyzed by glutaconate CoA-transferase (GctAB).

The enzyme is heterooctamer ($\alpha_4\beta_4$), consisting of two different subunits, α named as GctA and β named as GctB, with molecular masses 36 and 29 kDa, respectively.

Glutaconate CoA transferase was purified via ammonium sulfate precipitation, DEAE-Sephacel and Sephacryl S-300 columns from cell-free extract of *A. fermentans*, with specific activity of 65 U/mg (the specific activity in the cell-free extract is 4.2 U/mg) (Buckel et al., 1981).

The genes *gctA* and *gctB*, coding for glutaconate CoA transferase, form a cluster, 7.3 kb in length, with *gcdA* (carboxytransferase or α-subunit of glutaconyl-CoA decarboxylase) and *hgdCAB* genes (2-hydroxyglutaryl-CoA dehydratase and its activator) called hydroxyglutarate operon, since the enzymes encoding these genes are involved in the conversion of (*R*)-2-hydroxyglutarate to crotonyl-CoA in the pathway of glutamate fermentation in *A. fermentans* (figure 11).

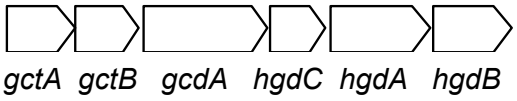
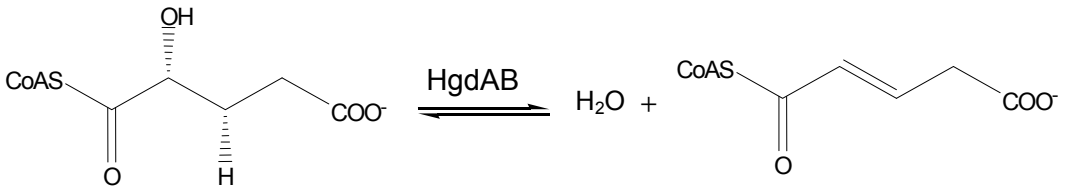


Figure 11. Hydroxyglutarate operon. The organization of genes encoding key enzymes involved in the fermentation of glutamate by the hydroxyglutarate pathway in *A. fermentans*. The genes *gctA* and *gctB* encode glutaconate CoA transferase, *gcdA* encodes the α-subunit (carboxytransferase) of glutaconyl-CoA decarboxylase, *hgdC* encodes the activator, *hgdA* and *hgdB* encode (*R*)-2-hydroxyglutaryl-CoA dehydratase.

1.4.3 (*R*)-2-Hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

(*R*)-2-Hydroxyglutaryl-CoA dehydratase belongs to the 2-hydroxyacyl-CoA dehydratase group. Seven different 2-hydroxyacyl-CoA dehydratases have been purified and characterised (table 1).

2-Hydroxyglutaryl-CoA dehydratase is involved in the reversible *syn*-elimination of water from (*R*)-2-hydroxyglutaryl-CoA, yielding (*E*)-glutaconyl-CoA. During this elimination, the non-active β-proton is removed.



(*R*)-2-Hydroxyglutaryl-CoA (*E*)-Glutaconyl-CoA

Figure 12. Reversible *syn*-dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA, catalyzed by (*R*)-2-hydroxyglutaryl-CoA dehydratase (HgdAB)

Table 1. Characteristics of 2-hydroxyacyl-CoA dehydratases

Organism	Substrate	Component A	Component D	Specific activity (s^{-1})	References
Acidaminococcus fermentans	(*R*)-2-Hydroxyglutaryl-CoA	HgdC, γ_2, 2x27 kDa; $[4Fe\text{-}4S]^{1+/2+}$	HgdAB, αβ, 54+42 kDa; $[4Fe\text{-}4S]^{2,+}$, FMN,riboflavin	10	(Hans et al., 2002; Schweiger et al., 1987)
Costridium symbiosum	(*R*)-2-Hydroxyglutaryl-CoA	Not purified[1] 2x27 kDa	HgdAB, αβ, 54+42 kDa; 2 $[4Fe\text{-}4S]^{2,+}$, FMN	50 80	(Hans et al., 1999)
Fusobacterium nucleatum	(*R*)-2-Hydroxyglutaryl-CoA	HgdC, γ_2, 2x28 kDa; $[4Fe\text{-}4S]^{1+/2+}$	HgdABD, αβδ, 49+39+24 kDa; $[4Fe\text{-}4S]^{2,+}$, riboflavin	< 50	(Klees et al., 1992)
Clostridium sporogenes	(*R*)-Phenyllactate	FldI, η_2, 2x29 kDa; $[4Fe\text{-}4S]^{1+/2+}$	FldABC, αβγ, 46+43+40 kDa; $[4Fe\text{-}4S]^{2,+}$, flavin not detected	1	(Dickert et al., 2002; Dickert et al., 2000)
Clostridium difficile	(*R*)-2-Hydroxyisocaproyl-CoA	HadI, η_2, 2x29 kDa; $[4Fe\text{-}4S]^{1+/2+}$	HadBC, βγ, 46+43 kDa 1-2 $[4Fe\text{-}4S]^{2,+}$, FMN?	150 (V_{max})	(Kim et al., 2005)
Clostridium propionicum	(*R*)-Lactyl-CoA	LcdC	LcdAB, αβ, 48+41 kDa 2 $[4Fe\text{-}4S]^{2,+}$, FMN,riboflavin	85	(Hofmeister and Buckel, 1992; Parthasarathy et al., 2010; Schweiger et al., 1987)
Megasphaera elsdenii	(*R*)-Lactyl-CoA	Not purified	LcdAB, αβ, 2 $[4Fe\text{-}4S]^{2,+}$?, FMN ?	Low	(Gokarn et al., 2004)

[1]Component A (HgdC) from *A. fermentans* was used.

The 2-hydroxyglutaryl-CoA dehydratase system from *C. symbiosum* consists of two components, the actual dehydratase (D) and its activator (A) (see 1.3.1).The gene *hgdC* from *A. fermentans* was expressed in *E. coli,* purified (Hans et al, 2002; Hans and Buckel, 2000) and the crystal structure has been solved (Locher et al., 2001).

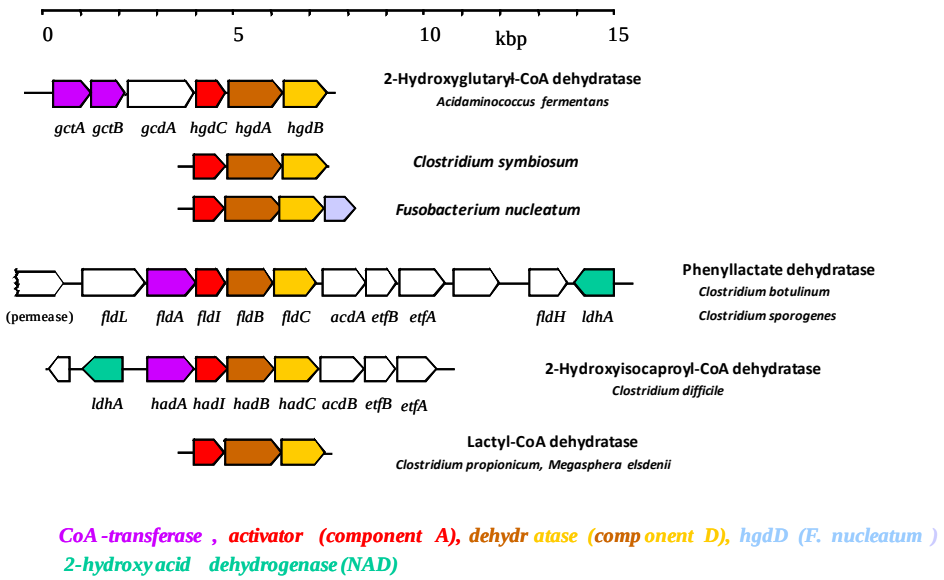


Figure 13. Genes encoding 2-hydroxyacyl-CoA dehydratases and accessory enzymes. Explanations of gene abbreviations are given in the text.

1.4.4 Glutaryl-CoA dehydrogenase from *S. aciditrophicus*

Acyl-CoA dehydrogenases are flavoproteins and catalyze the first step of the β-oxidation, whereby a *trans* double bond between C2 and C3 is formed (Thorpe and Kim, 1995). They can be categorized into three distinct groups based on their specificity for short-, medium-, or long-chain fatty acids. FAD is required as co-factor in the mechanism of the enzyme to act as electron and proton acceptor.

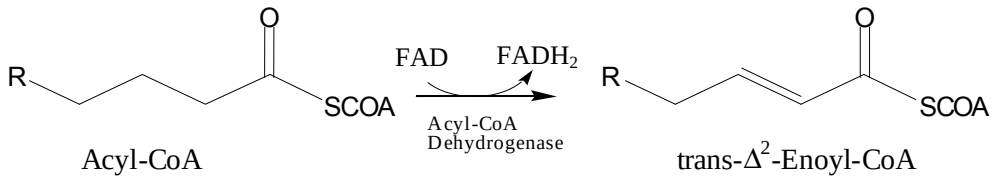


Figure 14. Beta-oxidation catalyzed by acyl-CoA dehydrogenase

There are two types of glutaryl-CoA dehydrogenases, decarboxylating and non-decarboxylating. Decarboxylating dehydrogenases catalyze the dehydrogenation and the decarboxylation of its substrate, from glutaryl-CoA crotonyl-CoA is formed. Non-decarboxylating dehydrogenases catalyze only the dehydrogenation of substrate, in this case glutaconyl-CoA is formed from glutaryl-CoA.

Acyl-CoA dehydrogenases are an important class of enzymes in mammalian cells, because of their role in the metabolism of fatty acids. The decarboxylating glutaryl-CoA dehydrogenase from human is well studied, with known crystal structure. Deficiency of this enzyme causes disease, known as glutaric acidemia type I (Hoffmann and Zschocke, 1999). This enzyme is involved in the aerobic degradation of lysine and tryptophan as well as in the anaerobic degradation of benzoate. The latter has been studied in denitrifying, facultative anaerobic *Thauera* and *Azoarcus* species. There is type II of glutaric academia, which is consequence of mutations in genes encoding electron transferring flavoprotein (EtfA or EtfB) or electron transfer flavoprotein-ubiquinone oxidoreductase (EtfDH) (Freneaux et al., 1992; Loehr et al., 1990).

Glutaryl-CoA dehydrogenase from *S. aciditrophicus* belongs to the non-decarboxylating group. It is composed of one subunit (41.8 kDa) and the gene coding for this protein is named as SYN_00480 (McInerney et al., 2007). From this group the only well studied protein is from the sulfate reducing *Desulfococcus multivorans* (Wischgoll et al., 2009), whose crystal structure was recently solved (Wischgoll et al., 2010).

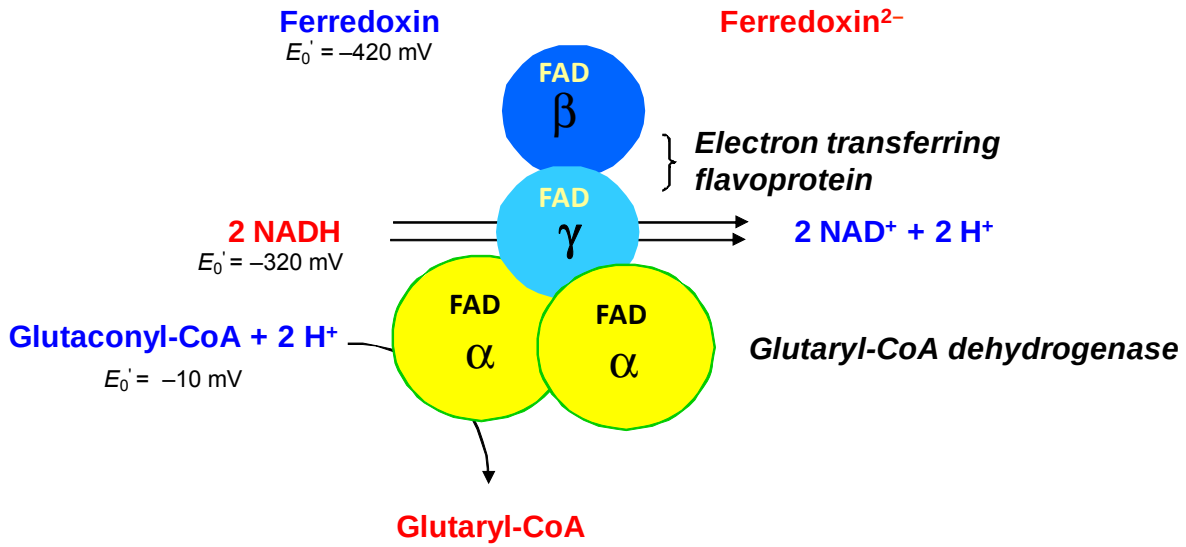
1.4.5 Electron-transferring flavoprotein from *S. aciditrophicus*

Electron-transferring flavoproteins (ETFs) of the “aerobic type” catalyze the electron transfer between other flavoproteins and quinones. Etf is composed of two different subunits (β and γ) and contains FAD, which is the redox center in the protein (O'Neill et al., 1998). Mutations in the human enzyme cause the disease glutaric aciduria type II, characterized by defects in the ability of the body to use fatty acids for energy (Freneaux et al., 1992; Loehr et al., 1990). ETF accepts electrons from acyl-CoA dehydrogenases, involved in the β-oxidation of fatty acids. ETF is reoxidized in mitochondria by ubiquinone of the respiratory chain, catalyzed by a membrane-bound ETF-quinone oxidoreductase. In anaerobic bacteria, such are members of the order Clostridiales, the “anaerobic type of ETF” together with butyryl-CoA dehydrogenase (BCD) catalyzes the NADH dependent reduction of crotonyl-CoA to butyryl-CoA (see 1.3.1).

