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Expression of *Ascaris suum* Malic Enzyme in a Mutant *Escherichia coli* Allows Production of Succinic Acid from Glucose

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ABSTRACT

The malic enzyme gene of *Ascaris suum* was cloned into the vector pTRC99a in two forms encoding alternative amino-termini. The resulting plasmids, pMEA1 and pMEA2, were introduced into *Escherichia coli* NZN111, a strain that is unable to grow fermentatively because of inactivation of the genes encoding pyruvate dissimilation. Induction of pMEA1, which encodes the native animoterminus, gave better overexpression of malic enzyme, approx 12-fold compared to uninduced cells. Under the appropriate culture conditions, expression of malic enzyme allowed the fermentative dissimilation of glucose by NZN111. The major fermentation product formed in induced cultures was succinic acid.

Index Entries: Metabolic engineering; succinic acid; *Escherichia coli*; malic enzyme; *Ascaris suum.*

Succinic acid and other dicarboxylic acids can be produced as end products in microbial fermentations of renewable carbohydrate feedstocks and are potential intermediates in the synthesis of commodity chemicals *(1)*. Chemical conversion of succinic acid can yield established commodity chemicals, such as 1,4-butanediol and tetrahydrofuran *(1)*, or new products such as biodegradable solvents or polymers.

Escherichia coli normally produces a mixture of fermentation products of which succinic acid is a minor component *(2,3)*. Previously, we reported that overexpression of phosphoenolpyruvate carboxylase in *E. coli* results in increased succinic acid formation *(4)*. This manipulation involves

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increasing the flux through the normal pathway to succinic acid. Other biochemically feasible routes to succinic acid do not occur naturally because of the regulation of crucial enzymes, either at the genetic or enzymatic level. Here we report that genetic manipulation of one such enzyme, malic enzyme, can at least in part overcome these regulatory limitations.

Physiologically malic enzyme catalyzes the conversion of malate and NAD^+ to pyruvate, NADH, and CO_2. However, their reverse reaction is favored thermodynamically *(5)*. Malic enzyme is regulated genetically—its synthesis is induced in the presence of malate *(6,7)*—and at the the level of catalysis. Allosteric activation and inhibition have been reported for malic enzyme from various sources *(8–10)* and kinetic parameters favor the physiological reaction. The K_ms for malic enzyme from *Ascaris suum*, the only example for which K_ms were determined for the reaction in both directions, were 45 m*M* for pyruvate compared to 0.4 m*M* for malate *(11)*. Under normal conditions, pyruvate would not accumulate to a sufficiently high concentration to allow malic enzyme to be effective in producing dicarboxylic acids.

Mutants of *E. coli* lacking pyruvate:formate lyase (*pfl*) and lactate dehydrogenase (*ldh*) are blocked in the metabolism of pyruvate and fail to grow fermentatively *(12)* (Fig. 1). Such strains accumulate pyruvate, excreting it into the medium to millimolar concentrations *(13)*. The authors have previously shown that an enzyme with a poor K_m for pyruvate, a genetically engineered malate dehydrogenase with lactate dehydrogenase activity *(13)*, can allow such a mutant to grow fermentatively, in that case by lactic acid fermentation. Here the authors evaluate the potential of malic enzyme expressed in the same strain to allow the production of succinic acid as the major fermentation product (Fig. 1).

The gene encoding the NAD^+-dependent malic enzyme of *A. suum* was cloned earlier under control of the *lac* promoter *(14)*. Because this promoter is repressed by glucose, we recloned the gene into the vector pTRC99a (Pharmacia Biotech) that employs the *trc* promoter and permits induction by isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of glucose. The malic enzyme gene was amplified by the polymerase chain reaction (PCR) using plasmid pME-2 *(14)* as template and primers based on the published sequence of the *A. suum* malic enzyme gene *(14)*. A truncated *N*-terminus was designed to match the *N*-terminus predicted for the *E. coli* enzyme based on its DNA sequence *(15)*. The primers were:

C-terminus; ATTTAGGTACCTTAACCATCCATGCTGTCAT
N-terminus; TTTCCTCCATGGTTAAAAGTGTCGCTCATCAT
truncated N-terminus; TTAAATCCATGGACGAAAAAGAGATG

Primers were combined at 1 μ*M* with approx 200 ng of pME-2 vector in a standard PCR using native Taq polymerase (Perkin-Elmer). Both sets of primers (generating products with alternate *N*-termini) gave products of 1.7 kb, the size expected for the malic enzyme gene *(14)*. The fragment was verified to be the malic enzyme gene by digestion with restriction enzymes.

