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Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid

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Abstract Malic acid, a petroleum-derived C4-dicarboxylic acid that is used in the food and beverage industries, is also produced by a number of microorganisms that follow a variety of metabolic routes. Several members of the genus *Aspergillus* utilize a two-step cytosolic pathway from pyruvate to malate known as the reductive tricarboxylic acid (rTCA) pathway. This simple and efficient pathway has a maximum theoretical yield of 2 mol malate/mol glucose when the starting pyruvate originates from glycolysis. Production of malic acid by *Aspergillus oryzae* NRRL 3488 was first improved by overexpression of a native C4-dicarboxylate transporter, leading to a greater than twofold increase in the rate of malate production. Overexpression of the native cytosolic alleles of pyruvate carboxylase and malate dehydrogenase, comprising the rTCA pathway, in conjunction with the transporter resulted in an additional 27 % increase in malate production rate. A strain overexpressing all three genes achieved a malate titer of 154 g/L in 164 h, corresponding to a production rate of 0.94 g/L/h, with an associated yield on glucose of 1.38 mol/mol (69 % of the theoretical maximum). This rate of malate production is the highest reported for any microbial system.

Keywords Malic acid · Metabolic engineering · Filamentous fungi · Transcriptomics · *Aspergillus oryzae*

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Introduction

Malic acid is a C4-dicarboxylic acid with an annual production estimated at 40,000 tons (Goldberg et al. 2006). It is used predominantly in the food and beverage industries as an acidulant and taste enhancer/modifier, particularly in combination with artificial sweeteners. Additionally, C4-dicarboxylic acids including malic, fumaric, and succinic acids have been proposed as potential bulk chemical precursors that could be produced from renewable resources by microbial fermentation (Werpy and Petersen 2004; Sauer et al. 2008), although it remains an open question as to whether production costs (including recovery and chemical conversion) can be reduced sufficiently to supplant current routes from petroleum.

Industrial production of malic acid as a racemic mixture is currently based on the catalytic conversion of maleic anhydride resulting from the oxidation of benzene or *n*-butane (Nexant 2009). However, the L-isomer of malic acid is a key intermediate in the citric acid cycle and is produced by a wide range of microorganisms including bacteria, yeast, and filamentous fungi. Four native metabolic pathways resulting in the biosynthesis of malic acid from glucose have been identified and analyzed (Zelle et al. 2008). Considering both yield and simplicity, the optimal pathway appears to be that starting from pyruvate (produced by glycolysis) and proceeding through carboxylation by pyruvate carboxylase to oxaloacetate, followed by reduction to malate by malate dehydrogenase (Fig. 1). From glucose to malate, this route is adenosine triphosphate (ATP) neutral, and it results in the fixation of 1 mol CO_2/mol malate, with a maximum theoretical yield of 2 mol malate/mol glucose. This cytosolic pathway, which is commonly designated as the reductive tricarboxylic acid (rTCA) pathway, appears to be the one utilized by several *Aspergillus* species, including *Aspergillus nidulans*, *Aspergillus oryzae*, and *Aspergillus flavus*, that natively produce malic

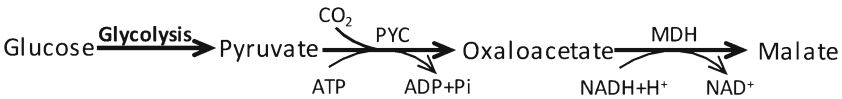

Fig. 1 Production of malic acid through the rTCA pathway. *PYC* pyruvate carboxylase, *MDH* malate dehydrogenase

acid (Osmani and Scrutton 1983; Peleg et al. 1988; Bercovitz et al. 1990). The same pathway, extended by the addition of a final step catalyzed by fumarase, is also implicated in fumaric acid production by the filamentous fungus *Rhizopus oryzae* (Peleg et al. 1989).

Aspergilli offer several potential advantages as production hosts that could be engineered to produce compounds such as malic acid with high yields and production rates at the industrial scale. They have been used for decades for the large-scale production of industrial enzymes, organic acids, and pharmaceuticals, and both *Aspergillus niger* and *A. oryzae* have achieved a "Generally Recognized As Safe" status (Abe et al. 2006; Andersen et al. 2009). The diverse metabolism found in strains from this genus should allow for the production of a wide range of useful compounds. An extensive genetic toolkit comprising various promoters, selection markers, and transformation and gene knockout methods is available, along with annotated genome sequences for a number of species, and methods for mutagenesis and high-throughput screening have been developed (Andersen et al. 2009; Lubertozzi and Keasling 2009; Schmid et al. 2009). An additional advantage may be that *Aspergillus* strains are able to utilize sugars derived from lignocellulosic materials including xylose and potentially produce the hydrolytic enzymes necessary to make these sugars available from various biomass sources (Begum and Alimon 2011; Duarte and Costa-Ferreira 1994; Prathumpai et al. 2003). However, Aspergilli also have some apparent disadvantages that may limit their use in this context. In contrast to *Escherichia coli* and *Saccharomyces cerevisiae*, no native replicating extrachromosomal vectors have been discovered, and the artificial vectors which have been developed are unstable even under selection, limiting their utility (Aleksenko et al. 1996; Aleksenko and Clutterbuck 1997; Aleksenko and Ivanova 1998). Transformation efficiencies are relatively low, and recombinant genes integrate chromosomally in a mostly ectopic fashion and with varying copy numbers, resulting in a pool of heterogeneous transformants that must be screened to find the highest producers (Hynes 1996; Verdoes et al. 1993; Fowler et al. 1993). The frequency of homologous recombination can be increased by deletion of genes comprising the nonhomologous end-joining pathway (Ninomiya et al. 2004; Mizutani et al. 2008), although this may result in reduced copy number and potentially expression level. Clearly, a number of challenges may be anticipated when considering Aspergilli for this application.

