Breeding of a Cyclic Imide-Assimilating Bacterium, \textit{Pseudomonas putida} s52, for High Efficiency Production of Pyruvate

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A succinimide-assimilating bacterium, \textit{Pseudomonas putida} s52, was found to be a potent producer of pyruvate from fumarate. Using washed cells from \textit{P. putida} s52 as catalyst, 400 mM pyruvate was produced from 500 mM fumarate in a 36-h reaction. Bromopyruvate, a malic enzyme inhibitor, was used for the selection of mutants with higher pyruvate productivity. A bromopyruvate-resistant mutant, \textit{P. putida} 15160, was found to be an effective catalyst for pyruvate production. Moreover, under batch bioreactor conditions, 767 mM of pyruvate was successfully produced in a 72-h reaction with washed cells from \textit{P. putida} 15160 as catalyst.

Key words: pyruvate; cyclic imide; succinimide; \textit{Pseudomonas putida}; bromopyruvate

Pyruvate is an effective precursor in the synthesis of various pharmaceuticals, such as L-3,4-dihydroxyphenylalanine (L-DOPA). It is commercially produced by fermentation, but the production yield of pyruvate with respect to glucose added to the medium during fermentation is relatively low. Thus the identification of new microbial systems for the efficient production of pyruvate is of considerable interest. We have reported a novel metabolic pathway for cyclic imides in the bacterium \textit{Blastobacter} sp. A17p-4. Through this pathway, cyclic imides are transformed to organic acids and they are further metabolized in the TCA cycle. The cyclic imide transformation pathway is distributed through a wide range of microorganisms, and we have found that a glutarimide-assimilating \textit{Pseudomonas} strain effectively produces pyruvate from fumarate, an intermediate of cyclic imide metabolism and a relatively inexpensive starting material for pyruvate production. Although the strain produced pyruvate at high yield (94 mM pyruvate from 100 mM fumarate), at higher concentrations fumarate inhibited production activity, resulting in insufficient pyruvate accumulation.

In this study, a potent pyruvate producer, \textit{Pseudomonas putida} s52, was isolated, and chemical mutagenesis was used to obtain mutant strains with higher productivity. It is generally recognized of microbial production systems that mutations conferring resistance to structural analogs of end products can prevent feedback inhibition of the synthetic pathway of the products, but such regulatory mutants for pyruvate overproduction are difficult to obtain, because pyruvate is a central intermediate in various metabolic pathways and production of it is intricately controlled by balance of biosynthesis and further anabolism. On the other hand, mutants resistant to inhibitors of specific enzymes in pyruvate synthesis pathways might show higher activity due to activation of mutant enzymes or an elevated alternative pathway. Bromopyruvate, a malic enzyme inhibitor, was found to be a potential inhibitor of pyruvate production from fumarate, and it was used here to obtain mutant strains that produced higher amounts of pyruvate than the parent strain, \textit{P. putida} s52.

Materials and Methods

Isolation of succinimide-assimilating microorganisms. The basal medium comprised 0.1% (w/v) KH2PO4, 0.1% (w/v) K2HPO4, 0.03% (w/v) MgSO4·7H2O, 0.01% (w/v) yeast extract, and 0.2% (w/v) NH4Cl in tap water, pH 7.0. Soil samples were cultivated with shaking at 28 °C for 2–4 d in the basal medium supplemented with 2% (w/v) succinimide as sole source of carbon. The soil cultures were streaked on 2% agar plates of the same medium to obtain isolated colonies. The soil isolates were cultured in the basal medium, collected by centrifugation at 2,500 × g for 10 min, and washed twice with 0.85% (w/v) NaCl to prepare washed cells used in subsequent experiments.

Screening of pyruvate-producing strains. The reaction mixture comprised 500 mM fumarate, 100 mM potassium phosphate buffer (pH 7.0), and 5% (w/v) washed cells obtained from agar plates as described above. The reaction mixtures were incubated at 28 °C for 24 h with shaking at 300 rpm and then centrifuged at 4,000 × g for 10 min. Pyruvate, fumarate, and other organic acids in the resulting supernatants were separated by HPLC using an AmineX HPX-87H packed column (7.8 × 30 mm; Bio Rad Laboratories, Boston, MA) with 0.4% (w/v) H2SO4 at a flow rate of 0.7 mL/min as eluent. Organic acids were quantified by UV absorbance at a wavelength of 220 nm.