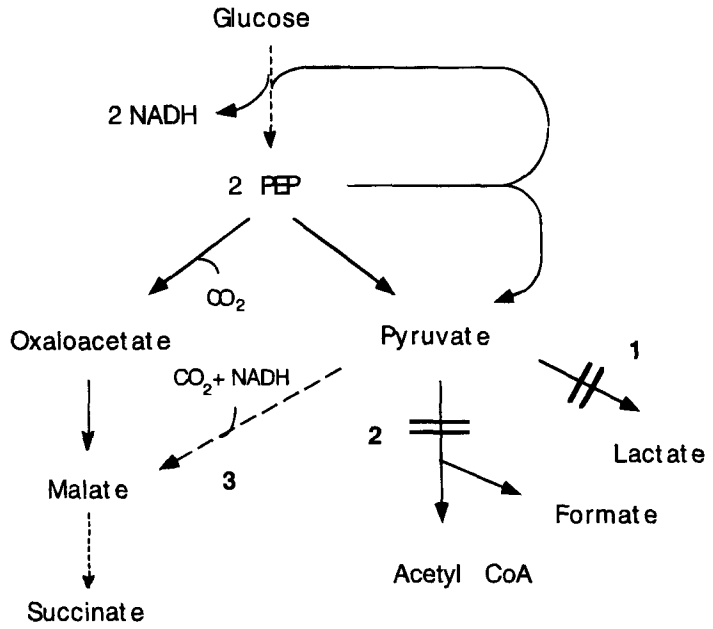

Fig. 1. Diversion of metabolites to succinic acid in *E. coli* NZN111. In the strain NZN111 fermentation is blocked by inactivation of the genes for lactate dehydrogenase (*ldh*, enzyme 1) and pyruvate:formate lyase (*pfl*, enzyme 2). In principle, introduction of malic enzyme (enzyme 3) can allow conversion of accumulated pyruvate to dicarboxylic acids.

The amplified gene was cloned into the NcoI and KpnI sites of pTRC99a by standard techniques *(16)*. PCR products were digested with NcoI and KpnI (Promega), gel-isolated, purified with the Qiaex Gel Extraction Kit (Qiagen), and ligated into pTRC99a that had been cleaved with NcoI and KpnI and dephosphorylated with calf intestinal phosphatase (Promega).

The *E. coli* strain NZN111, a generous gift from Dr. David Clark, contains insertionally inactivated *pfl* and *ldh* genes and is incapable of fermentative growth. An aerobically grown culture was made competent and transformed with the above ligation mixtures by standard methods, and the resulting colonies were screened for the malic enzyme gene by restriction fragment analysis. Four colonies containing a plasmid encoding the mature malic enzyme (called pMEA1) and nine containing the truncated form (called pMEA2) were obtained. Representative colonies were evaluated for expression of malic enzyme induced by IPTG. Cells were lysed enzymatically and assayed using Mn^{2+} as the activating metal ion *(11)*.

When induced at 37°C, the authors observed no evidence for malic enzyme overexpression. Denaturing polyacrylamide gel analysis of extracts confirmed that malic enzyme was not overexpressed. When grown at 30°C, on the other hand, moderate induction of malic enzyme activity occurred. Induction of a representative pMEA1- and pMEA2-containing strain with 1 m*M* IPTG resulted in maximal specific activities of 2.7 and 1.1

Table 1
Induction of *A. suum* Malic Enzyme in Transformants[a]

Strain	IPTG	Specific Activity (μmol/min/mg)	SD[b]	Induction (fold)
NZN111 (pMEA1)	–	0.44	0.13	
	+	5.26	1.49	11.9
NZN111 (pMEA2)	–	0.50	0.17	
	+	1.44	0.42	2.9

[a]Cultures were grown aerobically at 30°C in 25 mL LB medium containing 100 μg/mL ampicillin. At OD_{600} of 0.5, 1 m*M* IPTG was added. 2 mL aliquots were sampled and assayed for malic enzyme activity.

[b]Standard deviation.

μmol/min/mg, respectively, after 8 h compared to approx 0.3 μmol/min/mg for uninduced controls. Cells observed under the microscope were elongated and contained inclusion bodies. Denaturing gel electrophoresis of these samples revealed increased abundance of a protein of molecular weight 68,000, consistent with that expected for the *A. suum* malic enzyme (data not shown). Titration of the IPTG concentration indicated no difference in malic enzyme overexpression from pMEA1 between 0.25 and 4 m*M* IPTG. Representative transformants containing pMEA1 or pMEA2 were compared for overexpression induced by 1 m*M* IPTG in 6 h. Plasmid pMEA1 supported approx 12-fold overexpression of malic enzyme whereas pMEA2, which encodes a truncated form of the enzyme, consistently gave lower overexpression, approx threefold (Table 1).