In this study, we report work to successfully engineer *A. oryzae* NRRL 3488 for increased production of malic acid by overexpression of native genes comprising the rTCA pathway in conjunction with a C4-dicarboxylic acid transporter. Gene candidates for overexpression were identified by transcriptional analysis of RNA samples generated in fermentors under conditions supporting malic acid production. Recombinant strains were obtained by pooling up to three genes and transforming using a single selection marker. The highest producing transformants achieved a greater than threefold improvement in malic acid production rate compared to the starting strain, with a glucose yield near 70 % of the theoretical maximum.

Materials and methods

Construction of plasmids and transformation

Strains and plasmids are listed in Table 1. The expression host used in this work was *A. oryzae* NRRL 3488, which is identical to *A. oryzae* ATCC 56747, a known malic acid producer (Bercovitz et al. 1990). Expression constructs incorporated the phosphoglycerate kinase (*pgk*) promoter (see succeeding paragraphs and sections), the gene of interest, the acetamidase (*amdS*) marker (Kelly and Hynes 1985), the *glaA* terminator (Christensen et al. 1988), and an ampicillin resistance marker. The *pgk* promoter was amplified by polymerase chain reaction (PCR) from *A. oryzae* NRRL 3488 genomic DNA using primers PGK-1 and PGK-2 listed in Table 2. The malate dehydrogenase (*mdh3*) gene was amplified by PCR from *A. oryzae* NRRL 3488 genomic DNA using primers MDH3-1 and MDH3-2 listed in Table 2. The pyruvate carboxylase (*pyc*) gene was amplified by PCR from *A. oryzae* NRRL 3488 genomic DNA using primers PYC-1 and PYC-2 listed in Table 2.

Table 1 Plasmids and strains used in this study

Strain or plasmid	Relevant characteristics
Plasmids	
pShTh104	*pgk* promoter; C4T318 gene; *glaA* terminator; *amdS* marker; Amp^R
pSaMF21	*pgk* promoter; *mdh3* gene; *glaA* terminator; *amdS* marker; Amp^R
pRyan1	*pgk* promoter; *pyc* gene; *glaA* terminator; *amdS* marker; Amp^R
Strains	
A. oryzae NRRL 3488	Wild type
ShTh1040-22	NRRL 3488+pShTh104
SaMF2103a-68	NRRL 3488+pShTh104, pSaMF21, and pRyan1

Table 2 PCR primers

Primer	Sequence
PGK-1	5′-CGAATTGTTTAAACGTCGACGTTGTATATTGTCGTTGGGGTTCTG-3′
PGK-2	5′-CGCGTAGATCTGCGGCCGCACCAGGTATGGACGATGTTCTATCACACAAG-3′
PGK-3	5′-TGACCTTCCACGCTGACCAC-3′
C4T318-1	5′-GTGATAGAACATCGTCCATAATGCTGACACCTCCCAAGTT-3′
C4T318-2	5′-AGTCACCTCTAGTTAATTAATTACTAATCAGATACATCCTCAT-3′
C4T318-3	5′-CTAATCAGATACATCCTCA-3′
MDH3-1	5′-ACACAACTGGCCATGGTCAAAGCTGGTGAGTTAGCAATCCTTAACAGAT-3′
MDH3-2	5′-AGTCACCTCTAGTTAATTAATTATTACTTTGGTGGTGGGTTCTTAACGAA GTCGATGCCT-3′
MDH3-3	5′-GGGATTTGAACAGCAGAAGG-3′
PYC-1	5′-ACACAACTGGCCATGGCGGCTCCGTTTCGTCA-3′
PYC-2	5′-AGTCACCTCTAGTTAATTAATTATTACGCTTTGACGATCTTGCAG-3′
PYC-3	5′-GGAAACGTCAAGCGGCTTGC-3′

Protoplasts of *A. oryzae* NRRL 3488 were generated and transformed as described previously (Christensen et al. 1988), with the substitution of 5 mg/mL of GLUCANEX (Novozymes A/S, Bagsværd, Denmark) and 0.5 mg/mL chitinase from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO, USA) for Novozyme 234. Transformants were selected by spreading protoplasts on minimal plates (Cove 1966) containing 1.0 M sucrose, 10 mM acetamide as nitrogen source, and 15 mM CsCl to inhibit background growth. Prior to transformation, expression vectors were digested with *Pme*I and size-selected on a 0.8 % agarose–TBE gel to remove the ampicillin resistance marker.