Abbreviations: HPLC, high-performance liquid chromatography; NTG, N-methyl-N′-nitro-N-nitrosoguanidine; EDTA, ethylenediaminetetraacetic acid

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Optimization of pyruvate production by washed cells of *Pseudomonas putida* s52. To optimize culture conditions, basal medium supplemented with 2% (w/v) glucose, t-malate, succinimide, succinate, or fumarate was used. Cells were cultivated at 28 °C for 24–72 h and washed cells were prepared. The reaction was performed by combining 100–1,000 mM fumarate and 2.5–7.5% washed cells in various 100 mM buffers (potassium phosphate buffer at pH 5.5–8.0, Tris/HCl buffer at pH 7.5–9.5, and borate/NaOH buffer at pH 9.5–11.0), with shaking at 0–300 rpm. Reactions were carried out for 24 h, and then the mixtures were centrifuged. The resulting supernatants were analyzed for organic acids by HPLC, as described above.

Search of an inhibitor of pyruvate production by washed cells of *P. putida* s52. To find an effective inhibitor of pyruvate production, a reaction was carried out in the mixture of 5% washed *P. putida* s52 cells, 500 mM fumarate, and 2 mM inhibitor in 100 mM potassium phosphate buffer (pH 7.0) at 28 °C with shaking (300 rpm). Reactions were carried out for 12 h, and then the mixtures were centrifuged. The resulting supernatants were analyzed for organic acids by HPLC, as described above.

Preparation of bromopyruvate-resistant mutants of *P. putida* s52 and analysis of pyruvate production. Mutation of *P. putida* s52 was induced by N-methyl-N’-nitro-N-nitrosoguanidine (NTG) treatment. *P. putida* s52 was cultivated at 28 °C for 5–8 h in basal medium with 2% fumarate as sole carbon source. The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.03–0.3 mg/mL of NTG and were incubated for 2 h at 20 °C. The NTG-treated mutant cells were harvested, transferred into 50 mL of basal medium containing 0.03% (w/v) bromopyruvate and 2% fumarate, and cultivated at 28 °C with shaking at 300 rpm for 5–8 h. Cells were then harvested by centrifugation, diluted appropriately, spread onto 2% agar plates of the same medium, and incubated at 28 °C for 24 h. The resulting colonies were isolated and maintained on 2% agar plates of the same composition. The pyruvate production of the mutants was evaluated under the screening conditions described above with 1,000 mM fumarate.

Pyruvate production from fumarate under batch bioreactor conditions. Washed cells of *P. putida* strains cultivated for 48 h in basal medium supplemented with 2% fumarate were used as catalysts. The reaction mixture contained 1,000 mM fumarate, 5% washed cells, and 100 mM potassium phosphate buffer (pH 7.0). The reaction was carried out in a thermostatically controlled reaction vessel (200 mL, 28 °C) with vigorous stirring and aeration (1 vvm). Several drops of adecanol were added to prevent excessive foaming of the reaction mixture. The pH of the reaction mixture was maintained automatically at 7.0 with 2 mM H3PO4. Aliquots of the reaction mixture were sampled, and the reaction was stopped by removing the cells by centrifugation, and the resulting supernatant was analyzed for organic acids by HPLC as described above.

Results

Selection of pyruvate-producing strains from succinimide-assimilating microorganisms

Microorganisms were isolated with succinimide as sole carbon source, and were used in reactions with 500 mM fumarate as substrate. Among 72 succinimide-assimilating microorganisms, six strains produced high amounts of pyruvate under the screening conditions imposed. Strain s52, which showed the highest pyruvate production, was selected for further investigation. It was identified as *Pseudomonas putida* by the results of physiological and characteristics tests: NO3 reduction, –; indole production, –; arginine dihydrolase, +; urease, –; aesculin hydrolysis, –; gelatin hydrolysis, –; β-galactosidase, –; assimilation (glucose, +; l-arabinose, –; d-mannose, –; d-mannitol, –; N-acetyl-d-glucosamine, –; maltose, –; gluconate, +; n-caproate, +; adipate, –; dl-malate, +; citrate, +; phenylacetate, +). Based on a metabolite analysis of succinimide assimilation, the metabolic pathway of succinimide into pyruvate in *P. putida* s52 as well as those of other reported cyclic imide-assimilating microorganisms is proposed to be as shown in Fig. 1.