The effect of overexpression of malic enzyme on fermentative growth was investigated using a transformant containing plasmid pMEA1. As a control we used NZN111 containing the parent vector, pTRC99a. Duplicate cultures of each strain were grown aerobically at 30°C in LB medium containing ampicillin and 10 g/L glucose. When the OD_{600} reached 0.5, one culture of each pair was induced with 1 m*M* IPTG. After 4 h (OD_{600} of 2–4), cells were centrifuged under sterile conditions, washed once with fresh medium and resuspended in sufficient medium to give an OD_{600} of 2.0 for each culture. One mL of these suspensions was immediately injected into sealed, stoppered serum tubes containing 10 mL of experimental medium—LB with 18 g/L glucose, 100 μg/mL ampicillin, 1 m*M* IPTG (for the induced cultures only), and 0.5 g of $MgCO_3$ to maintain pH—under an atmosphere of air:CO_2 (1:1) at 14 psi. The gas composition and pressure were established prior to inoculation by use of a gassing manifold *(17)*. Cultures were incubated on their side at 30°C and agitated at 100 rpm.

The metabolism of the cultures was analyzed by high-pressure liquid chromatography. Aliquots of 1 mL were removed with a syringe, centrifuged, and fractionated on a Bio-Rad Aminex HPX-87H column (7.8 × 300 mm) using a Shimadzu LC-10A chromatographic system with UV

Table 2
Effect of Expression of *A. suum* Malic Enzyme on Product Distribution[a]

Strain	IPTG	Amt (g/liter) of product					
		Glucose	Succinate	Pyruvate	Lactate	Acetate	Ethanol
NZN111 (pTRC99a)	–	2.58	2.63	4.02	0.34	0.72	2.18
	+	3.31	2.45	3.85	0.29	0.73	2.00
NZN111 (pMEA1)	–	5.50	2.06	2.50	0	0.61	1.11
	+	0	7.07	2.83	0	0.00	1.31

[a]Cultures were grown at 30°C in sealed serum tubes containing 10 mL of LB medium containing 18 g/L glucose, 100 μg/mL ampicillin, and, where indicated, 1 m*M* IPTG. The headspace was air:CO_2 (1:1) at 14 psi. Metabolites were analyzed by high pressure liquid chromatography after consumption of glucose ceased (24 h).

absorbance and refractive index detection. The column was eluted isocratically with 5 m*M* sulfuric acid, and data were analyzed with an EZChrom chromatographic data system (Scientific Software). Quantification was based on comparison to standards of known concentration.

After 24 h, metabolism of glucose had ceased. At this point, control cultures had partially metabolized glucose and generated small amounts of fermentation products (Table 2). The major fermentation product formed in these cultures was pyruvic acid, as expected for NZN111 because of the lack of functional pyruvate:formate lyase and lactate dehydrogenase. The authors assume that adequate pools of NADH were available for reduction of pyruvate; one NADH is produced per every three carbons metabolized via glycolysis and mutants blocked in pyruvate dissimilation fail to ferment glucose because of their inability to achieve electron balance *(12)*. Only the culture in which malic enzyme was induced—NZN111(pMEA1) containing IPTG—consumed all the glucose. Succinic acid was the major fermentation product in this culture, present at approximately three times the concentration observed in the control cultures. Acetic acid was present at lower levels. The amounts of other fermentation products were comparable to those in control cultures. The formation of succinic acid instead of malic or fumaric acid requires an additional reductive step and creates an electron imbalance relative to the NADH produced in glycolysis if succinic acid were the sole product. The production of acetate and pyruvate in this culture may be a consequence of the need to maintain electron balance in the fermentation *(2)*.

For all the cultures, part of the glucose consumption can be attributed to aerobic respiration using the air initially present in the culture tubes. If the authors provided a fully anaerobic atmosphere initially, none of the cultures grew. They attribute this failure to the demands put on the cells

by simultaneous induction of malic enzyme, the transition to anaerobic growth, and the accumulation of pyruvate.

These results demonstrate both the potential and limitations of the use of malic enzyme to manipulate fermentative metabolism in *E. coli*. Whereas expression of malic enzyme does channel metabolites to succinic acid, it does so inefficiently. This inefficiency is a result in part because of the poor K_m of the *A. suum* malic enzyme for pyruvate, but may also be because of the poor expression obtained for the heterologous *A. suum* enzyme. Some of these issues can be addressed by the use of alternative malic enzymes or the development of improved enzymes through genetic engineering. The authors have initiated cloning of the NAD^+-dependent malic enzyme from *E. coli* in anticipation that it will give better over-expression and, perhaps, possess a better K_m for pyruvate.

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