In *C. kluyveri* and *C. tetanomorphum* ETF and BCD form a tight complex, unlike in *A. fermentans* and *Megasphaera elsdenii,* where these two proteins separate during purification (O'Neill et al., 1998; Zhen, 2008)

In *S. aciditrophicus* only two genes (SYN_02637 and SYN_02638) are found encoding the two subunits of ETF (33.6 kDa and 26.7 kDa) (figure 16). This enzyme has not been characterized until now.

Like the reduction of crotonyl-CoA to butyryl-CoA (see above) that of glutaconyl-CoA to glutaryl-CoA ($E^{\circ\prime}$ = -10 mV) by NADH ($E^{\circ\prime}$ = -320 mV) is a highly exergonic reaction. Therefore, it could be also coupled to ferredoxin reduction ($E^{\circ\prime}$ = -420 mV) by NADH (figure 15). We suppose that Gdh together with EtfAB catalyzes the two-NADH-dependent reduction of glutaconyl-CoA to glutaryl-CoA coupled to the reduction of ferredoxin.



Hydrogenase: Ferredoxin^{2-} + 2 H$^+$ → Ferredoxin + H_2

Sum: Glutaconyl-CoA + 2 NADH + 2 H$^+$ → Glutaryl-CoA + 2 NAD$^+$ + H_2

Figure 15. Reduction of glutaconyl-CoA to glutaryl-CoA with NADH, coupled with reduction of ferredoxin

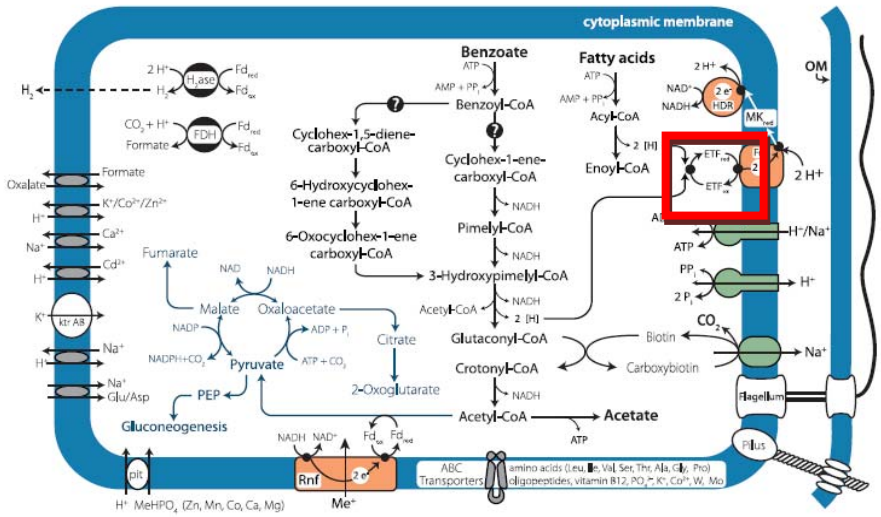


Figure 16. Metabolism of *S. aciditrophicus SB*. Red square: Electron-transferring protein (ETF).

Aims of the work

The aim is to convert *E. coli* to a glutaconate and glutarate producer.

In order to convert *E. coli* to a glutaconate producer, six genes encoding 2-hydroxyglutarate dehydrogenase, glutaconate CoA-transferase, 2-hydroxyglutaryl-CoA dehydratase and its activator have to be introduced.

To reduce glutaconate and to obtain glutarate as a main product, genes encoding glutaryl-CoA dehydrogenase and electron transferring flavoprotein have to be cloned and expressed.

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

All chemicals and biochemicals were from Roche (Mannheim, Germany), Sigma (Deisenhofen, Germany), and AppliChem. The enzymes for DNA manipulation, DNA size markers, protein molecular mass markers and the molecular mass standard for SDS/PAGE were from Fermentas GmbH (St. Leon-Rot, Germany). Sequencing primers were purchased from MWG-Biotech AG (Ebersberg, Germany). Coenzyme A is from MP Biomedicals. CoA-esters of glutaric and acetic acids were prepared from the corresponding anhydrides (Simon and Shemin, 1953).

2.1.1.1 Glutaconyl-CoA synthesis

Glutaconyl-CoA was obtained by enzymatic synthesis with glutaconyl-CoA transferase (GctAB) (Buckel et al., 1981). 20 µmol acetyl-CoA, 200-400 µmol glutaconate, and 5 U GctAB were mixed in a volume of 3-5 ml 50 mM potassium phosphate pH 7.0 and the mixture reacted at 37°C for 1 hour, after which time the mixture was acidified to pH 2.0 and filtered through a 10 kDa cut-off membrane (Amicon, Amersham Biosciences).

The CoA thioesters were purified by reverse phase chromatography through Sep-Pak C18 columns (Waters, Massachusetts USA). The column was washed with methanol and equilibrated with 0.1% TFA (v/v). The reaction mixture was loaded on the column and washed with 3 volumes 0.1% TFA . Elution was performed with 0.1% TFA containing 50% acetonitrile (v/v). The eluted CoA ester was freed from acetonitrile by drying in Speed-Vac concentrator (Bachofer, Germany). It was then refrozen and lyophillized (Alpha1-4, Christ Instruments, USA). The obtained powder was stored at -80°C.

2.1.2 Instruments and columns

Beckman (München, Germany) supplied the ultra-centrifuge, and Sorvall (München) the cooling centrifuges. All spectrophotometric assays were performed on Ultrospec 1100 *pro* spectrophotometers from Amersham Biosciences installed under aerobic or anaerobic conditions as needed, or on an Uvikon 943 double beam spectrophotometer from Kontron Instruments, Switzerland. Quartz cuvettes were used for measurements below 320 nm and disposable plastic cuvettes for measurements above 320 nm, all of which had a path length of 1 cm and a volume of 0.5 to 1 ml. HPLC columns were from Merck (Darmstadt). Strep-Tag II columns were purchased from IBA GmbH (Göttingen).

2.1.3 Anaerobic work

Anaerobic experiments have been done in an anaerobic glove box supplied by Coy Laboratories, Ann Arbor MI, (USA). The enzymatic assay for determination of 2-hydroxyglutaryl-CoA dehydratase activity was performed at 20 ^{o}C in the glove box under a nitrogen atmosphere containing 5% H_2. Buffers for the assay were prepared by boiling and cooling under vacuum. Afterwards the buffers were flushed with nitrogen and transferred to the anaerobic chamber. Enzyme activity was determined inside the anaerobic chamber with an Ultrospec 1000 *pro*.

2.1.4 Bacteria and culture media

E. coli was grown at 25°C in Standard I medium (Merck: 1.5% Peptone, 0.3 % yeast extract, 100 mM NaCl, 5 mM Glucose) and in LB medium (1% Tryptone, 1% NaCl, 0.5% yeast extract) containing antibiotic(s) depending on the harbored plasmid. The strains DH5α [F^- φ80 Δ lacZΔM15Δ(lacZYA-argF)U169, deoR, recA1, end A1,hsdR17(rk^-, mk^+), phoA, supE44,λ^-, thi-1, gyrA96, relA1] and α-competent (Bioline GmbH) were used for gene cloning and BL21 (DE3) for the gene expressions.

2.1.5 Plasmids

pACYCDuet™-1 (Novagen) (T7 promoters, His•Tag and S•Tag, Cm^R) was used for the *hgdH* and *gctAB* expression, pASK-IBA3plus (IBA GmbH) (tet promoter/operator, C-terminal Strep-tag II, cytosolic localization of the recombinant protein, Amp^R) for the *hgdC* and *hgdAB* expression, pASK-IBA7plus (IBA GmbH) (tet promoter/operator, N-terminal Strep-tag II, cytosolic localization of the recombinant protein, Amp^R) for the gdh expression, pCDFDuet™-1 (Novagen) (T7 promoters, His•Tag and S•Tag, Sm^R) for the etfAB expression.

2.1.6 Antibiotics

The stock of antibiotics was prepared and used as described below.

Antibiotic	Stock	Final concentration
Carbenicillin	100 mg/ml H_2O sterilized by filtration (0.2 µm)	100 µg/ml
Chloramphenicol	50 mg/ml 70 % ethanol	50 µg/ml
Spectinomycin	50 mg/ml H_2O sterilized by filtration (0.2 µm)	50 µg/ml

2.2 Methods for DNA work

2.2.1 Plasmid DNA isolation

Plasmid DNA isolation was done by alkaline lysis methods using GeneJET™ Plasmid Miniprep kit (Fermentas). LB medium (5 ml) containing antibiotic(s) was inoculated with a bacterial colony and incubated with gyration overnight at 37 °C. The culture was transferred into an Eppendorf tube and harvested at 13000 *g* in a microcentrifuge for 2 minutes. The bacterial pellet was suspended in 250 µl Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl pH 8.0) then lysed by adding 250 µl Solution II (0.2 M NaOH, 1% SDS), and neutralized with 300 µl Solution III (3 M potassium acetate / glacial acetic acid pH 4.8). The soluble supernatant was separated from cell debris by centrifugation for 5 minutes and transferred into a new Eppendorf tube.

2.2.2 Genomic DNA isolation from *S. aciditrophicus SB*

Genomic DNA from *S. aciditrophicus SB* used in this work was kind gift from Marie Kim (Philipps-Universität Marburg).

For genomic DNA isolation, 2 g of *S. aciditrophicus* SB cells were suspended in 3 ml Tris-sucrose buffer (10 mM Tris/HCl pH 8.0, 25% sucrose). The suspended cells were incubated at 37 °C for 90 minutes with gentle shaking after adding 100 mg lysozyme. Then, 4 ml 10 mM Tris/HCl pH 8.0, 25 mM EDTA was added and incubated on ice for 15 minutes. After adding 20 mg proteinase K and 100 mg RNase, the mixture was incubated at 37 °C for 3 hours. The protein was removed by extraction with 3 x saturated phenol and 1 x chloroform/isoamylalcohol (24:1). The aqueous phase was transferred to a dialysis bag for overnight dialysis in TE (10 mM Tris/HCl, 1 mM EDTA pH 8.0) buffer.

2.2.3 Agarose gel electrophoresis

Agarose powder was mixed with electrophoresis TAE-buffer (40 mM Tris/acetate, 1 mM EDTA) to the desired concentration, and then heated in a microwave oven until it completely melted. After cooling the solution to about 60°C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel had solidified, the comb was removed and the gel was inserted horizontally into the electrophoresis chamber just covered with buffer. DNA samples mixed with 6X *MassRuler*™ Loading Dye Solution (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 60% glycerol and 60 mM ETDA) were then pipetted into the sample wells, and a voltage was applied. Bromophenol blue dye migrates through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration had occurred, DNA fragments were stained with ethidium bromide and placed on an ultraviolet transilluminator.

2.2.4 Elution of DNA fragments from agarose gel

DNA bands were exposed on an UV-illuminator (using short wavelength) and rapidly cut out from the agarose gel. Extraction was performed following the manual of the QIAquick Gel Extraction Kit (QIAGEN GmbH).

2.2.5 DNA restriction and ligation

Restriction reactions were usually performed following the enzyme insert manual. For ligations of double stranded DNA, T4-DNA ligase (Fermantas GmbH, St. Leon-Rot, Germany) were used following the enzyme insert manual.

2.2.6 Dialysis of ligation mixtures

The ligation mixture was dialysed before electro transformation. The ligation mixture was pipetted on a Millipore-Membrane (#VSWP 02500) which was floating on water.

After 30 minutes of dialysis, the ligation mixture was carefully recovered from the membrane and used for electrotransformation.

2.2.7 Preparation of competent *E. coli* cells for electro transformation

An overnight Standard I medium culture (5 ml) inoculated with a fresh single *E. coli* colony from a plate was used to inoculate a 500 ml main culture and grown into the exponential phase (OD_{578} = 0.5 – 0.8). The cells were harvested by a pre-cooled (4 °C) high-speed centrifuge at 6000 *g* for 20 minutes. The harvested cells were washed two times with 500 ml ice-cold sterile H_2O and once with 20 ml 10% glycerol. The washed competent cells were suspended in 1 ml 10% glycerol and 40 µl aliquots in thin-wall 500 µl tubes were stored at –80 °C.

2.2.8 Electrotransformation

The dialyzed ligation mixture was added to 40 µl competent cells and transferred to a Gene-Pulser cuvette (BioRad, München). A pulse was given to the cuvette using the following settings: 25 µF, 1.8 kV and 200 Ohm. The cuvette was washed with 300 µl LB medium and transferred to a sterile 1.5 ml Eppendorf tube. The transformation mixture was incubated for 30 minutes at 37°C before plating on LB agar, containing antibiotic(s). The agar plate was incubated overnight at 37 °C to get the colonies.

2.2.9 DNA concentration and purity determination

DNA concentration and purity were determined by measuring OD_{260} and OD_{280}.
OD_{260} = 1 corresponds to 50 µg/ml of dsDNA
$OD_{260}/OD_{280} < 1.8$ indicates contamination with protein or phenol
$OD_{260}/OD_{280} > 1.8$ indicates contamination with RNA
$OD_{260}/OD_{280} \approx 1.8$ indicates pure dsDNA

2.2.10 PCR reactions

PCR reactions were performed using a High Fidelity DNA polymerase, Phusion (Finnzymes), and the reaction mixtures were made with following concentration of the ingredients and cycling program:

Concentration of ingredients

	Final concentration
dNTP	200 µM
Forward primer	500 nM
Reverse primer	500 nM
Template DNA	20 to 200 pg/µl (plasmid DNA) 1 to 2 ng/µl (genomic DNA)
DNA polymerase	1 U

Cycling program

1.	98 °C	3 min
2.	98 °C	10 sec
3.	69 °C (depending on primer)	20 sec
4.	72 °C	50 sec (depending on the length of target gene)
5.	72 °C	5 min

29 cycles from 2. to 4.

2.2.11 PCR primers

Restriction site in the primer is underlined.