Pyruvate production from fumarate by *P. putida* s52

The optimal conditions for pyruvate production from fumarate with washed cells of *P. putida* s52 were investigated. To obtain active cells for pyruvate production, the effects of carbon sources in the culture medium were examined. Glucose and an intermediate of succinimide metabolism, succinimide, succinate, l-malate, or fumarate, were added to the basal medium as sole source of carbon, and the activity of the washed cells was evaluated in a resting cell reaction. *P. putida* s52 showed poor growth with glucose, while it grew well when cultured with succinimide, succinate, l-malate, or fumarate. When fumarate was used as carbon source for cell growth, washed cells of *P. putida* s52 showed the highest pyruvate production in the resting cell reaction (Fig. 2). Aeration was a critical factor in the reaction, and vigorous shaking (300 rpm) brought about the production of a large amount of pyruvate. With gentle shaking, an accumulation of l-malate, an expected intermediate in pyruvate production from fumarate, was observed. Pyruvate production increased with increasing concentrations of fumarate up to 500 mM, but remained constant at higher fumarate concentrations up to 1,000 mM. The other reaction conditions, including amounts of washed cells, pH, and buffer, were optimized (data not shown). Under optimized reaction conditions with 5% washed cells of *P. putida* s52 as catalyst, in 100 mM potassium phosphate buffer (pH 7.0) at 28 °C with shaking (300 rpm), 286 mM of pyruvate was produced in 24 h (Fig. 3). At the initial stage of the reaction, the highest amount of the intermediate l-malate accumulated at 13 h, and a small amount of oxaloacetate, a byproduct, accumulated throughout the reaction (Fig. 3).

Bromopyruvate-resistant mutants from *P. putida* s52

In order to obtain strains with increased pyruvate production, we isolated mutants of *P. putida* s52 that were resistant to inhibitors of the enzymes involved in
fumarate metabolism to pyruvate. Conversion of malate was a rate-limiting step in the pyruvate production pathway, because significant amounts of malate accumulated in the reaction mixture of \textit{P. putida} s52 cells with a high concentration of fumarate (data not shown). Hence effects of specific inhibitors of malate-metabolizing enzymes on the pyruvate production of \textit{P. putida} s52 were examined. Three malic enzyme inhibitors, bromopyruvate,\textsuperscript{11) }L-aspartic acid, \textsuperscript{12) }and ethylenediaminetetraacetic acid (EDTA), \textsuperscript{13) }and an L-malate dehydrogenase inhibitor, gossypol\textsuperscript{14) }were tested. Bromopyruvate clearly inhibited pyruvate production (Fig. 4), and its growth inhibition on \textit{P. putida} s52 was further examined. The addition of 0.1 mM of bromopyruvate strongly inhibited the growth of \textit{P. putida} s52 in the culture medium with fumarate as sole carbon source (Fig. 5). Growth inhibition by up to 1 mM bromopyruvate was effectively cancelled by the addition of pyruvate to the fumarate medium. Mutant strains of \textit{P. putida} s52 that were resistant to high levels (2 mM) of bromopyruvate in the culture medium were obtained, and were used to produce pyruvate from 1,000 mM fumarate. Among the approximately 2,200 bromopyruvate-resistant mutants assayed, 20 mutant strains produced larger amounts of pyruvate than the parent strain, \textit{P. putida} s52. Even with 1 mM bromopyruvate, one strain, \textit{P. putida} 15160, grew in the fumarate medium as well as \textit{P. putida} s52 did in the fumarate medium containing pyruvate (Fig. 5). Pyruvate production from 1,000 mM fumarate by washed cells of \textit{P. putida} s52 was carried out using 5% washed \textit{P. putida} s52 cells cultured in basal medium containing 2% of various carbon sources: fumarate, L-malate, succinate, succinimide, and glucose. Data shown are results of a pyruvate-producing reaction carried out under optimized conditions with 500 mM fumarate over 24 h. Concentrations of pyruvate (dark-gray bars), L-malate (light-gray bars), and fumarate (white bars) are shown.