Shake flask cultivation

C4 acid production by *A. oryzae* strains was assessed in shake flasks using a two-stage protocol. Seed medium was composed of 30 g/L glucose, 3 g/L Bacto Peptone, 560 mg/L KH_2PO_4, 560 mg/L K_2HPO_4, 925 mg/L $NaH_2PO_4{\cdot}H_2O$, 820 mg/L Na_2HPO_4, 75 mg/L $MgSO_4{\cdot}7H_2O$, 75 mg/L $CaCl_2{\cdot}H_2O$, and 0.75 mL/L 1,000× Micronutrient Solution (5 g NaCl, 5 g $FeSO_4{\cdot}7H2O$, and 1 g citric acid/L water). Acid production medium was composed of 100 g/L glucose, 80 g/L $CaCO_3$, 6 g/L Bacto Peptone, 150 mg/L KH_2PO_4, 150 mg/L K_2HPO_4, 100 mg/L $MgSO_4{\cdot}7H_2O$, 100 mg/L $CaCl_2{\cdot}H_2O$, and 1 mL/L 1,000× Micronutrient Solution.

Strains were plated onto potato dextrose agar and allowed to sporulate at 34 °C for 5 to 7 days. Spores were collected in 0.05 % TWEEN® 80. Seed cultures were prepared in 250 mL unbaffled flasks containing 100 mL of seed medium and inoculated with 300 μL of spore stock. Seed cultures were grown for approximately 22 h at 30 °C with shaking at 200 rpm. Acid production cultures were prepared in 250 mL unbaffled flasks containing 50 mL of acid production medium and 3 mL of the 17-h seed cultures. Cultures were incubated at 30 °C with shaking at 200 rpm for 3 days.

Fermentations

All fermentations were run in 2-L Applikon fermentors (Applikon Biotechnology, Foster City, CA, USA). Seed medium was composed of 40 g/L glucose, 6 g/L Bacto Peptone, 0.75 g/L of KH_2PO_4, 0.75 g/L of K_2HPO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.005 g/L $FeSO_4{\cdot}7H_2O$, and 0.005 g/L NaCl. The fermentor batch medium used to generate cells for microarray experiments consisted of 100 g/L glucose, 40 g/L $CaCO_3$, 6 g/L Bacto Peptone, 0.150 g/L KH_2PO_4, 0.150 g/L K_2HPO_4, 0.10 g/L $MgSO{\cdot}7H_2O$, 0.10 g/L $CaCl_2{\cdot}2H_2O$, 0.005 g/L $FeSO_4{\cdot}7H_2O$, 0.005 g/L NaCl, and 0.5 mL/L Pluronic L61. For the more productive recombinant strains ShTh1040-22 and SaMF2103a-68, the batch medium was enriched by increasing the glucose concentration to 160 g/L, the Bacto Peptone concentration to 9 g/L, and the initial $CaCO_3$ concentration to 120 g/L; a 25 % *w/w* glucose solution was also fed at 8 g/L/h starting at 20 h postinoculation, and an additional 50 g/L sterile $CaCO_3$ was added to each fermentor on day 5. Fermentor temperature was controlled at 34±0.1 °C. Inlet air flow was maintained at 1 *v/v/m* and the agitation rate was set at 500 rpm. No acid or base additions were used for pH control other than $CaCO_3$. Fermentor seed flask inoculations were carried out as described previously for shake flask seed inoculations. One hundred forty-four milliliters of inoculum was used per 2-L fermentor.

Dry cell weight determinations

In order to dissolve excess $CaCO_3$ and precipitated acids, whole broth samples for dry cell weight (d.c.w.) analysis were diluted with 0.5 volume of 2 N HCl. Biomass was recovered from acidified broth by centrifugation, washed with water and recentrifuged, and dried at 85 °C for a minimum of 48 h before weighing.

Quantitation of C4 acids by HPLC

Quantitation of C4 acids was performed by reverse phase high-performance liquid chromatography (HPLC) using an Agilent 1200 Series Binary LC System equipped with a Phenomenex Aqua 5 μm C18 125 Å 250×4.6 mm ID column and Phenomenex AQ C18 4×3.0 mm SecurityGuard Cartridge. The mobile phase consisted of 10 % methanol (HPLC grade) and 90 % 145 mM phosphate pH 1.5 buffer. Whole culture samples were diluted into the mobile phase and filtered through a 0.45-μm Durapore PVDF membrane prior to injection.

Cell extract preparation

Mycelia from acid flasks were filtered through cheesecloth, washed repeatedly with water to remove precipitates, and frozen at −20 °C. Frozen cell mass was ground to a smooth paste using a chilled mortar and pestle and suspended by addition of pH 7.4 phosphate-buffered saline. The suspension was spun at 4 °C at 16,000×*g* for 10 min, and the resulting supernatant was used for intracellular enzyme activity assays.

Enzyme assays

The assay mixture for pyruvate carboxylase contained 100 mM Tris buffer (pH 8.0), 45 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.20 mM NADH, 1 mM acetyl-CoA, 1 mM ATP, and 1 mM pyruvate. The assay mixture for malate dehydrogenase contained 100 mM Tris buffer (pH 8.0), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.2 mM NADH, and 0.67 mM oxaloacetate. Enzyme activities were measured at 25 °C by monitoring NADH oxidation at 340 nm. Protein concentrations in cell extracts were determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard.