For ***gctAB***
Forward (NdeI): 5´-
ATGGTACATATGTGAGTAAAGTAATGACGTTAAAAGACGCAATCG-3´
Reverse (XhoI): 5´-ATGGTACTCGAGTTATTTTGCTTCCGTGACCTG-3´

For ***gdh*** for pASK-IBA7plus vector
Forward (KpnI): 5´-ATGGTAGGTACCATGGATTTTGCTTTA-3´
Reverse (HindIII): 5´-ATGGTAAAGCTTTTTCTGGGCCAGCAG-3´

For ***gdh*** for pCDFDuet-1 vector
Forward (PstI): 5´-ATGGTACTGCAGATGGATTTTGCTTTA-3´
Reverse (HindIII): 5´-ATGGTAAAGCTTTTTCTGGGCCAGCAG-3´

For ***etfAB***
Forward (NdeI): 5´-ATGGTACATATGGTGAATATTGTTGCGTGTGTAAAG-3´
Reverse (XmaJI):5´-ATGGTACCTAGGACCAACAAGTTTCTTGCACTCTTTTGC-3´

2.2.12 Cloning of the genes

The genes *hgdH* (996 bp) and *gctAB* (1766 bp) were subcloned into pACYCDuet-1 vector from pETDuet-1 and pJF118HE (Mack et al, 1994), respectively. The gene *hgdH* was restricted and ligated into *EcoN*I and *BamH*I restriction sites and the genes *gctAB* were amplified by PCR using primers with recognition sites for *Nde*I and *XmaJ*I, downstream of *hgdH*. For cloning of the *hgdAB* and its activator *hgdC* the pASK-IBA3plus vector was used. These genes were restricted and ligated into the *Eco47*III and *Mls*I restriction sites, respectively. The gene *gdh* was restricted and ligated into the *Kpn*I and *Hind*III restriction sites of pASK-IBA7plus vector (provides N-terminal *Strep-tag* II peptide, Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) and into *Pst*I and

*Hind*III restriction sites of pCDFDuet-1 vector. For cloning of the *etfAB* pCDFDuet-1 vector was used. The genes *etfAB* were restricted and ligated into *Nde*I and *XmaJ*I restriction sites.

2.2.13 Sequencing of the cloned genes

Primers described below were used to check sequence of the cloned genes.

Primers for pASK-IBA vectors

pASK-IBA forward: 5'-AGA GTT ATT TTA CCA CTC CCT-3'
pASK-IBA reverse: 5'-GCT CCA TCC TTC ATT ATA GC-3'

Primers for Duet vectors

ACYCDuetUP1: 5´-GGA TCT CGA CGC TCT CCC T-3´
DuetDOWN1: 5´-GAT TAT GCG GCC GTG TAC AA-3´
DuetUP2: 5´-TTG TAC ACG GCC GCA TAA TC-3´
T7 Terminator: 5´CCG CTG AGC AAT AAC TAG C-3´

2.3 Methods for protein work

2.3.1 Gene expressions

2.3.1.1 Expression in *E. coli* of the genes encoding the 2-hydroxyglutarate dehydrogenase, glutaconate CoA-transferase, 2-hydroxyglutaryl-CoA dehydratase and activator

The plasmids pACYCDuet-1, harbouring genes *hgdH* and *gctAB*, and pASK-IBA3plus, harbouring genes *hgdCAB*, were transformed into *E. coli* BL21 (DE3). An overnight anaerobic preculture (100 ml) of a fresh single colony was used to inoculate 1 L Standard I medium, described above, containing antibiotics (carbenicillin, 100 $\mu g \cdot ml^{-1}$; chloramphenicol, 50 $\mu g \cdot ml^{-1}$) and grown at the same conditions, at 25˚C. When the culture reached OD_{578} = 0.2, gene expression was induced with isopropyl-1-thio-β-D-galactoside, IPTG (240 $mg \cdot L^{-1}$) and anhydrotetracycline, AHT (200 $\mu g \cdot L^{-1}$). Cells were harvested 3 hours after induction (at OD_{578} = 0.573), washed and suspended in 20 ml of buffer (50 mM MOPS, 5 mM $MgCl_2$ and 2 mM DTT) under anoxic conditions. The induced *E. coli* cells were lysed by French press and cell debris was removed by ultracentrifugation at 100,000 *g* at 4°C for 1 hour.

2.3.1.2 Expression of recombinant activator of 2-hydroxglutaryl-CoA dehydratase from *A. fermentans*

The plasmid pASK-IBA3plus (Hans et al., 1999) harboring the gene *hgdC,* encoding the activator of 2-hydroxglutaryl-CoA dehydratase from *A. fermentans,* was transformed into the *E. coli* BL21 (DE3). The preculture was grown in Standard I medium, buffered with 10 mM MOPS pH 7.4 overnight at 37 °C under anaerobic conditions. For the production of recombinant protein, the cells were grown in 2 L of the same medium under same conditions. When the optical density of the culture reached 0.25, the cells were induced with 100 µM AHT for 3 hours and harvested under anaerobic conditions. The cells were kept at –80 °C.

2.3.1.3 Expression of the genes encoding glutaryl-CoA dehydrogenase and electron-transferring flavoprotein from *S.aciditrophicus*

Both proteins were produced by the same method.

The plasmids pASK-IBA7plus, harbouring *gdH*, and pCDFDuet-1, harbouring *etfAB*, were separately transformed into *E. coli* BL21 (DE3). An overnight aerobic preculture (200 ml) of a fresh single colony, grown at 30°C, was used to inoculate 2 L LB medium, containing riboflavin (10 $mg \cdot l^{-1}$) and appropiate antibiotics (*gdh* : carbenicillin, 100 $\mu g \cdot ml^{-1}$; *etfAB*: spectinomycin, 50 $\mu g \cdot ml^{-1}$) and grown at the 37°C. When the culture reached OD_{578} = 0.6-0.8, gene expression was induced with anhydrotetracycline, AHT (200 $\mu g \cdot L^{-1}$- for *gdh*) and with isopropyl-1-thio-β-D-galactoside, IPTG (240 $mg \cdot L^{-1}$ for *etfAB*). Cells were harvested 16 hours after induction.

2.3.2 Protein purification

2.3.2.1 Methods of cell disruption

Prior to purification of the activator of 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans*, oxygen was removed from buffers, columns, centrifuge tubes and other materials. All purifications steps were carried out anoxically in a glove-box (Coy Labs, Ann Arbor, USA). The homogeneity of all these proteins was established by SDS-PAGE.

Ultrasonic disintegration: Cells, suspended in the appropriate buffer, were filled into glass Rosetta cell, kept on ice and broken by ultrasonication on a Branson 250 Sonifier (Heinemann, Germany). The duty time was 5-10 minutes at 50% duty cycle. The process was repeated several times.

French press: The suspended cells were filled into a serum bottle under anoxic conditions. The bottle was sealed with a rubber stopper and an aluminium lid. Anoxic conditions were maintained by connecting the pressure cell via a needle to the serum bottle. The cell suspension was sucked into a pre-cooled French pressure cell (American Instruments, Maryland, USA) and the cells were disrupted by applying a

pressure of 150 MPa. The broken cells were collected into the serum bottle and refilled into the pressure cell via the needle for another cycle of disrupting. The cycle was repeated 4 times and the cells were observed under a microscope to verify optimal cell opening.

2.3.2.2 Determination of protein concentration

Protein concentration was determined by the Bradford method (Bradford, 1976). The assay is based on the shift of the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm upon binding of protein. Standards with 0 – 7 µg of BSA were made up to an 800 µl volume with water and 200 µl Coomassie Brilliant Blue G-250 reagent. The reactions were incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 595 nm.

2.3.2.3 SDS PAGE procedure

The samples were mixed with SDS-sample buffer (125 mM Tris/HCl, pH 6.8, 10% glycerol, 10% mercaptoethanol, 4% SDS, 0.2% bromophenol blue) in the ratio of 1:1 and boiled for 10 minutes to denature the proteins. Each well was loaded with 10 µl sample . The running buffer was 25 mM Tris pH 8.8, 190 mM glycine, 0.1% SDS. Electrophoresis was run at constant voltage of 200 mV until the bromphenolblue marker reached the end of the gel. The proteins were stained by heating the gel with 0.1% Coomassie Brillant blue R-250 in methanol/water/glacial acetic acid (4:5:1) shortly and leaving it at room temperature for 5 minutes. The gel was distained by heating it with ethanol/water/glacial acetic acid (4:5:1) and incubating overnight on a shaker.

Table 2. SDS PAGE gel content

Stock solutions	Separating gel	Stacking gel
1 M Tris/HCl pH 8.8	3000 µl	-
1 M Tris/HCl pH 6.8	-	470 µl
H_2O	1370 µl	2900 µl
10% SDS	85 µl	40 µl
Acrylamide/Bisacrylamide (40%/1.6%)	2750 µl	585 µl
5% TEMED	85 µl	40 µl
10% Ammonium peroxodisulfate	115 µl	80 µl

2.3.2.4 Purification of recombinant activator of 2-hydroxglutaryl-CoA dehydratase from *A. fermentans*

Cells of *E. coli* BL21 (DE3), harboring the plasmid with the activator gene (*hgdC*) from *A. fermentans,* were suspended in the anaerobic buffer A (50 mM MOPS pH 7.4, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT). The cells were opened by French pressure cell as described above (3.2.1.) and centrifuged for 1 hour at 100,000 *g* at 4°C in the ultracentrifuge.

The cell-free extract was applied onto a 5 ml Strep-Tactin affinity column, which was already equilibrated and washed with buffer A. Elution was done with buffer E (buffer A, 3 mM desthiobiotin and 2 mM ADP). 1 ml fractions were collected and the brown fractions were concentrated by Centricon 30 kDa cut-off membranes. The purified protein was stored at -80°C.

2.3.2.5 Partial purification of recombinant glutaconate CoA-transferase from *A. fermentans*

Cells of *E. coli* BL21 (DE3), harboring the plasmid with glutaconate CoA transferase from *A. fermentans*, were suspended in 15 ml 20 mM potassium phosphate, pH 7.4

and sonicated for 15 min (divided in three intervals) with a Branson sonifier. Cell debris was removed by centrifugation at 100 000 *g* for 1 hour at 4°C. To the cell-free extract ammonium sulfate was added to achieve 50% saturation. After centrifugation at 25,000 *g* for 30 minutes, the supernatant was brought to 80% ammonium sulfate saturation and centrifuged as described above. The protein pellet was dissolved in 40 ml 20 mM potassium phosphate buffer, pH 7.4 and partially purified with Centricon cut-off membrane of 100 kDa. The sample was stored at 4°C.

2.3.2.6 Purification of recombinant glutaconyl-CoA dehydrogenase from *S. aciditrophicus*

Cells of *E. coli* BL21 (DE3), harbouring the plasmid with the gene encoding glutaconyl-CoA dehydrogenase from *S. aciditrophicus*, were suspended in 20 ml 50 mM Tris/HCl , pH 8.0 and disrupted with a Branson sonifier for 15 min (divided in three intervals). Cell debris was removed by centrifugation at 100,000 *g* for 1 hour at 4°C. For protein isolation a 5 ml Strep-Tactin affinity column was used, previously equilibrated with buffer W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl). Elution was done with buffer E (buffer W, 2.5 mM desthiobiotin). 1 ml fractions were collected. The purified protein was stored at -80°C.

2.3.2.7 Purification of recombinant electron-transferring flavoprotein from *S. aciditrophicus*

E. coli BL21 (DE3), harboring the plasmid with the genes encoding the electron-transferring flavoprotein from *S. aciditrophicus,* were suspended in 50 mM potassium phosphate, pH 7.0 and disintegrated with a Branson sonifier for 15 min (divided in three intervals). Cell debris was removed by centrifugation at 100,000 *g* for 1 hour at 4°C. Etf was isolated via DEAE Sepharose (GE Healthcare) column. The column was first equilibrated with buffer A (50 mM potassium phosphate, pH 7.0, 8 µM FAD), after applying the cell-free extract to the column, protein was eluted with buffer B

(buffer A, 1 M NaCl). The yellow fractions were concentrated by Centricon 30 kDa cut-off membranes. The purified protein was stored at -20°C.

2.3.3 Enzyme activity assays

2.3.3.1 (*R*)-2-Hydroxyglutarate dehydrogenase from *A. fermentans*

(*R*)-2-Hydroxyglutarate dehydrogenase activity was measured aerobically at ambient temperature in a cuvette of 0.5 ml total volume, containing 0.1 M Tris pH 8.0, 0.2 mM NADH and (*R*)-2-hydroxyglutarate dehydrogenase. The reaction was started with 1 mM 2-oxoglutarate. The decrease of absorbance, caused by oxidation of NADH (ε = 6.3 $mM^{-1}cm^{-1}$), was monitored at 340 nm (Bresser, 1997).

2.3.3.2 Glutaconate CoA-transferase from *A. fermentans*

Glutaconate CoA-transferase activity was assayed aerobically at room temperature. The increase of absorbance was followed at 412 nm (ε = 14.2 mM^{-1} cm^{-1}). Reagents used in assay are 0.1 M potassium phosphate pH 7.0, 0.2 M sodium acetate, 1 mM oxaloacetate, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 20µg citrate synthase, 0.1 mM glutaryl-CoA, total volume 0.5 ml (Buckel et al., 1981; Jacob et al., 1997).

2.3.3.3 (*R*)-2-Hydroxyglutaryl-CoA dehydratase from *Clostridium symbiosum*

(*R*)-2-Hydroxyglutaryl-CoA dehydratase activity was measured under anoxic conditions at ambient temperature in cuvette of 0.5 ml total volume, containing 50 mM 3-(N-morpholino)propanesulfonic acid (Mops) pH 7.0, 10 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.1 mM dithionite, 0.4 mM ATP, and recombinant 2-hydroxyglutaryl-CoA dehydratase with activator. After 10 minutes incubation, 2 mM acetyl-CoA and 2 mM 2-hydroxyglutarate were added and the reaction was started with 1.8 U glutaconate CoA transferase. The increase of absorbance due to the formation of glutaconyl-CoA was followed at 290 nm (ε = 2.2 $mM^{-1}cm^{-1}$) (Kim et al., 2008).