**Fig. 2.** Effects of Carbon Sources on Pyruvate Production by \textit{P. putida} s52 Cells.

Pyruvate production was carried out using 5% washed \textit{P. putida} s52 cells cultured in basal medium containing 2% of various carbon sources: fumarate, L-malate, succinate, succinimide, and glucose. Data shown are results of a pyruvate-producing reaction carried out under optimized conditions with 500 mM fumarate over 24 h. Concentrations of pyruvate (dark-gray bars), L-malate (light-gray bars), and fumarate (white bars) are shown.

**Fig. 3.** Time-Course of Pyruvate Production from Fumarate by \textit{P. putida} s52 Cells.

Pyruvate production using 5% washed \textit{P. putida} s52 cells was carried out under optimized conditions with 500 mM fumarate over 24 h. The concentrations of pyruvate ( ), L-malate ( ), fumarate ( ), and oxaloacetate ( ) are shown.

**Fig. 4.** Effects of Inhibitors on Pyruvate Production by \textit{P. putida} s52 Cells.

Pyruvate production using 5% washed \textit{P. putida} s52 cells was carried out for 12 h under optimized conditions with 500 mM fumarate, without inhibitor, and in the presence of 2 mM of inhibitors L-aspartate, EDTA, gossypol, or bromopyruvate. The concentrations of pyruvate (dark-gray bars), L-malate (light-gray bars), and fumarate (white bars) are shown.

**Fig. 5.** Effects of Bromopyruvate on the Growth of \textit{P. putida} s52 and \textit{P. putida} 15160.

The microorganisms were cultured in media containing 0, 0.5, 1, or 2 mM bromopyruvate. \textit{P. putida} s52 was cultured in fumarate medium ( ) and in fumarate medium containing 0.4% pyruvate ( ). \textit{P. putida} 15160 was cultured in fumarate medium ( ). Cell growth was measured by the absorbance at 590 nm.
P. putida 15160 or the parental P. putida s52 was then examined (Fig. 6). Using P. putida s52 cells, about 400 mM of pyruvate accumulated in 36 h, compared to about 560 mM of pyruvate when P. putida 15160 cells were used. In both reactions, L-malate accumulated at the initial stage. The P. putida 15160 cells converted L-malate to pyruvate more efficiently than the cells of P. putida s52 (Fig. 6).

Pyruvate production from fumarate by P. putida 15160 under batch bioreactor conditions

The washed cells of P. putida 15160 were used for the pyruvate production from 1,000 mM fumarate at a controlled pH (Fig. 7). This strain accumulated high concentrations of pyruvate, and maximum production by it was 767 mM at 72 h. In this case, L-malate accumulated at the initial stage of the reaction, but disappeared completely by 48 h.

Discussion

Pyruvate was effectively produced from fumarate through an active metabolic pathway of succinimide-assimilating microorganisms. A prolific pyruvate-producing mutant derived from succinimide-assimilating strain P. putida s52 was selected based on resistance to bromopyruvate. Bromopyruvate, a malic enzyme inhibitor, was found to prevent pyruvate production from fumarate and to inhibit the growth of P. putida s52 in the medium when fumarate was used as sole carbon source, indicating that it played a crucial role in pyruvate synthesis from fumarate in this strain (Fig. 1). The recovery of P. putida 15160 in the bromopyruvate-containing medium might have been due to increased activity of the malic enzyme or to compensatory induction of other enzymes for the conversion of L-malate into pyruvate. Indeed, without bromopyruvate, accumulated L-malate was successfully converted to pyruvate in the reaction mixture of P. putida 15160 as compared to P. putida s52 (Fig. 6). Under batch bioreactor conditions with P. putida 15160, 767 mM pyruvate was produced from fumarate. The concentration of pyruvate produced with P. putida 15160 was much higher than with Torulopsis glabrata, a strain used in the industrial production of pyruvate, which produced 676 mM pyruvate from glucose.15) This higher productivity of pyruvate with P. putida 15160 should be helpful in increasing total amount and facilitating downstream processes of pyruvate production.

Biosynthesis of L-valine and L-leucine is one of the major pyruvate assimilation pathways.15) Hence we assumed that L-valine/L-leucine auxotrophs derived from P. putida 15160 would become more prolific pyruvate-producing strains. In a preliminary study, we obtained auxotrophic mutants and tested them for pyruvate production, but they produced just a little more pyruvate than the parent strain. These results indicate that further introduction of auxotrophy for other compounds produced from pyruvate, such as L-alanine, enhances pyruvate production in these strains.

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