Cell harvest for RNA isolation

Samples for RNA isolation were harvested from three independent fermentation vessels at 6 h, 24 h, then at roughly 24 h intervals through 144 h. Harvested samples were treated as follows: one volume (15 mL) of whole broth was combined with two volumes of RNAlater® (Life Technologies, Carlsbad, CA, USA), mixed by inversion, and poured through a wire mesh kitchen strainer to collect mycelia. Mycelia were rinsed with a mixture of TE buffer and RNAlater® (1:2) and quickly frozen in liquid nitrogen. Samples were stored at −80 °C until used for RNA extraction.

RNA purification, quality control, and labeling

Total cellular RNA was extracted from each fermentation sample by grinding a portion of frozen mycelia (approximately 1 g) with mortar and pestle in liquid nitrogen until a fine powder was obtained. Samples were then processed using the Invitrogen TRIzol Plus® RNA Purification and PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). EDTA was added to the TRIzol® reagent at a concentration of 25 mM for chelation of residual calcium. All total RNA samples were then further purified using the Qiagen RNeasy MinElute® Cleanup Kit (Qiagen, Inc., Valencia, CA, USA). The RNA concentration and quality were evaluated using the NanoDrop spectrophotometer and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Biotin-labeled cRNA was prepared according to instructions provided by Affymetrix (http://www.affymetrix.com/support/technical/manual/expression manual.affx).

Microarray hybridization and scanning

The microarrays used in this study were custom-designed *Aspergillus* Affymetrix GeneChips (Andersen et al. 2008a). Hybridization on the GeneChips (one sample per chip) and scanning were done by the UC Davis Genome Center. The resulting data files (.DAT and .CEL files) were provided for subsequent analyses.

Data normalization and statistical analyses

CEL files were processed using RMAExpress (Bolstad et al. 2003). Normalization of the microarray data involved the following steps: (a) background adjustment using the robust multi-array average method on all probes, (b) cross-chip quantile normalization, and (c) obtaining gene expression values using median polish approach with a robust linear model to summarize probe-level expression values (Irizarry et al. 2003). Normalized expression values were filtered in Excel using a threshold value of 100 to eliminate genes that were transcribed at a very low level. Intensity ratios (also referred to as fold-change values) were converted to $\log_2$ ratios as necessary for some software applications.

Results

Fermentations for microarrays

Representative analytical and process data from one of the three independent fermentations subjected to microarray analysis are shown in Fig. 2a, b. Dissolved oxygen remained at or above 80 % for the duration of the fermentation. The fermentation pH was above 6.5 until roughly 69 h, when it began a gradual decline due to $CaCO_3$ consumption resulting from malic acid production. Fermentor sampling for the measurement of metabolite and biomass levels and recovery of

samples for array analysis was delayed to allow for adequate cell growth. Biomass levels, which were measured starting at 72 h, did not exceed 5 g/L d.c.w. Residual glucose analysis was not performed for these fermentations, but results from previous shake flask studies indicate that glucose would not have been fully consumed at the observed acid and biomass amounts. Malic acid production was relatively linear between 44 and 92 h, with a production rate of 0.28 g/L/h. Final acid titers at 140 h were malate 31.8 g/L, succinate 4.3 g/L, and fumarate 0.4 g/L. No other metabolites were detected in significant amounts.

Selection of *pgk* promoter based on microarray analysis

The most commonly used promoters for recombinant expression in *A. oryzae* are those linked to expression of amylase genes as exemplified by the Taka-amylase (*amyB*) gene and promoter. These promoters are induced by starch or maltose (Tsuchiya et al. 1992) and subject to catabolite repression by glucose through a CreA-mediated mechanism (Kato et al. 1996). In order to avoid the need for inducer addition to drive malic acid production and to facilitate production on glucose, expression of a range of genes was examined using microarray analysis. The expression profile of the 3-phosphoglycerate kinase (*pgk*) gene (AO090038000395), shown in Fig. 3a, indicates that this promoter is well suited to the expression of potential malic acid boosting genes, as it shows high and consistent transcript levels over the fermentation timecourse. Since the pyruvate that is ultimately converted to malic acid by the rTCA pathway is generated by glycolysis, linkage of glycolytic regulation with the expression of other pathway genes through the *pgk* promoter appears to be a sensible strategy. For these reasons, the *pgk* promoter was used for all expression constructs incorporating genes utilized to improve the production of malic acid in *A. oryzae* NRRL 3488.

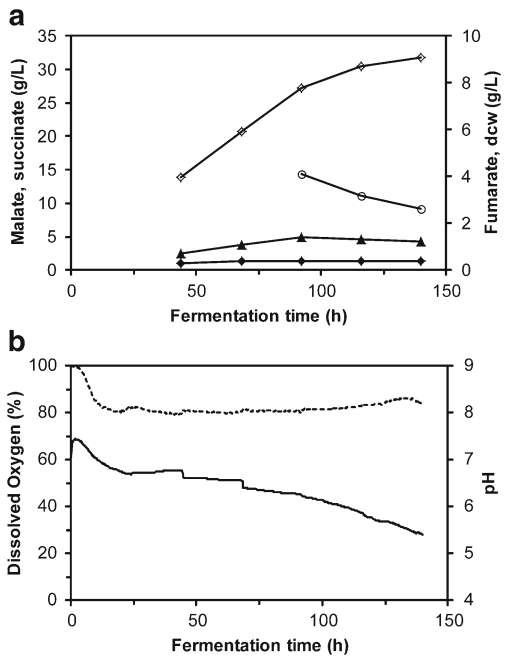

Fig. 2 Representative time profiles from one of the 2-L fermentations of *A. oryzae* NRRL 3488 used for transcriptional analysis. **a** Malate (*open diamonds*), succinate (*filled triangles*), fumarate (*filled diamonds*), and d.c.w. (*open circles*). **b** Dissolved oxygen (*dashed lines*) and pH (*solid lines*)