2.3.3.4 Glutaryl-CoA dehydrogenase from *S. aciditrophicus*

Glutaryl-CoA dehydrogenase activity was measured with ferricenium hexafluorophosphate ($FcPF_6$) as artificial electron acceptor. The assay measured the decrease of absorbance at 300 nm in a reaction containing 0.1 mM and 10 mM glutaryl-CoA in 100 mM Tris/HCl pH 8.0. A solution of 2 mM $FcPF_6$in 10 mM HCl was previosly prepared (Lehman and Thorpe, 1990).

During the enzymatic dehydrogenation of glutaryl-CoA, ferricenium ions of blue colour get reduced to ferrocene displaying a decrease in the absorbance (ε_{300nm} = 3.6 $mM^{-1}cm^{-1}$). The second assay used for determination of glutaryl-CoA dehydrogenase activity measured the decrease of absorbance at 300 nm in a reaction containing 0.1 mM $FcPF_6$, 10 mM glutarate, 0.1 mM acetyl-CoA, 1.8 U glutaconate CoA transferase, and glutaryl-CoA dehydrogenase in 100 mM Tris/HCl pH 8.0.

The second assay used for determination of glutaryl-CoA dehydrogenase activity measured the decrease of absorbance at 300 nm in a reaction containing 0.1 mM $FcPF_6$, 10 mM glutarate, 0.1 mM acetyl-CoA, 1.8 U glutaconate CoA transferase, and glutaryl-CoA dehydrogenase in 100 mM Tris pH 8.0. Ferricenium hexafluorophosphate was prepared as explained above.

2.3.2.5 Electron-transferring flavoprotein from *S.aciditrophicus*

The activity of electron-transferring flavoprotein was measured aerobically with iodonitrosotetrazolium chloride (INT) at ambient temperature. The reaction, containing 50 mM Tris/HCl pH 7.5, 10 mM NADH, and 5 mM INT, was started by addition of ETF. The formation of red formazan was followed at 492 nm (ε = 19.2 $mM^{-1}cm^{-1}$) (Möllering et al., 1974).

2.3.3.6. Gdh/Etf with NAD(P)H and glutaconyl-CoA

The assay was performed under anaerobic conditions at ambient temperature. The reaction mixture contained 50 mM potassium phosphate pH 7.0, 125 µM glutaconyl-

CoA, 25 µM NAD(P)H, 10 µM ferredoxin, 0.4 U hydrogenase and the Gdh/Etf complex in final volume of 1 ml. The oxidation of NADH was followed at 340 nm (ε = 6.3 $mM^{-1}cm^{-1}$).

2.3.4 Glutaconate determination

Glutaconate was enzymatically determined with 2 mM acetyl-CoA, 50 mM potassium phosphate pH 7.0, 0.25 mM NADPH, glutaconate, and a catalytic amount of the enzymes 1.8 U glutaconate-CoA transferase, 2.4 U glutaconyl-CoA decarboxylase (Buckel, 1986, 2001) and 0.4 U crotonyl-CoA carboxylase/reductase (Li et al., 2008). The formation of $NADP^+$ was measured spectrophotometrically at 340 nm (ε = 6.3 $mM^{-1}cm^{-1}$). In the range from 0-100 µM glutaconate, the change of absorbance was linear.

Glutaconate was also determined by HPLC (High-performance liquid chromatography) at 50 °C and at 205 nm using a C18 reverse-phase column (GE Healthcare) in 20 mM sulfuric acid.

2.3.5 Glucose and glutamate consumption

Glucose concentration in the medium was determined at room temperature with the coupled enzymatic assay. The reaction mixture in total volume of 1 ml consisted of 0.1 M sodium phosphate pH 7.0, 5 mM 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 0.15 U horseradish peroxidise, 1 mM potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) and 10 U glucose oxidase. The oxidation of ABTS was followed at 725 nm (ε = 19 $mM^{-1}cm^{-1}$). $K_3[Fe(CN)_6]$ was used to oxidize the reducing agent cysteine of the medium, which bleached the blue-green oxidized ABTS ($ABTS^+$) (Bergmeyer and Bernt, 1974). In the range from 0-100 µM glucose, the change of absorbance was linear.

The concentration of glutamate in the medium was measured at ambient temperature. The assay contained 100 mM potassium phosphate pH 7.4, 1 mM oxaloacetate, 0.2 mM NADH, 4 U (*R*)-2-hydroxyglutarate dehydrogenase and up to

0.15 mM (*S*)-glutamate. The reaction was started with 2 U aspartate aminotransferase. The commercial enzyme was obtained as suspension in 2 M ammonium sulfate and 2 mM 2-oxoglutarate. Prior to the assay, the enzyme was precipitated by centrifugation (Eppendorf centrifuge) and dissolved in the assay buffer. The oxidation of NADH was followed at 340 nm. In the range from 0-100 µM glutamate, the change of absorbance was linear.

2.3.6 Determination of ethanol production

The reaction mixture contained 75 mM $Na_4P_2O_7$, 74 mM semicarbazide HCl, 22 mM glycine, 1 mM NAD, and up to 300 µM ethanol, pH 8.8. The reaction was initiated with 0.075 U alcohol dehydrogenase. In the range from 0-300 µM ethanol, the change of absorbance at 340 nm (ε = 6.3 $mM^{-1}cm^{-1}$) was linear.

2.4 Determination of kinetic parameters

The K_m values were determined by fitting the data to Michaelis-Menten curves, using the GraphPad Prism software (GraphPad software, Inc., La Jolla, CA).

The inhibition constant K_i can be calculated by fitting the data to a modified Michaelis-Menten equation for non-competitive inhibition by product.

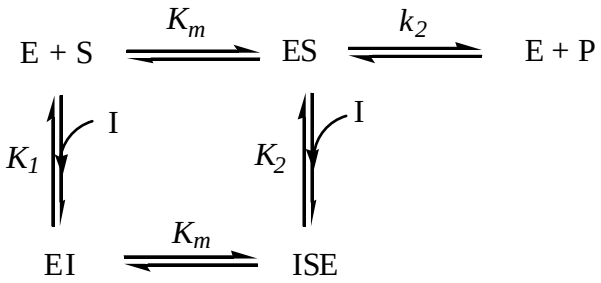


Figure 17. A non-competitive inhibitor binds to both enzyme (E) and enzyme-substrate complex (ES) with identical affinities ($K_1 = K_2$). Non-competitive inhibition does not change K_m, but decreases V_{max}.

$$\frac{1}{v} = \frac{1}{vmax} \times \left(1 + \frac{[I]}{K2}\right) + \frac{Km}{vmax[S]} \times (1 + \frac{[I]}{K1})$$

$$K_2 = K_1 = K_i$$

$$v = \frac{vmax}{\left(1 + \frac{[I]}{Ki}\right)} \times \frac{1}{1 + \frac{Km}{[S]}}$$

$$v = \frac{vmax}{1 + \frac{[I]}{Ki}} \times \frac{[S]}{[S] + Km}$$

$$v'max = \frac{vmax}{1 + \frac{[I]}{Ki}}$$

equation 4

$$Ki = \frac{[I]}{\frac{vmax}{v'max} - 1}$$

Results

3.1 Glutaconate production

3.1.1 Coexpression of the genes encoding (*R*)-2-hydroxyglutarate dehydrogenase, glutaconate CoA transferase, (*R*)-2-hydroxyglutaryl-CoA dehydratase and its activator

The gene *hgdH* encoding (*R*)-2-hydroxyglutarate dehydrogenase obtained in pETDuet-1 was introduced into a pACYCDuet-1 vector with *EcoNI* and *BamHI* restriction enzymes. The genes *gctAB* encoding glutaconate CoA transferase from *A. fermentans* obtained in pJF118HE (Mack et al., 1994) were amplified by PCR using primers with recognition sites for *Nde*I and *XmaJ*I. Then they were subcloned into the same pACYCDuet-1 vector downstream of *hgdH*, pID-3 (figure 18)

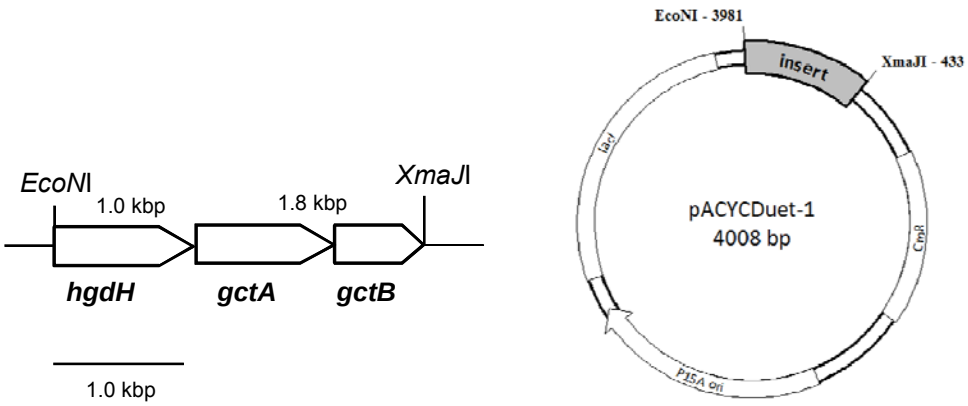


Figure 18 Construction of recombinant plasmid pID-3. P15A ori: origin of replication, lacI: lactose repressor, CmR: chloramphenicol resistance, *hgdH*: gene of (*R*)-2-hydroxyglutarate dehydrogenase, *gctAB*: genes of glutaconate CoA transferase, *Eco*NI and *XmaJ*I are restriction enzymes.

For cloning of *hgdAB* (2-hydroxyglutaryl-CoA dehydratase) from *C. symbiosum* and its activator *hgdC* from *A. fermentans,* the pASK-IBA3plus vector (IBA GmbH, Göttingen, Germany) was used (figure 19). Genes *hgdAB* were introduced with and *Eco47III* restriction enzymes and gene *hgdC* was introduced in the recognition sites for *MlsI*. The obtained plasmid was named pID-4.

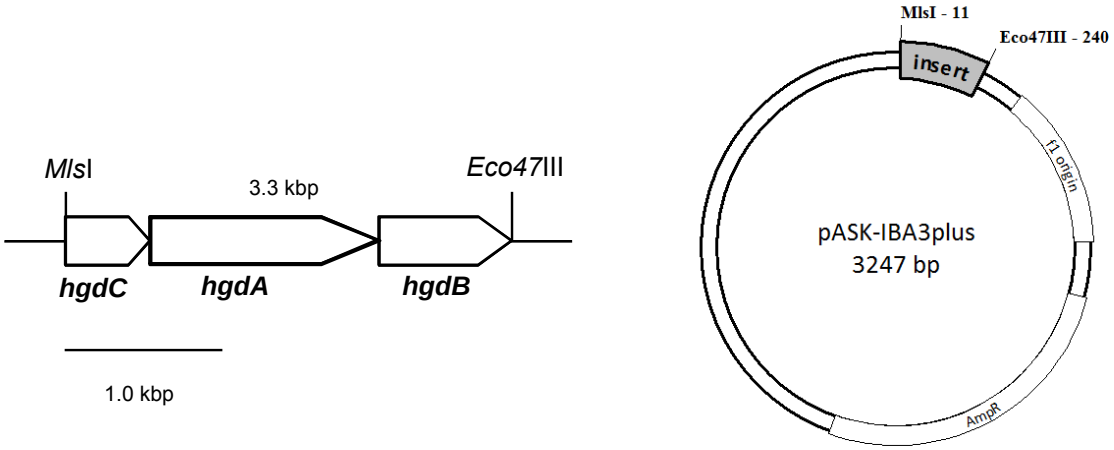


Figure 19. Construction of the recombinant plasmid pID-4. F1 origin: origin of replication, AmpR: ampicillin resistance, insert: inserted DNA (hgdCAB), *Mls*I and *Eco47*III are restriction enzymes.

E. coli cells harbouring plasmids pID-3 and pID-4 were grown anaerobically at 25°C and induced with IPTG and AHT at 3 h. After further growth for 3 h, the cells were harvested and opened as described in Materials and Methods. The cell-free extract (100.000 *g* supernatant) but also the pellet showed thick protein bands as analysed by SDS PAGE (figure 20). Without induction these bands were absent.

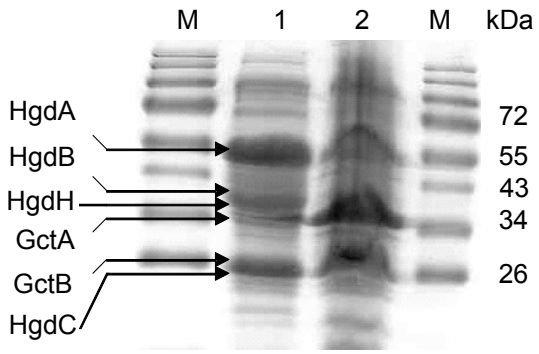
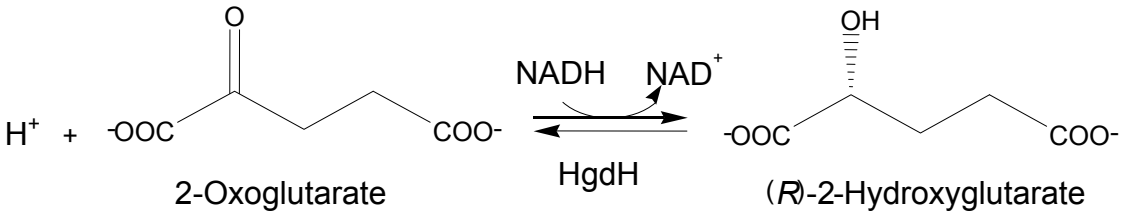


Figure 20. SDS PAGE gel with produced HgdH (2-hydroxyglutarate dehydrogenase, 36.5 kDa), GctAB (glutaconate CoA transferase, 35.7 kDa and 29.2 kDa), HgdAB (2-hydroxyglutaryl-CoA dehydratase, 53.9 kDa and 41.9 kDa) and HgdC (activator of 2-hydroxyglutaryl-CoA dehydratase, 27.3 kDa). The SDS-PAGE gel (15%) was stained with Coomassie Brilliant Blue. M: molecular mass marker, 1: supernatant, 2: pellet

3.1.2 Enzyme assays

3.1.2.1 (*R*)-2-Hydroxyglutaryl-CoA dehydrogenase from *A. fermentans*

For determination of the 2-hydroxyglutarate dehydrogenase activity an NADH-dependent assay was used with 2-oxoglutarate as substrate. Formation of NAD^+ was followed at 340 nm.



The determined specific activity of the 2-hydroxyglutarate dehydrogenase in the cell free extract was 150 U/mg. For the determination of V_{max} = 164 U/mg and K_m = 0.094 mM 2-oxoglutarate was used, the concentration of which varied from 0 to 2.0 mM (double measurment) (figure 21).

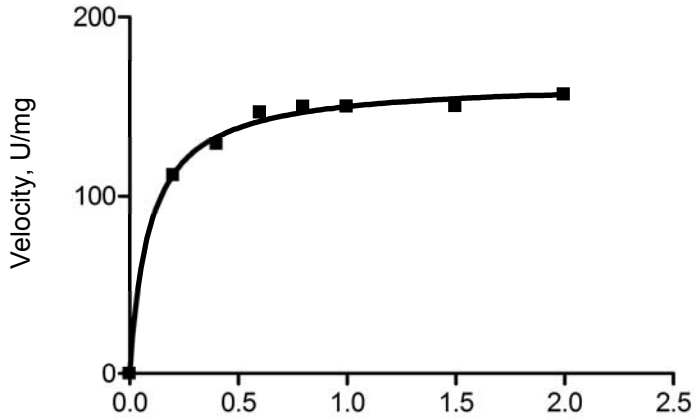
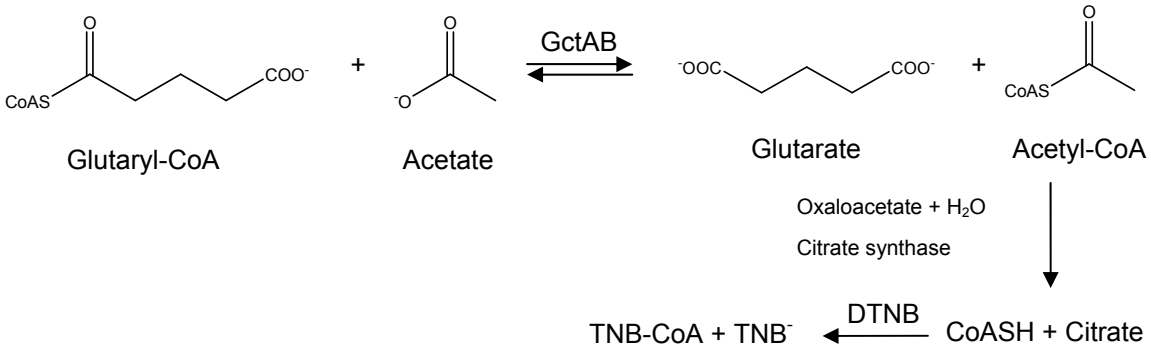


Figure 21. Michelis-Menten curve. Determinations of V_{max} and K_m were done with NADH and 2-oxoglutarate in varied concentrations.

3.1.2.2 Glutaconate CoA transferase from *A. fermentans*



To determine the activity of glutaconate CoA transferase the DTNB assay was used. This assay is based on the reaction of a thiol with DTNB, giving the mixed disulfide and yellow TNB.

The highest specific activity of the glutaconate CoA transferase in the cell free extract was 0.25 U/mg. For determination of V_{max} and K_m the glutaryl-CoA concentration varied from 0 to 1.0 mM (double measurment) (figure 22). The obtained saturation curve is biphasic and can hardly fitted with the Michaelis-Menten equation. A similar observation has been made with the pure enzyme (Buckel et al., 1981).

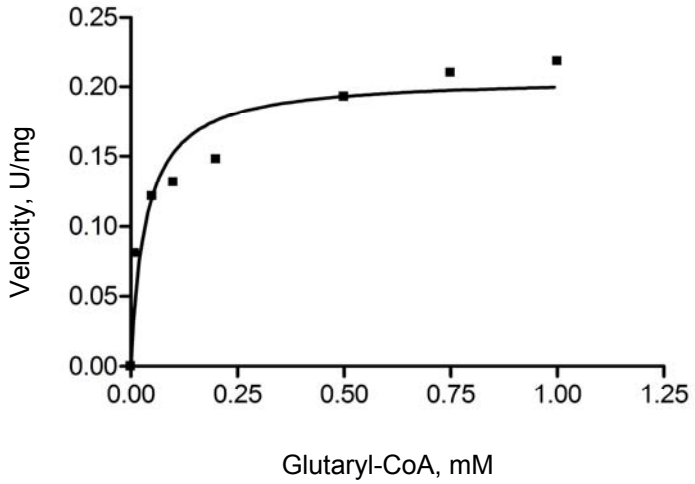
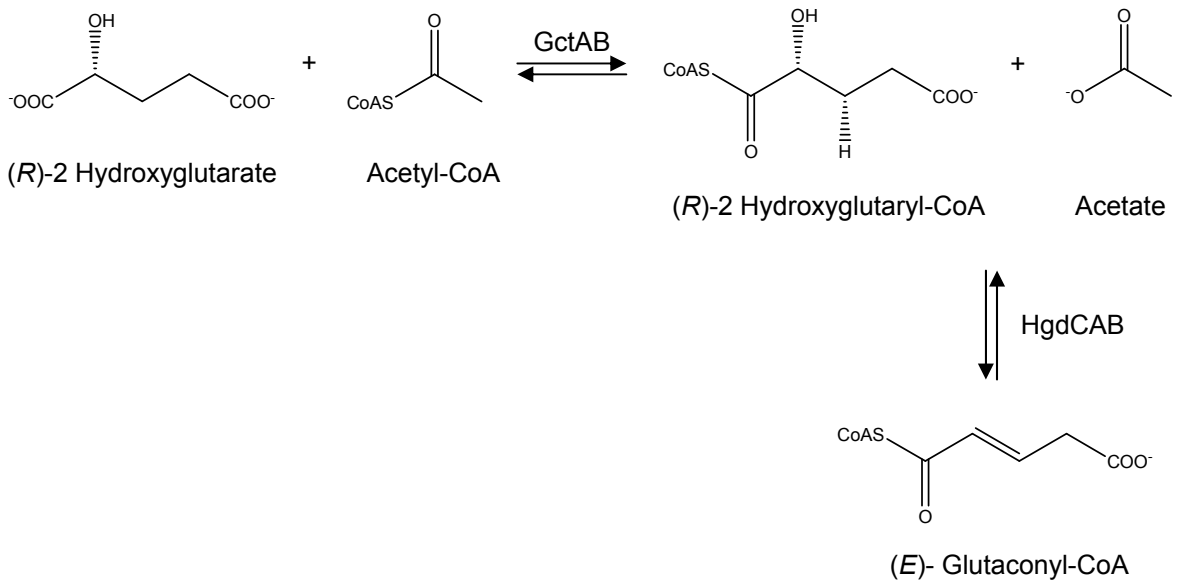


Figure 22. Dependence on the rate of CoA-transfer from glutaryl-CoA to acetate.

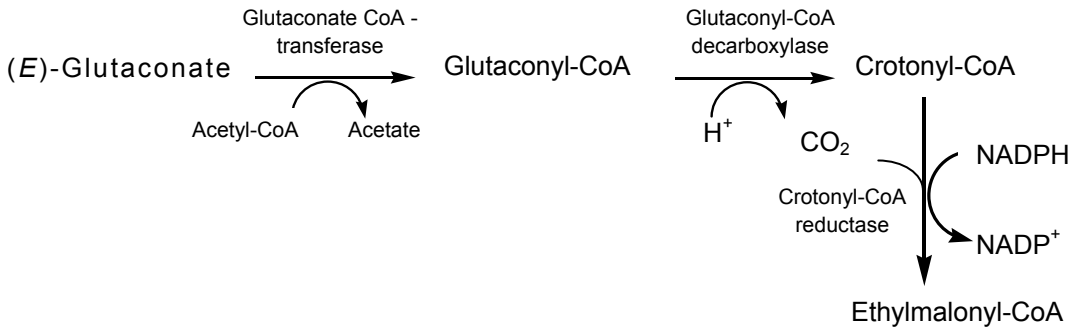
3.1.2.3 2-Hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

2-Hydroxyglutaryl-CoA dehydratase activity was measured with a coupled assay under anoxic conditions at ambient temperature. The increase of absorbance due to the formation of glutaconyl-CoA was followed at 290 nm (ε = 2.2 $mM^{-1}cm^{-1}$). In cell free extracts the highest measured specific activity was 0.46 U/mg. When riboflavin and iron(III)citrate were omitted in the Standard I medium, the activity was almost 6-fold lower, 0.08 U/mg.



3.1.3 Glutaconate determination

After production of all three enzymes involved in glutaconate production, the next step was glutaconate determination in order to prove our proposed pathway. For measuring glutaconate a coupled assay was used, in which the consumption of NADPH was followed. Glutaconate was also identified and determined by HPLC yielding identical results.



Sum: Glutaconate^{2-} + Acetyl-CoA + NADPH + H^+ = Ethymalonyl-CoA$^-$ + Acetate$^-$ + $NADP^+$

After growth of recombinant *E. coli* the glutaconate concentration in the medium was 0.30 ± 0.05 mM; when glutamate was omitted, the concentration decreased to 0.1 mM. Hence, glutaconate indeed was produced indicating that the enzymes work also *in vivo*. The original strain *E. coli* BL21 (DE3) was grown and analysed under the same conditions as the recombinant strain and glutaconate was not detected. Addition of riboflavin and iron(III)citrate in the Standard I medium increased the concentration of glutaconate almost 10-fold up to 2.7 ± 0.2 mM (Table 3). In order to get an idea how glutaconate was exported, a cell-free extract was prepared from 590 mg wet packed cells (approx. 118 mg dried cells) in 20 ml 50 mM MOPS pH 7.4. Assuming a volume of 2.5 ml/g dried cells (Brock and Buckel, 2004), the concentration of glutaconate measured in the cell free extract as 0.23 mM rises to 16 mM in the cells. Hence, 2-hydroxyglutaryl-CoA dehydratase, which contains Fe and riboflavin-5'-phosphate, as well as glutaconate export appear to be rate-limiting steps in glutaconate production.

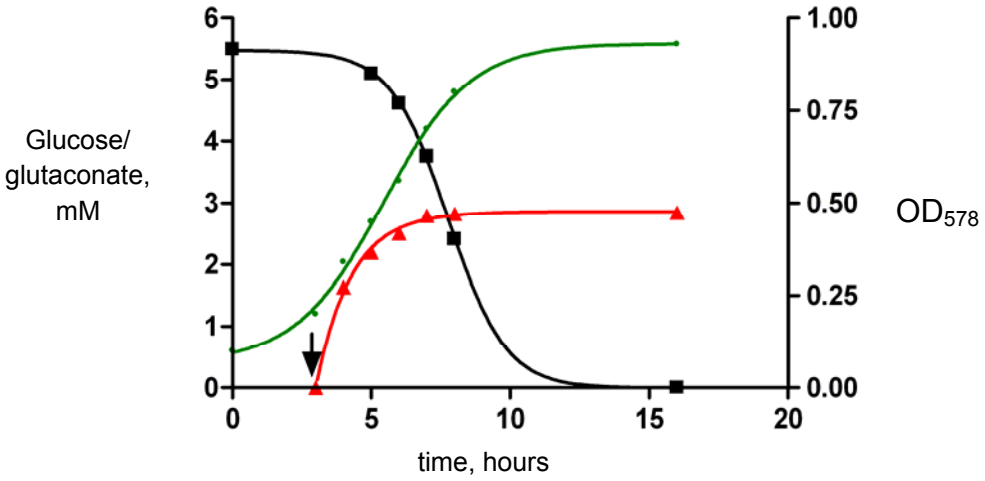


Figure 23. Glucose vs glutaconate vs cell growth. Glucose consumption (black), glutaconate formation (red) and cell growth (green). The arrow indicates the time of induction with IPTG and AHT.

Table 3. Glutaconate concentration in the medium and in the cells of recombinant *E. coli* strain with different suplements

	glutaconate in the medium, mM	**glutaconate in the cell, mM**
without suplements	0.10	
10 mM glutamate	0.30 ± 0.05	
10 mM glutamate, 2 mM Fe(III)citrate	2.5 ± 0.2	~15
10 mM glutamate, 2 mM Fe(III)citrate, 0.2 mM riboflavin	2.7 ± 0.2	~16
10 mM glutamate, 2 mM Fe(III)citrate, 0.4 mM riboflavin	1.6	~8

3.1.4 Consumption and production of the other compounds

The expected uptake of glucose, as the main carbon source, during growth was demonstrated with the glucose oxidase assay and by measuring the production of ethanol with the alcohol dehydrogenase assay (figure 24). The data show that fermentation of glucose and production of ethanol correlate with growth of the organism but apparently not with glutaconate production. The final concentration of ethanol in the medium of the original *E coli* BL21 (DE3) was 10.2 ± 0.2 mM, whereas that of the recombinant strain only reached 8.7 ± 0.2 mM.