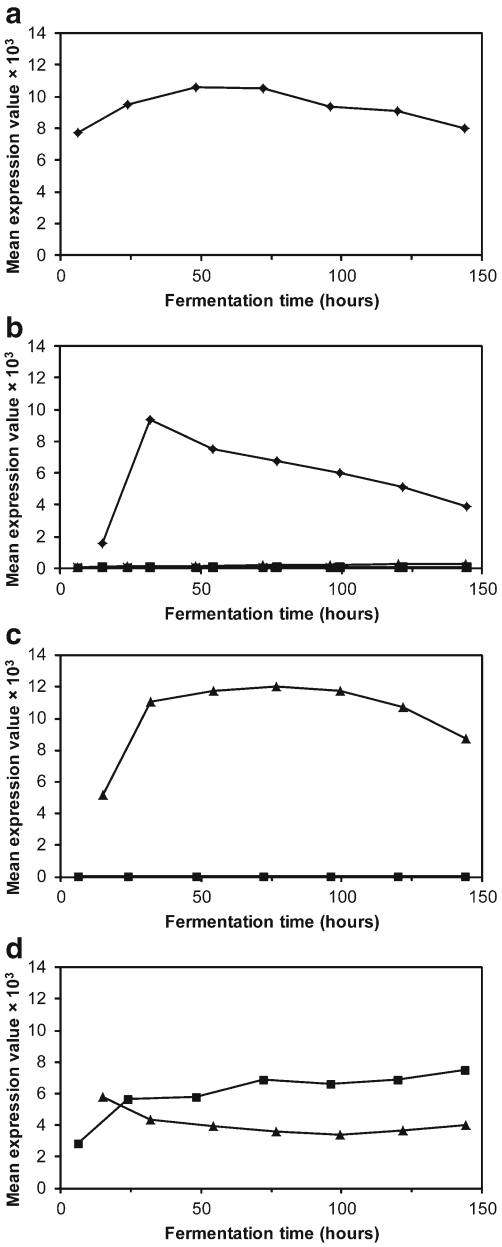

Fig. 3 Expression profiles of several genes during the fermentation of *A. oryzae* NRRL 3488 shown in Fig. 1. **a** 3-Phosphoglycerate kinase gene AO090038000395. **b** *S. pombe mae1* homologs AO090023000318 (*filled diamonds*), AO090005000361 (*filled squares*), AO090206000038 (*filled triangles*), AO090038000553 (*asterisks*), AO090701000480 (*filled circles*), and AO090138000098 (*plus signs*). **c** Pyruvate carboxylase genes AO090023000801 (*filled triangles*) and AO090701000666 (*filled squares*). **d** Malate dehydrogenase genes AO090701000013 (*filled squares*) and AO090005000438 (*filled triangles*)

Transcription of genes encoding putative C4-dicarboxylate transporters

The mechanism of malate transport from the cytosol to the extracellular environment in *Aspergillus* is not known. However, a gene encoding a dicarboxylate transporter active towards malic acid and designated as *mae1* has been identified from *Schizosaccharomyces pombe* (Grobler et al. 2005), and the expression of this gene in *S. cerevisiae* increases malic acid production threefold (Zelle et al. 2008). An amino acid BLAST search using the *S. pombe mae1* gene sequence to probe the *A. oryzae* RIB40 genome (Machida et al. 2005) resulted in the identification of six potential orthologues of the *mae1* gene (AO090023000318, AO090005000361, AO090206000038, AO090038000553, AO090701000480, and AO0901380000098). Data from microarray analysis shows that AO090023000318 is transcribed at a high level at 24 h of fermentation, declining almost linearly to the final time point; expression levels of the other *mae1* orthologues are near or below the detection value (Fig. 3b). Based on this analysis, expression of the AO090023000318 (C4T318) gene from the strong constitutive *pgk* promoter was tested as a means for improving malic acid production by *A. oryzae* NRRL 3488.

Transcription of pyruvate carboxylase (*pyc*) and malate dehydrogenase (*mdh*) genes

The rTCA pathway is a two-step cytosolic pathway that proceeds from pyruvate through oxaloacetate to malate, utilizing the enzymes pyruvate carboxylase (*pyc*) and malate dehydrogenase (*mdh*). Transcripts encoding two pyruvate carboxylases were detected by microarray analysis (Fig. 3c). The first *pyc* gene, AO090023000801, is transcribed at a high level in these fermentations. The second, AO090701000666, is transcribed at a very low level. Sequences for both genes were analyzed by WoLF PSORT software (Horton et al. 2007) for protein localization prediction. This software predicts two different subcellular localizations for the *pyc* genes, with AO090701000666 (low transcription) localized in the mitochondria and AO090023000801 (high transcription) localized in the cytosol. These results suggest that the AO090023000801 *pyc* gene is a component of the cytosolic rTCA pathway, and overexpression of this gene was tested as a means of increasing malic acid production in *A. oryzae* NRRL 3488.