Glutamate increased glutaconate production and therefore the next imposed question was the relation of glutamate consumption and glutaconate production. The concentration of glutamate was measured in a coupled assay with aspartate aminotransferase and 2-hydroxyglutarate dehydrogenase. As it shown in figure 24,

glutamate was utilized before glutaconate formation started and – surprisingly – resynthesized before the production of glutaconate had ceased.

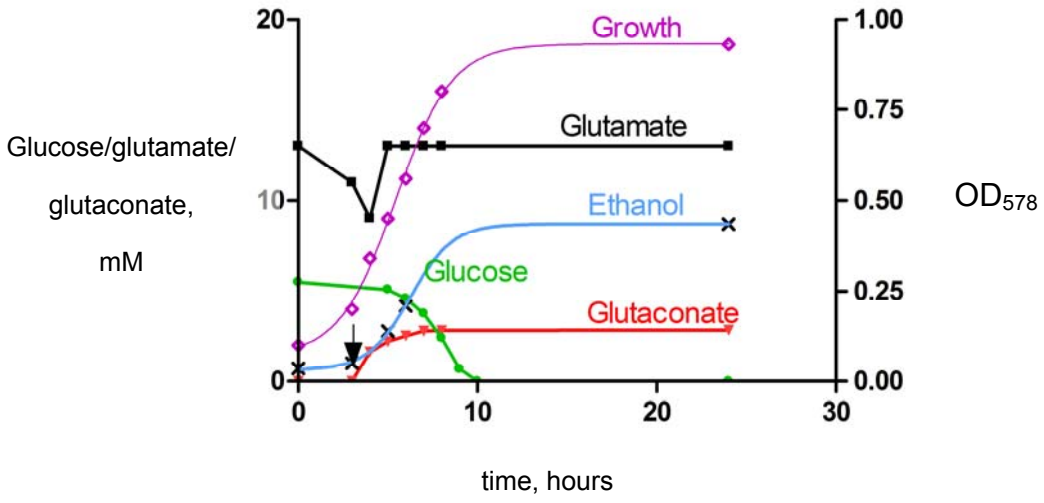


Figure 24. Glucose vs glutamate vs glutaconate. Glucose consumption (green), glutamate utilization and synthesis (black), glutaconate production (red), ethanol synthesis (blue), cell growth (purple). Arrow indicates induction time.

Synthesis of glutamate in recombinant strain opened a new question: why is glutamate after few hours consuming synthesized again? In order to get a more precise answer to this question, the original strain *E. coli* BL21 (DE3) was grown under same conditions as recombinant strain and analysed.

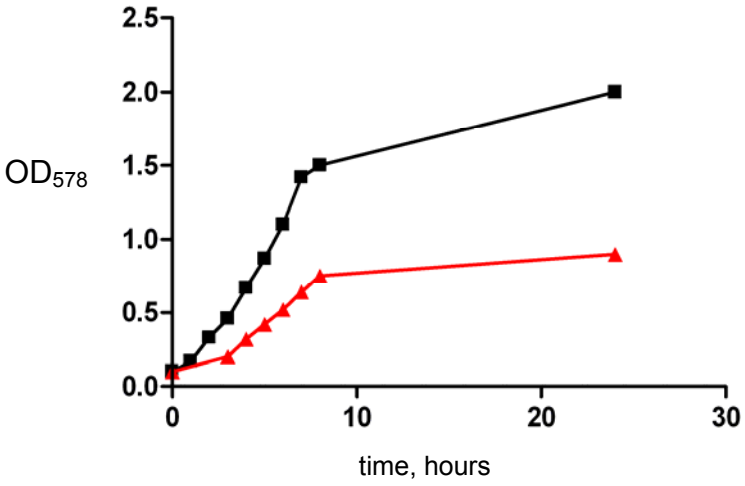


Figure 25. Growth curve for original (black) and recombinant *E. coli* BL21 (DE3) (red).

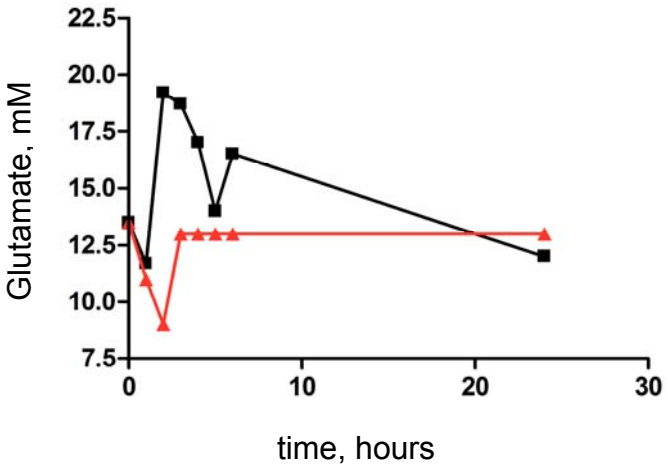


Figure 26. Glutamate consumption and synthesis in original *E. coli* BL21 (DE3) (black) and recombinant *E. coli* BL21 (DE3) (red).

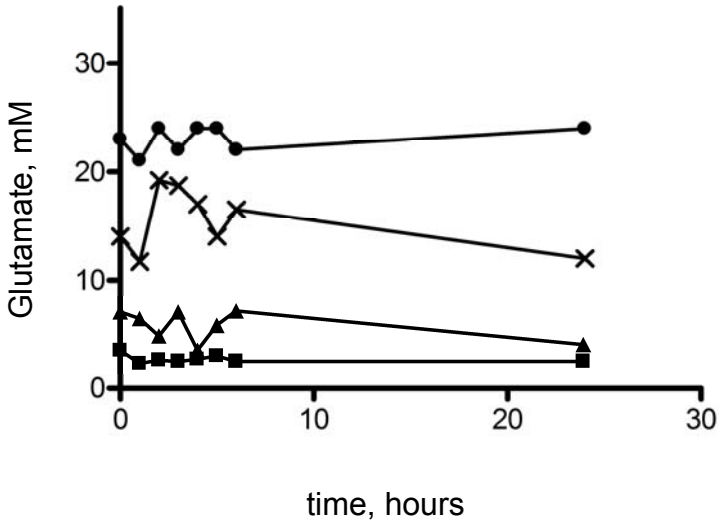


Figure 27. Change of glutamate concentration during growth in original *E. coli* BL21 (DE3) with different start concentrations of glutamate in the medium: 3 mM (■), 7 mM (▲), 13 mM (x), 23 mM (●).

Considering that the overnight preculture was in the stationary phase, it can be an additional stress for the cells to adapt after inoculation and this fact can be the reason why the glutamate concentration is fluctuating. The experiment was repeated with an overnight preculture in the exponential phase (Dr Peter Lenz, personal communication). The repeated experiment shows the same results as the previous one, so the glutamate consumption and synthesis in *E coli* BL21 (DE3) under anaerobic conditions remains inexplicable.

3.2 Glutaryl-CoA dehydrogenase from *S. aciditrophicus*

The restriction enzyme *Kpn*I and *Hind*III sites were introduced into the primers for cloning of the expresion vector pASK-IBA7plus. The amplified DNA fragment (*gdh*) was cut with *Kpn*I and *Hind*III and ligated into the expression vector, which contains an N-terminal fused *Strep*-tag II peptide for one-step purification.

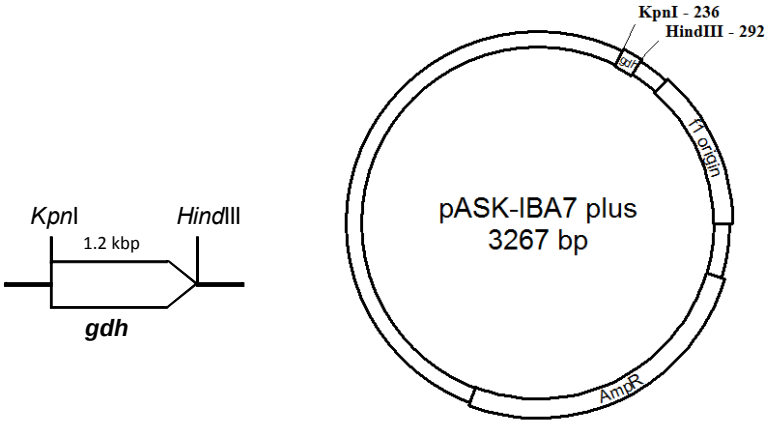


Figure 28. Construction of recombinant plasmid. F1 origin: origin of replication, AmpR: ampicillin resistance, gdh: glutaryl-CoA dehydrogenase, *Kpn*I and *Hind*III are restriction enzymes

The plasmid construct, shown in figure 28, was transformed into *E. coli* BL21 (DE3) strain. The culture was grown under aerobic conditions at 37 °C and induced with anhydrotetracycline (200 $\mu g \cdot L^{-1}$) in the exponential phase (A_{578} = 0.6 – 0.8). After overnight growth, cells were harvested. The cell free extract was obtained after centrifugation of cells opened by sonication. Pure protein was obtained by purification using *Strep*-Tactin affinity chromatography (figure 29) as described in Methods for protein work.

$$\text{Glutaryl-CoA} + 2\ \text{Ferricenium}^{+} \xrightarrow{\text{Gdh}} \text{Glutaconyl-CoA} + 2\ \text{Ferrocene} + 2\ H^{+}$$

Glutaryl-CoA dehydrogenase activity was determined by following initial decrease in absorbance at 300 nm (ε = 3.6 $mM^{-1}cm^{-1}$) due to reduction of ferricenium ion. The specific activity of glutaryl-CoA dehydrogenase, purified via *Strep*Tag column, was 8 U/mg. For determination of V_{max} = 38 U/mg and K_m = 0.092 mM the glutaryl-CoA concentration varied from 0 to 0.4 mM (double measurment) (figure 30).

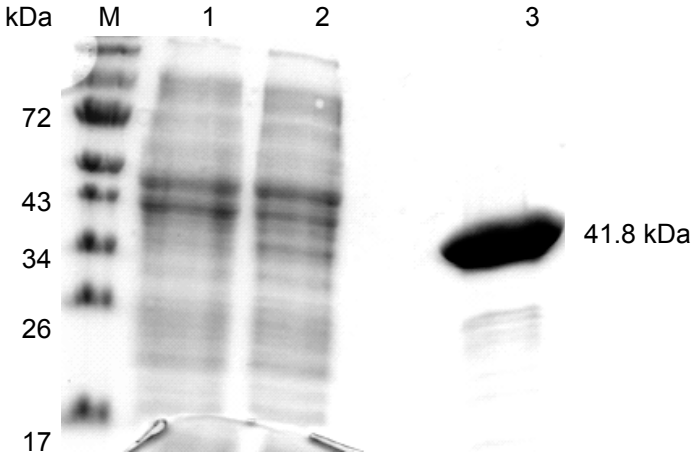


Figure 29. SDS PAGE gel for purified Gdh (glutaryl-CoA dehydrogenase). The SDS-PAGE gel (15%) was stained with Coomassie Brilliant Blue. M: molecular mass marker, 1: cell free extract of induced cells, 2: flow through, 3: purified Gdh via *Strep*-Tactin column

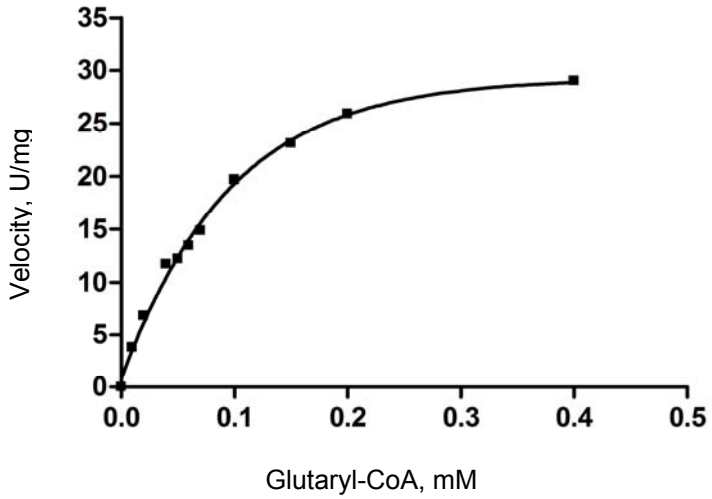


Figure 30. Kinetics of glutaryl-CoA dehydrogenase with ferricenium as electron acceptor.

3.2.1 Inhibition by glutaconyl-CoA

Glutaryl-CoA dehydrogenase from *Desulfococcus multivorans* is inhibited by its product, glutaconyl-CoA (Wischgoll et al., 2009). The kinetic parameters of the enzyme from *S. aciditrophicus* were determined with the assay, described in

Materials and Methods, in the presence of glutaconyl-CoA at varied concentrations (figure 31).

Increasing the concentration of glutaconyl-CoA, the maximum velocity decreased, while the K_m value remained constant. These results indicate that glutaconyl-CoA acts as a non-competitive inhibitor. Using equation 4 (see Materials and Methods) a K_i value of 50 ± 4 µM was calculated.