Two putative malate dehydrogenase (*mdh*) genes were found in the *A. oryzae* RIB40 genome (AO090701000013-*mdh3*, AO090005000438-*mdh*), and transcripts corresponding to these two genes were also detected in *A. oryzae* microarray experiments (Fig. 3d). Sequences for both genes were also subjected to WoLF PSORT analysis for protein localization prediction. This analysis predicts two different subcellular localizations for the *mdh* genes, with AO090701000013 more likely localized to the cytosol and AO090005000438 more likely localized to the mitochondria. Since the mitochondrial compartment contains the enzymes of the TCA cycle, the AO090005000438 *mdh* gene product presumably functions as a component of the TCA cycle, while the AO090701000013 *mdh3* gene product most likely participates in the cytosolic rTCA pathway. Based on this analysis, the effect of overexpressing *mdh3* on malic acid production by *A. oryzae* NRRL 3488 was also evaluated. Both *pyc* and *mdh3* were overexpressed in combination with the C4T318 transporter.

Overexpression of the C4-dicarboxylic acid transporters in *A. oryzae* NRRL 3488

The impact of the overexpression of the native C4T318 dicarboxylic acid transporter on malic acid production was tested by transformation of *A. oryzae* NRRL 3488 with plasmid pShTh104 (see Table 1) and selection of transformants as described previously. A total of 49 transformants were recovered and tested in shake flasks. Malic acid concentrations at 3 days ranged from near wild-type levels (27 g/L) to roughly twofold wild-type levels (60 g/L). As was reported for *A. flavus* (Battat et al. 1991), the majority of the malic acid produced was found as a precipitate, presumably the calcium salt.

The top producing transformant in shake flasks, ShTh1040-22, was evaluated in 2-L fermentors using the method described, and the results can be seen in Fig. 4a, b. Glucose is present in excess over the course of fermentation, and the

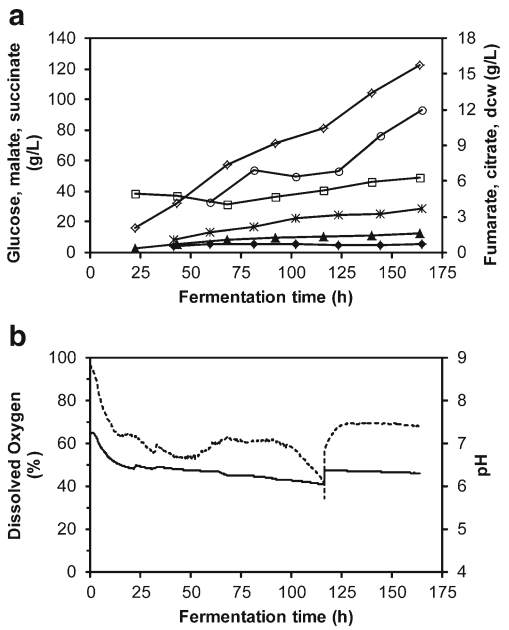

Fig. 4 Time profiles from a 2-L fermentation of recombinant strain ShTh1040-22 which overexpresses the native C4T318 transporter. **a** Glucose (*open squares*), malate (*open diamonds*), succinate (*filled triangles*), fumarate (*filled diamonds*), citrate (*asterisks*), and d.c.w. (*open circles*). **b** Dissolved oxygen (*dashed lines*) and pH (*solid lines*)

dissolved oxygen is maintained above 50 %. After a drop from the initial value of 7.2, the pH is kept near 6.4 until the initial $CaCO_3$ is consumed at approximately 115 h, when it drops briefly to 6.0 before rising again to 6.4 due to $CaCO_3$ addition. Biomass levels do not exceed 12 g/L d.c.w. The malic acid concentration at 164 h was 122 g/L, while those of succinic acid and fumaric acid were 12 and 0.7 g/L, respectively. This corresponded to an overall malic acid production rate of 0.74 g/L/h, while the production rate based on total dicarboxylic acids (malate+succinate+fumarate) was 0.82 g/L/h. The malate yield on glucose was 1.17 mol/mol, and the total dicarboxylic acid yield on glucose was 1.29 mol/mol. In contrast with fermentations of wild-type *A. oryzae* NRRL 3488, a small amount of citric acid was produced, with 3.7 g/L detected at 164 h.

In order to validate the results from microarray analysis indicating that five of the *mae1* homologs were not expressed and, therefore, not contributors to malic acid production, expression constructs were made with each gene and introduced into *A. oryzae* NRRL 3488 using the same methods followed for the C4T318 transporter. No increase in the production of malic acid or any other C4-dicarboxylic acid was detected in any of these transformants (data not shown).

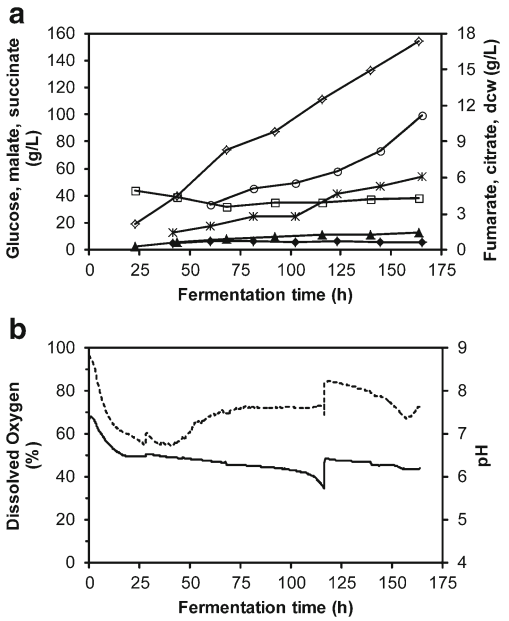

Fig. 5 Time profiles from a 2-L fermentation of recombinant strain SaMF2103a-68 which overexpresses the native C4T318 transporter along with pyruvate carboxylase and malate dehydrogenase. **a** Glucose (*open squares*), malate (*open diamonds*), succinate (*filled triangles*), fumarate (*filled diamonds*), citrate (*asterisks*), and d.c.w. (*open circles*). **b** Dissolved oxygen (*dashed lines*) and pH (*solid lines*)

Overexpression of C4T318, *mdh3*, and *pyc* in *A. oryzae* NRRL 3488

In an attempt to further improve malic acid production, the C4T318, *mdh3*, and *pyc* genes were used to transform *A. oryzae* NRRL 3488, using plasmids pShTh104, pSaMF21, and pRyan1, respectively (see Table 1), which were linearized to remove the ampicillin resistance marker as described previously. The three linearized constructs were simultaneously transformed into NRRL 3488, and this transformation procedure was performed 3 times, yielding 82 independent transformants. These transformants were tested in shake flasks, and several transformants producing malic acid at levels of 1.1-fold to 1.3-fold greater than strain ShTh1040-22 were found. Fermentor testing of the top 6 transformants from shake flasks identified the highest producing transformant as SaMF2103a-68, and the fermentation profiles of this strain are shown in Fig. 5a, b. Glucose is once again present in excess over the course of fermentation, and the dissolved oxygen is maintained above 50 %. Biomass levels are reduced slightly relative to strain ShTh1040-22, reaching a maximum of 11 g/L d.c.w. The pH profile was qualitatively similar to the ShTh1040-22 fermentation, although it dropped somewhat lower, to pH 5.7, before recovering to pH 6.4 as a result of $CaCO_3$ addition. The malic acid concentration at 164 h was 154 g/L, while those of succinic acid and fumaric acid were 13 and 0.6 g/L, respectively. This corresponded to an overall malic acid production rate of 0.94 g/L/h, while the production rate based on total dicarboxylic acids (malate+succinate+fumarate) was 1.0 g/L/h. The malate yield on glucose was 1.38 mol/mol, and the total dicarboxylic acid yield on glucose was 1.50 mol/mol. As shown, a small amount of citric acid was also produced by this strain, reaching a maximum level of 6 g/L at 164 h.

The distribution of recombinant expression cassettes in 37 randomly selected triple transformants was evaluated by PCR of genomic DNA using probe pairs PGK-3+C4T318-3, PGK-3+MDH3-3, and PGK-3+PYC3-3 specified in Table 2. As expected, due to selection, all transformants contained at least one recombinant gene construct. Over half of the transformants (20 out of 37) contained all 3 expression cassettes, while only 14 % (5 out of 37) contained just 1 expression cassette. All three expression cassettes were present in SaMF2103a-68. Measurements of intracellular malate dehydrogenase and pyruvate decarboxylase activities were performed on cell extracts obtained from shake flasks of *A. oryzae* NRRL3488, as well as ShTh1040-22 and SaMF2103a-68, and the results are shown in Table 3. The intracellular activities of both malate dehydrogenase and pyruvate carboxylase from strain SaMF2103a-68 were significantly increased over those of the wild-type and ShTh1040-22 strains. These results suggest that overexpression of the *mdh3* and *pyc* genes in addition to the C4T318 transporter gene contributes to increased malic acid production by strain SaMF2103a-68 relative to strain ShTh1040-22, which only overexpresses the C4T318 transporter.

Table 3 Intracellular specific activity measurements of wild-type and recombinant strains grown in shake flasks

Strain (genotype)	Malic acid (g/L)	Specific activity (U/mg)	
		Malate dehydrogenase	Pyruvate carboxylase
NRRL 3488 (WT)	23.1	2,010	11.6
ShTh1040-22 (C4T318)	45.2	1,940	11.7
SaMF2103a-68 (C4T318/MDH3/PYC)	59.1	4,261	56.0

Discussion

Several previous reports have described metabolic engineering efforts to improve malic acid production in both *S. cerevisiae* (Zelle et al. 2008) and *E. coli* (Moon et al. 2008; Zhang et al. 2011) using various combinations of gene overexpression and deletion. The malate titer of 154 g/L and production rate of 0.94 g/L/h achieved with strain SaMF2103a-68 in this study exceed the maxima which have been previously disclosed (Table 4). The reported glucose yield of 1.42 mol/mol achieved with *E. coli* XZ658 in a two-stage process appears to be slightly higher than the 1.38 mol/mol reached by SaMF2103a-68. However, the yield in this two-stage process has apparently been calculated by only considering the second stage of the process, and it will be <1.42 mol/mol when considering both stages together since additional glucose is consumed in the first stage. In contrast with these other engineered strains, citric acid was the only non-C4-dicarboxylic acid produced in measurable quantities by recombinant *A. oryzae* strains ShTh1040-22 and SaMF2103a-68.