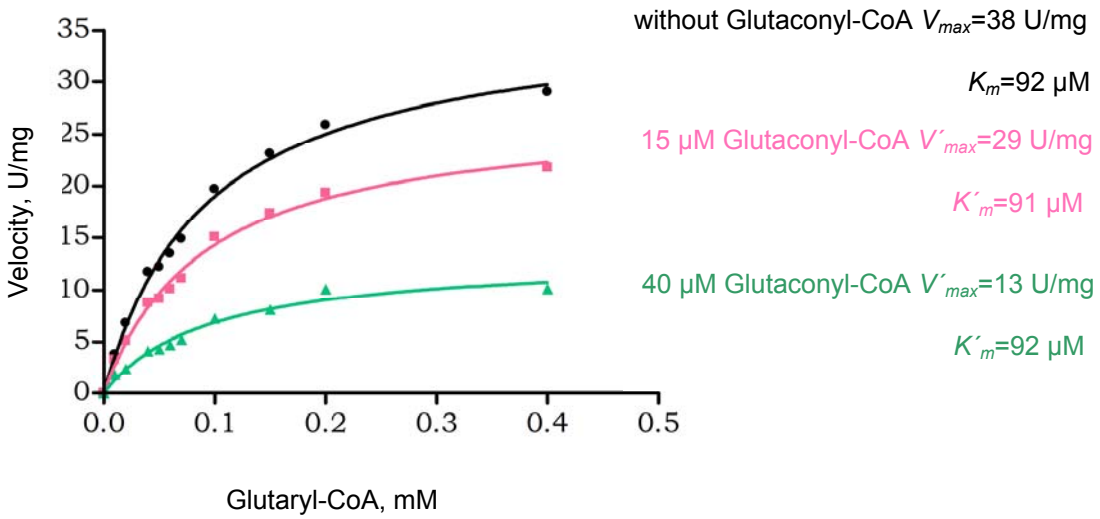


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Figure 31. Michaelis- Menten plots for inhibition of glutaryl-CoA dehydrogenase with 15 and 40 µM glutaconyl-CoA

3.3 Electron-transferring flavoprotein from *S. aciditrophicus*

For cloning the *etfAB* genes into the pCDFDuet-1 vector, restriction sites for *Nde*I and *Avr*II were introduced. The amplified DNA fragment (*etfAB*) was cut with *Nde*I and *Avr*II restriction enzymes and ligated into the multiple cloning site 1 of pCDFDuet-1 vector (provides His•Tag, 6 x His, and S•Tag, Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser), whereby the S•Tag was eliminated by introduction of the *etfAB* genes.

The activity of electron-transferring flavoprotein was measured by oxidation of NAD(P)H with INT. The formation of red formazan was followed at 492 nm (ε = 19.2 $mM^{-1}cm^{-1}$). The determined specific activity was 0.4 U/mg, when NADH was used, and 2 U/mg, when NADPH was used as electron donor.

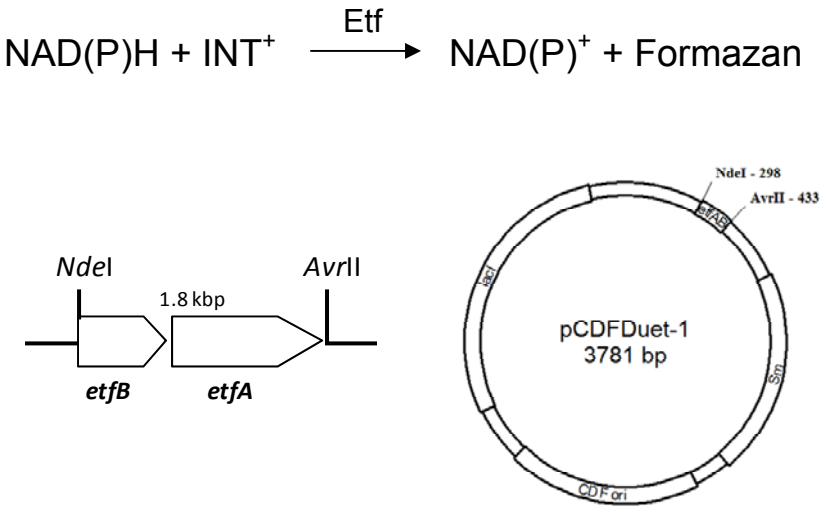

Figure 32. Construction of the recombinant plasmid. CDF ori: origin of replication, *lacI*: lactose repressor, SmR: streptomycin/spectinomycin resistance, *etfAB*: inserted DNA (electron-trasferring flavoprotein), *Nde*I and *Avr*II are restriction enzymes.

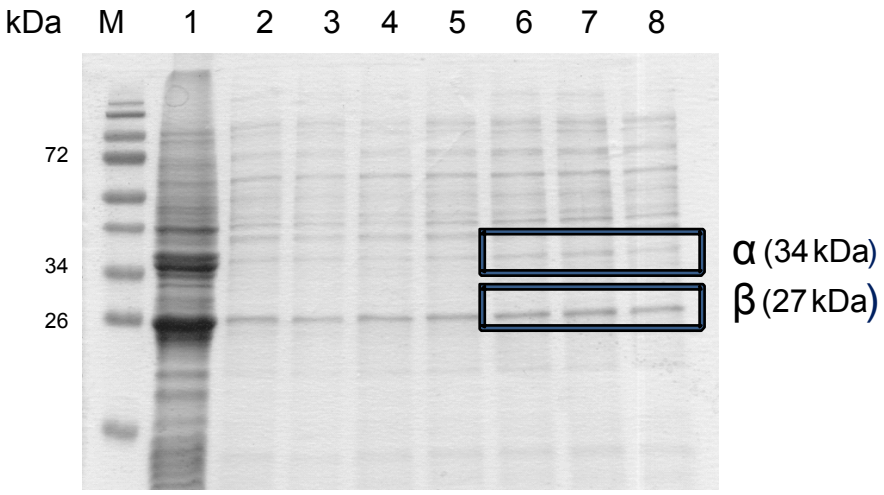

Figure 33. SDS PAGE gel of purified EtfAB (electron-trasferring flavoprotein, 34 kDa and 27 kDa). The SDS-PAGE gel (15%) was stained with Coomassie Brilliant Blue. M: molecular mass marker, 1: cell free extract of induced cells, 2-8: fractions after purification via DEAE Sepharose, fractions 6-8 were concentrated and used in enzyme analysis.

3.4 Coexpression of the genes encoding the glutaryl-CoA dehydrogenase and electron-transferring flavoprotein from *S. aciditrophicus*

In order to convert *E. coli* to a glutarate producer, pCDFDuet-1 vector harbouring *gdh* and *etfAB* genes, should be introduced in the expression strain together with two other vectors harbouring *hgdH*, *gctAB*, *hgdAB* and *hgdC* genes.

Restriction sites for *Pst*I and *Hind*III were introduced in the primers for cloning the gene into the pCDFDuet-1 vector. The *gdh* gene encoding glutaryl-CoA dehydrogenase was introduced into the multiple cloning site 1 of the pCDFDuet-1 vector, upstream of *etfAB* genes, as described above (see 3.1.3).

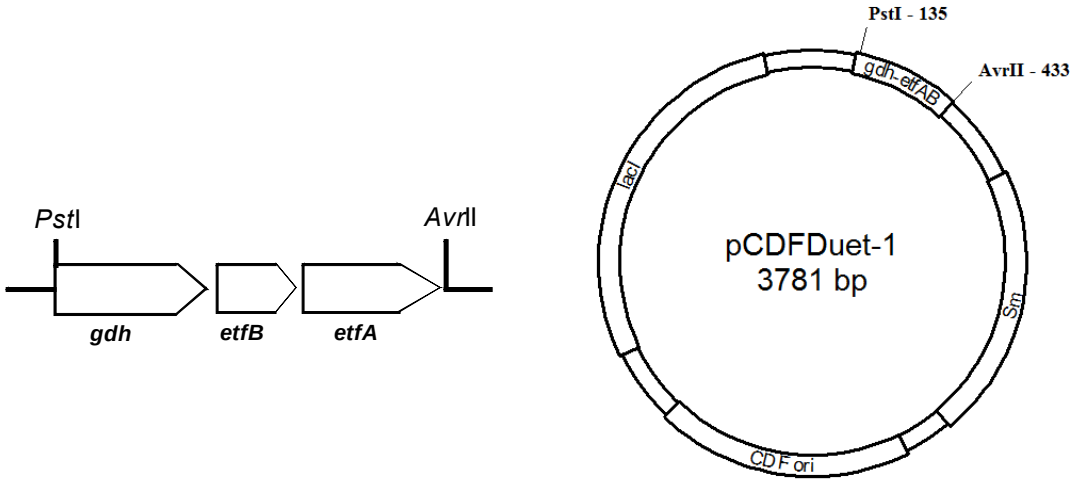

Figure 34. Construction of recombinant plasmid. CDF ori: origin of replication, *lacI*: lactose repressor, SmR: streptomycin/spectinomycin resistance, *gdh*: inserted DNA (glutaryl-CoA dehydrogenase), *etfAB*: inserted DNA (electron-trasferring flavoprotein), *Pst*I and *Avr*II are restriction enzymes.

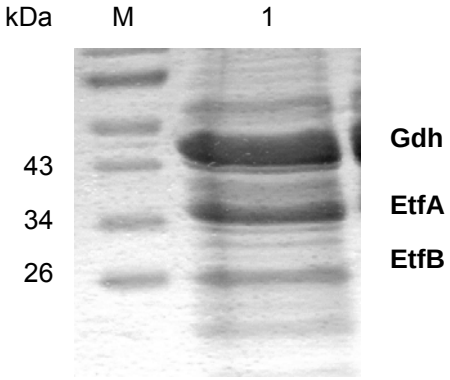

Figure 35. SDS PAGE gel of partially purified Gdh (glutaryl-CoA dehydrogenase, 41.8 kDa) and EtfAB (electron-transferring flavoprotein, 34 kDa and 27 kDa). The SDS-PAGE gel (15%) was stained with Coomassie Brilliant Blue. M: molecular mass marker, 1: purified proteins via DEAE column.

In the introduction it has been proposed that Gdh together with EtfAB can catalyze the reduction of glutaconyl-CoA to glutaryl-CoA with 2 NAD(P)H and ferredoxin. In order to prove this hypothesis, an assay was performed under anaerobic conditions at ambient temperature. The determined specific activity of the Gdh/Etf complex was 10 mU/mg (figure 36). The control assays were carried out in the absence of ferredoxin or glutaconyl-CoA.

$$\text{Glutaconyl-CoA} + 2\ \text{NADH} + 2\ \text{H}^+ \xrightarrow{\text{Gdh/Etf}} \text{Glutaryl-CoA} + 2\ \text{NAD}^+ + \text{H}_2$$

$$\text{Ferredoxin}^{2-} + 2\ \text{H}^+ \xrightarrow{\text{Hydrogenase}} \text{Ferredoxin} + \text{H}_2$$

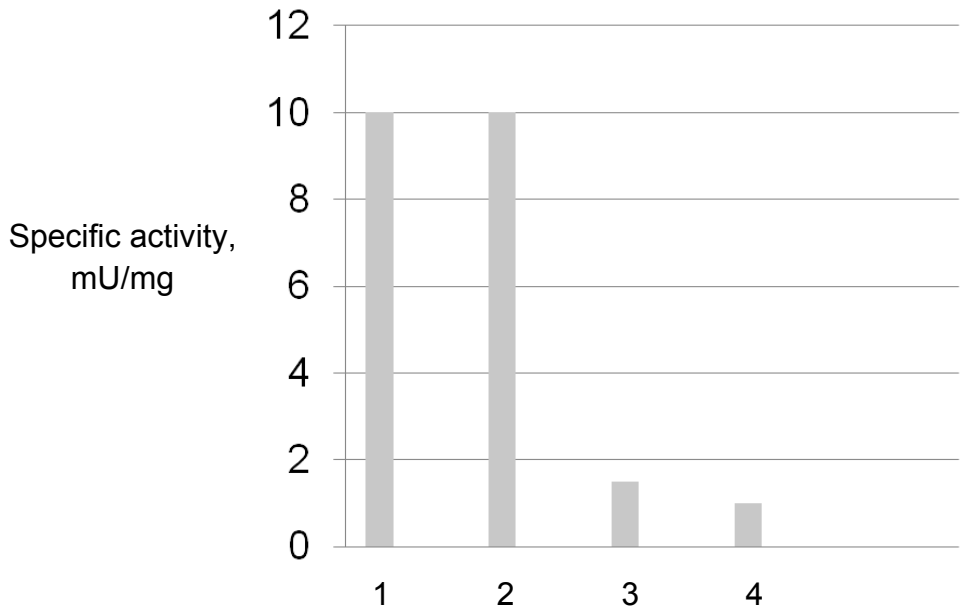

Figure 36. Specific activity of Gdh/Etf complex. 1: complete assay with NADH, 2: complete assay with NADPH, 3: control, carried out without ferredoxin, 4: control carried out without glutaconyl-CoA. Complete assay means that all componenets are added.

Discussion

4.1 Glutaconate biosynthesis

Six genes encoding enzymes that together catalyze the conversion of 2-oxoglutarate to glutaconate were constructed on two plasmids and transformed into *E. coli*. All genes were expressed and yielded active enzymes. The data indicate that the recombinant *E. coli* BL21 (DE3) strain indeed produced glutaconate, although at the initial low level of 0.1 mM. The three-fold enhancement of glutaconate production, from 0.1 to 0.3 mM, by the addition of 10 mM glutamate suggested that this amino acid rather than glucose served as carbon source. The cells were grown on Standard I medium, which contained 5 mM glucose and 3 mM glutamate, derived from yeast extract and peptone. Further addition of 10 mM glutamate, 0.2 mM riboflavin and 2 mM ferric citrate raised the yield of glutaconate from 0.3 mM to 2.7 ± 0.2 mM (table 3), as well as the activity of the 2-hydroxyglutarate dehydratase from *C. symbiosum* by a factor of six. The iron requirement stems from the two [4Fe-4S] clusters in this enzyme. The slight improvement with riboflavin is probably due to 0.2 riboflavin and 1.0 riboflavin-5'-phosphate (FMN) as prosthetic groups of the dehydratase (Hans et al., 1999). A further rise in glutaconate production could be achieved by improving the expression of the genes encoding glutaconate CoA-transferase. The data also indicate that the concentration of glutaconate inside the cells (16 mM) is about 6 times higher than that in the medium (2.7 mM). Therefore, the export of glutaconate, probably mediated by the succinate transporter (Janausch et al., 2001), appears to limit its production. The export could be easier with more flexible glutarate, whose production requires glutaconyl-CoA reductase. Most likely this enzyme is involved in the synthesis of cyclohexane carboxylate and benzoate by *S. aciditrophicus*. when axenically grown on crotonate.