Overexpression of the C4T318 transporter alone in *A. oryzae* NRRL 3488 increases production of malic acid by greater than twofold. The overexpression of *pyc* and *mdh* in addition to the C4T318 transporter results in a 27 % increase in malate production rate and an 18 % increase in glucose yield relative to overexpression of the transporter. These genetic modifications parallel those utilized to improve malate production in *S. cerevisiae* strain RWB525 (Zelle et al. 2008), yet the modified *A. oryzae* strain shows greater productivity and yield. Unmodified *A. oryzae* NRRL 3488 produces approximately 24-fold higher levels of malate than the unmodified *S. cerevisiae* host strain TAM, suggesting that *A. oryzae* may have a greater intrinsic malate production potential. Additionally, RWB525 produces significant amounts of both glycerol and pyruvate, reducing the carbon available for malate production by this strain.

As noted, a small amount of citric acid is also produced by these improved strains. *A. oryzae* has not previously been reported to produce significant amounts of citric acid, although production of small amounts by a related species, *A. flavus*, during malate fermentations has been reported (Battat et al. 1991). The exact metabolic route leading to the production of citric acid in strains ShTh1040-22 and SaMF2103a-68 is not known. It has been reported that malate accumulation may trigger citric acid excretion in *A. niger* through a process mediated by mitochondrial tricarboxylate transporters (Karaffa and Kubicek 2003; de Jongh and Nielsen 2008), and it may be that increased malate production by *A. oryzae* NRRL 3488 induces citrate production through a similar mechanism. Genome searches to identify putative mitochondrial tricarboxylate transporters coupled with transcriptional analysis examining the expression of these candidate genes may help elucidate the means of citrate production in the recombinant strains and allow the development of a strategy to redirect this carbon flow to the desired product, malic acid.

Previous efforts to implement or enhance C4-dicarboxylic acid production by filamentous fungi have been relatively unsuccessful. For example, attempts to redirect citric acid biosynthesis by *A. niger*, which is reportedly >100 g/L in the industrial scale (Magnuson and Lasure 2004), to production of succinic acid have met with only limited success—although the production of succinate was increased over threefold, the final concentration achieved was <1 g/L (Meijer et al. 2009). Efforts to improve the production of fumaric acid by *R. oryzae*

Table 4 Performance comparison of recombinant malate producers

Organism	Genetic modifications	Titer (g/L)	Productivity (g/L/h)	Yield (mol/mol)	Ref.
E. coli WGS-10	Δ*pta*+*pckA*[a]	9.25	0.74	0.56	Moon et al. (2008)
S. cerevisiae	+*mae1*, +*pyc*, +*mdh3*	59	0.29[b]	0.42	Zelle et al. (2008)
E. coli XZ658	Δ*ldhA* Δ*ackA* Δ*adhE* Δ*pflB* Δ*mgsA* Δ*poxB* Δ*frdBC* Δ*sfcA* Δ*maeB* Δ*fumB* Δ*fumAC*	22	0.15	1.0	Zhang et al. (2011)
E. coli XZ658[c]	See above	34	0.47	1.42	Zhang et al. (2011)
A. oryzae ShTh1040	+C4T318	122	0.74	1.17	This study
A. oryzae SaMF2103a-68	+C4T318, +*pyc*, +*mdh*	154	0.94	1.38	This study

[a] From *Mannheimia succiniciproducens* MBEL55E

[b] Calculated over the glucose consumption phase

[c] Two-stage fermentation with rate (and possibly yield) calculated based only on second stage

using metabolic engineering have resulted in relatively small improvements, largely due to difficulties encountered developing efficient methods for expressing or deleting genes in this organism (Skory 2004, 2005; Skory and Ibrahim 2007). The tendency of *R. oryzae* strains to clump and attach to surfaces under fermentation conditions is an added complication (Roa Engel et al. 2008). In contrast, metabolic engineering of *A. oryzae* NRRL 3488 has led to a significant increase in production rates and titers of malic acid. The successful introduction of three genes into this strain using a single selection marker suggests that additional genes could be incorporated through a single transformation, conserving other selection markers for subsequent gene addition or deletion. It is tempting to view the ectopic integration of transformed genes in varying copy numbers as a potential advantage for this application, as the result may be a pool of transformants with naturally varying expression levels of each gene that can be screened to find the optimal combination resulting in the best producer.

Further improvements in malic acid production by this organism may be realized using a systems biology approach, with integration of genome sequence, transcriptional analysis, and metabolite production data into a metabolic pathway model as has been carried out for *A. niger* (Andersen et al. 2008b). A preliminary metabolic model for *A. oryzae* which might be used in this context has already been published (Vongsangnak et al. 2008). Mutagenesis and screening will also be a likely route for the improvement of malic acid production, as it has been the major contributor to improvement of citric acid production by *A. niger* (Hjort 2006). The high level of malate production by *A. oryzae* NRRL 3488 suggests that this strain may also be useful as a platform host for the production of other pyruvate-derived or oxaloacetate-derived compounds, with the potential for sustainable and lower-cost production from lignocellulose-derived carbon sources.

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