No efforts were made yet to develop a defined growth medium to increase glucose concentration or to use mutant strains to attenuate ethanol production.

Glucose was completely consumed and almost stoichiometrically fermented to two ethanol. Only the small difference in ethanol production (1.5 ± 0.4 mM) from 5 mM

glucose between *E. coli* ‘wild type’ (10.2 ± 0.2 mM) and recombinant *E. coli* (8.7 ± 0.2 mM) indicated that a minor part of glucose gave rise to glutaconate.

The concentration of glutamate decreased initially, but was resynthesized to the same level one hour after induction. We have to assume that at the start of the fermentation, glutamate but later other amino acids present in the peptone-yeast extract medium are the precursors of glutaconate. Aspartate and asparagine can be converted into oxaloacetate, whereas alanine, serine, and cysteine could be oxidized via pyruvate to acetyl-CoA. Oxaloacetate and acetyl-CoA funnel into the Krebs cycle to form 2-oxoglutarate, the precursor of either glutamate or glutaconate (figure 1).

4.2 Glutamate synthesis

As it shown in results, during growth of recombinant *E. coli*, glutamate is utilized and after five hours re-synthesized (figure 24). Unexpectedly, we found a similar course in the ‘wild type’ strain depending on the initial glutamate concentration. To our knowledge, this is the first measurement of glutamate during anaerobic growth of *E. coli*. Considering that glutamate is one of the most abundant metabolites in the cell, *E. coli* must regulate its concentration very well (Bennett et al., 2009). The regulation may be simpler during anaerobiosis, because 2-oxoglutarate dehydrogenase is repressed, which means that the TCA cycle ceases to be a cycle, functioning as two linear pathways: an oxidative pathway from citrate to 2-oxoglutarate and a reductive pathway from oxaloacetate to succinate (Amarasingham and Davis, 1965; Buckel and Barker, 1974; Iuchi and Lin, 1988; Smith and Neidhardt, 1983). Further, *E. coli* is not able to ferment glutamate via neither 2-hydroxyglutarate nor 3-methylasparate (Buckel and Barker, 1974) (W. Buckel, unpublished observations). A possible third pathway, which has been recently discovered in mammalian cells (Yoo et al., 2008), would lead via 2-oxoglutarate, isocitrate and citrate, to acetyl-CoA and oxaloacetate. In *Hydrogenobacter thermophilus* the first step of the reductive carboxylation of 2-oxoglutarate to isocitrate catalyses an ATP-dependent biotin-containing carboxylation factor (Aoshima and Igarashi, 2006). This ATP-requirement probably prevents *E. coli* from the fermentation of glutamate as observed. During growth, glutamate serves as precursors for several amino acids, nucleosides, and all proteins, resulting in a small

decline of their concentrations (ca. 0.5 mM), which replenishes. The observed `chaotic` behaviour of the glutamate levels (figure 27), which happens in the recombinant strain as well as in the wild type, cannot be explained yet.

4.3 Glutaryl-CoA dehydrogenase

McInerney et al. proposed that dicarboxyl-CoA intermediates are formed during alicyclic or aromatic metabolism. Indeed, glutaconyl-CoA and glutaryl-CoA are the key intermediates during the fermentation of aromatic benzoate, as well as in the reduction of aliphatic crotonate (McInerney et al., 2007). Genome investigation and the cell free extract analysis of *S. aciditrophicus* proposed that gene SYN_00480 encodes glutaryl-CoA dehydrogenase, located between genes encoding the biotin carboxy carrier of glutaconyl-CoA decarboxylase and the α-subunit of glutaconyl-CoA decarboxylase.

In this work it was shown that the recombinant protein glutaryl-CoA dehydrogenase catalyzes the oxidation of glutaryl-CoA to glutaconyl-CoA with ferricenium hexafluorophosphate, which is an electron acceptor generally used to screen acyl-CoA dehydrogenase activity. Considering that glutaconyl-CoA, not crotonyl-CoA, is released as a final product, which was confirmed with a coupled assay including glutaconyl-CoA decarboxylase, we can say that this enzyme belongs to the group of nondecarboxylating glutaryl-CoA dehydrogenases.

The amino acid sequence identities of Gdh from *S. aciditrophicus* compared to human and *D. multivorans* Gdh are 29 % and 72 %, respectively. With high sequence similarity and known crystal structure, it is possible to reveal conserved amino acids, required for substrate binding. The amino acid alignment shows that the conserved arginine (R85 in *S.aciditrophicus*, R94 in human, R87 in *D. multivorans*) and glutamate (E365 in *S.aciditrophicus*, E414 in human, E367 in *D. multivorans*) which are involved in the carboxylate binding of the substrate, are present in all glutaryl-CoA dehydrogenases. The presence of the following amino acids E87, S95, T170 and Y369 in human, was assumed as the reason for the decarboxylation or releasement of glutaconyl-CoA intermediate. Probably the key amino acid of the

different catalytic behavior is the conserved tyrosine in decarboxylating Gdhs (Y369 in human), which is replaced with valine (V364 in *S.aciditrophicus,* V366 in *D. multivorans*). Probably tyrosine stabilizes the transient dienolate by π-interactions with the aromatic ring. The sequence further reveald that FAD should be bound via asparagine 371 that is the case in Gdh from *D. multivorans* (N369).

During oxidation, the activity of glutaryl-CoA dehydrogenase is reduced by its product, glutaconyl-CoA. By increasing the concentration of glutaconyl-CoA, the maximum velocity decreased, while the K_m value remained constant. Figure 31 with Michaelis-Menten plots clearly shows that glutaconyl-CoA acts as a non-competitive inhibitor. The inhibition was also observed in the case of glutaryl-CoA dehydrogenase from *D. multivorans.* but described as competitive (Wischgoll 2009). These experiments, however, have to be repeated (Matthias Boll, personal communication).

BLAST investigation revealed high sequence similarities of Gdh from *S. aciditrophicus* with proteins from sulfate-reducing bacteria other than *D. multivorans* DSM 2059 (72 %): *Desulfobacterium autotrophicum* HRM2 (72 %) and *Desulfatibacillum alkenivorans* AK-01 (70 %) annotated as acyl-CoA dehydrogenases. Most likely, both latter organisms also contain nondecarboxylating Gdhs, which may be involved in the oxidation benzoate by sulfate.

```
S.aciditrophicus      ------------------------------------------------------MDFAL 5
D.multivorans         ------------------------------------------------------MDFNL 5
H.sapiens             MALRGVSVRLLSRGPGLHVLRTWVSSAAQTEKAGRTQSQLAKSSRPEFDWQDPLVLEEQL 60
                                                                           ::  *

S.aciditrophicus      SEELEMLRSMARDFAAEKIAPFADKWDEEHYFPYEEVVKPMGELGFFGTVIPEEYGG--T 63
D.multivorans         SKELQMLQKEVRNFVNKKIVPFADQWDNENHFPYEEAVRPMGELGFFGTVIPEEYGGEGM 65
H.sapiens             TTDEILIRDTFRTYCQERLMPRILLANRNEVF-HREIISEMGELGVLG---PTIKGYGCA 116
                      : :  :::.  * :  ::: *     :.:. * :.* :  *****.:*   *   *

S.aciditrophicus      NMGWLAAMILTEEISRASSALRVQINMEGLGCAYTIWKYGTEEAKKKYVQKLVSAEYLGG 123
D.multivorans         DQGWLAAMIVTEEIARGSSALRVQLNMEVLGCAYTILTYGSEALKKKYVPKLSSAEFLGG 125
H.sapiens             GVSSVAYGLLARELERVDSGYRSAMSVQSSLVMHPIYAYGSEEQRQKYLPQLAKGELLGC 176
                      . . :*  :::.*: * .*. *  :.::     :.*  **:*  ::**: :* ..* **

S.aciditrophicus      FGITEFNAGSDVMSMKSTAEDKGD--YYLLNGSKTWISNANCADVIIYYAYTDKAAKGKG 181
D.multivorans         FGITEPDAGSDVMAMSSTAEDKGD--HWLLNGSKTWISNAAQADVLIYYAYTDKAAGSRG 183
H.sapiens             FGLTEPNSGSDPSSMETRAHYNSSNKSYTLNGTKTWITNSPMADLFVVWARCEDGCIRGF 236
                      **:** ::***  :*.: *. :..   : ***:****:*:  **::: :*  :...
```

```
S.aciditrophicus      LSAFAVELKNNPGIRTTDLDKMGSKSSPTGEIYLDNVKVPKENLLGKPGDGAKIVFGSLN 241
D.multivorans         LSAFVIEPRNFPGIKTSNLEKLGSHASPTGELFLDNVKVPKENILGKPGDGARIVFGSLN 243
H.sapiens             LLEKGMRGLSAPRIQG----KFSLRASATGMIIMDGVEVPEENVLPG-ASSLGGPFGCLN 291
                      *    :.  . * *:     *:. ::*.** : :*.*:**:**:*   ...    **.**

S.aciditrophicus      GTRLSAAAGGIGLAQACLDAAIKYANEREQFGKPIGTFQANQFLIGEMATELEAARLMVY 301
D.multivorans         HTRLSAAAGGVGLAQACLDAAIKYCNERRQFGKPIGDFQMNQDMIAQMAVEVEAARLLAY 303
H.sapiens             NARYGIAWGVLGASEFCLHTARQYALDRMQFGVPLARNQLIQKKLADMLTEITLGLHACL 351
                       :* . * * :* :: **.:* :*. :* *** *:.  *  *  :.:* .*:  .

S.aciditrophicus      RAAWQKDQGNLGNTVETAQAKYLAGEVAYKCAIGAMRIFGAYGYSTEYPVNRYYRDAPTY 361
D.multivorans         KAAAAKDEGRLNNGLDVAMAKYAAGEAVSKCANYAMRILGAYGYSTEYPVARFYRDAPTY 363
H.sapiens             QLGRLKDQDKAAPEMVSLLKRNNCGKALD-IARQARDMLGGNGISDEYHVIRHAMNLEAV 410
                      : .  **:..    :     :  .*:.    *  *  ::*. * * ** * *.  :  :

S.aciditrophicus      AMVEGSTNVCKMIVGGALLAQK------ 383
D.multivorans         YMVEGSANICKMIIALDQLGVRKANR-- 389
H.sapiens             NTYEGTHDIHALILGRAITGIQAFTASK 438
                         **: ::  :*:.    . :
```

Figure 37. Sequence alignment of glutaryl-CoA dehydrogenases from *S.aciditrophicus*, *D. multivorans* and *H. sapiens*. Conserved amino acids, required for substrate binding are arginine (R) and glutamate (E) (red) and for FAD binding are asparagine (N) or aspartate (D) (green).

4.4 Gdh/Etf complex

During degradation of benzoate by *S. aciditrophicus*, the formed glutaryl-CoA has to be oxidized to glutaconyl-CoA and H_2. Despite the low hydrogen pressure, enabled by methanogenic bacteria, the oxidation is still endergonic. This energetic barrier could be overcome by a reverse electron bifurcation, i.e., the endergonic reduction of NAD^+ with glutaryl-CoA could be driven by the exergonic reduction of another NAD^+ with reduced ferredoxin. *S. aciditrophicus* grows on crotonate without methanogenic partner. During crotonate fermentation, where acetate and cyclohexanecarboxylate are the end-products, glutaconyl-CoA is converted to glutaryl-CoA (figure 8).

Based on idea to use the system similar to the ETF/BDC system, studied in Prof. Buckel's and Thauer's groups (Herrmann et al., 2008; Li et al., 2008), we propose that Gdh together with EtfAB can catalyze the reduction of glutaconyl-CoA to glutaryl-CoA with 2 NADH and ferredoxin. Ferredoxin can be reoxidized by NAD^+ or protons mediated by Rnf (Herrmann et al., 2008) or hydrogenase, respectively. The genome

of *S. aciditrophicus* SB reveals the presence of a set of seven genes (SYN1664–1661, SYN1659, SYN1658, and SYN3073) with similarity to the genes encoding the Rnf ion-translocating electron transport complex found in *Rhodobacter capsulatus* (McInerney et al., 2007), what can additionally support our idea. Alternatively the reductant could be menaquinol, also present in *S. aciditrophicus*.

Gdh and EtfAB were produced and purified as complex, what makes this system similar to ETF/BCD systems from *C. kluyveri* and *C. tetanomorphum,* where the electron transferring flavoprotein makes a stable complex with butyryl-CoA dehydrogenase.

In this work it is shown that the glutaryl-CoA dehydrogenase/ETF complex catalyzed the oxidation of NAD(P)H with glutaconyl-CoA with specific activity of 10 mU/mg. When ferredoxin was omitted, a change in absorbance was not observed. This experiment showed that ferredoxin was required as an electron acceptor. NAD(P)H, as electron carrier, provides the reducing equivalents in the reductive part of the process and reduces ferredoxin, which must be reoxidised in order to maintain equilibrium. The hydrogen determination remains as one of the future experiments.

Butyryl-CoA dehydrogenase (crotonyl-CoA reductase) has been suggested to participate in energy conservation based on the redox potential difference between the glutaconyl-CoA/glutaryl-CoA ($E°'$ = -10 mV) and NAD^+/NADH ($E°'$ = -320 mV). Considering the assay described above and the similarity between purified complexes of BCD/ETF and Gdh/ETF, it can be proposed that also glutaconyl-CoA reductase may participate in energy conservation.

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Erklärung

Ich versichere, dass ich meine Dissertation mit dem Titel

Production of glutaconic acid in recombinant *Escherichia coli*

Selbständig, ohne unerlaubte Hilfe angefertigt und mich debei keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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