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Inducible Overexpression of the *FUM1* Gene in *Saccharomyces cerevisiae*: Localization of Fumarase and Efficient Fumaric Acid Bioconversion to L-Malic Acid

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Cloning of the *Saccharomyces cerevisiae FUM1* gene downstream of the strong *GAL10* promoter resulted in inducible overexpression of fumarase in the yeast. The overproducing strain exhibited efficient bioconversion of fumaric acid to L-malic acid with an apparent conversion value of 88% and a conversion rate of 80.4 mmol of fumaric acid/h per g of cell wet weight, both of which are much higher than parameters known for industrial bacterial strains. The only product of the conversion reaction was L-malic acid, which was essentially free of the unwanted by-product succinic acid. The *GAL10* promoter situated upstream of a promoterless *FUM1* gene led to production and correct distribution of the two fumarase isoenzyme activities between cytosolic and mitochondrial subcellular fractions. The amino-terminal sequence of fumarase contains the mitochondrial signal sequence since (i) 92 of 463 amino acid residues from the amino terminus of fumarase are sufficient to localize fumarase-*lacZ* fusions to mitochondria and (ii) fumarase and fumarase-*lacZ* fusions lacking the amino-terminal sequence are localized exclusively in the cytosol. The possibility that both mitochondrial and cytosolic fumarases are derived from the same initial translation product is discussed.

Fumarase (EC 4.2.1.2) catalyzes the interconversion of fumaric and L-malic acids in the tricarboxylic acid cycle. The enzyme has been purified from a number of sources, but the mammalian enzymes have been the most studied (1, 12). Several fumarase genes have been cloned, including those from *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (19, 25, 26). In *S. cerevisiae*, a single nuclear gene (*FUM1*) has been shown to encode the two fumarase isoenzymes, with subunit molecular weights roughly estimated at 48,000 and 53,000 for the mitochondrial and cytosolic species, respectively (2, 26). It has been speculated that the mitochondrial isoenzyme is processed by removal of a signal peptide during translocation of the protein from the cytosol through the mitochondrial membranes (2, 26). The ease with which *S. cerevisiae* can be genetically manipulated together with the extensive experience with yeast growth and fermentations make this organism an obvious tool for biotechnological processes.

Biological production of L-malic acid can be accomplished by two main routes. The first is the direct fermentation of carbohydrates by various *Aspergillus* species (e.g., *Aspergillus flavus*, *A. oryzae*, *A. wentii*, and *A. parasiticus*) able to accumulate L-malic acid (4, 21). Due to the low yield and productivity of L-malic acid obtained, this fermentation route is not used. The alternative route is by a biotransformation process that uses immobilized cells of *Brevibacterium* species (e.g., *Brevibacterium ammoniagenes* and *B. flavum*) containing high fumarase activity and fed fumaric acid, transforming it to L-malic acid (24, 27). This industrial process accounts for the production of about 15% of the total malic acid world production. A total yield of 70% of the theoretical maximum is obtained with immobilized bacteria (24, 27). Immobilized cells of *Candida rogosa* are also being used in China for the industrial production of L-malic acid from fumaric acid (28).

In the present communication, we describe the construction of a shuttle vector containing the *FUM1* gene under the control of a strong inducible yeast promoter. We examine both the level of expression and localization of the *FUM1* gene products. One of our constructed yeast strains is shown to convert fumaric acid to L-malic acid by a highly efficient process.

MATERIALS AND METHODS

Strains, plasmids, and media. *S. cerevisiae* DMM1-15A (*LEU2 URA3 ADE2 HIS5*) and plasmid YEp51 have been described previously (3, 23). Plasmids pG5/T2 and pG5/ST1 (26) containing the *FUM1* gene were generously provided by A. Tzagoloff, Columbia University, New York, N.Y. The *lacZ* gene was obtained from plasmid pMC1403 (6). The growth medium (SD) used in all experiments contained 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories) and 2% (wt/vol) glucose or galactose. It was supplemented with the appropriate amino acids. Lithium acetate DNA-mediated transformation was used for *S. cerevisiae* (11). Standard cloning techniques have been described previously (16).

Preparation of cell extracts and cell fractions. For the fumarase assay, 2 ml of yeast cultures grown to about 1.5 optical density units at 600 nm on SD medium containing galactose was harvested by centrifugation and suspended in 300 μl of TE buffer (10 mM Tris hydrochloride buffer [pH 8.0], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride. Cells were broken by vigorous mixing with glass beads for 1 min and centrifuged. The supernatant fraction

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obtained was used for enzyme assays. Cell growth for the β-galactosidase assay was carried out as described above in SD medium containing galactose which was buffered to pH 7 (7). Intact cells were used for the assay as described previously (7, 18).

For preparation of the mitochondrial and post-mitochondrial supernatant (cytosolic) fractions, 600 ml of yeast cultures grown to 1.5 optical density units was used. Yeast mitochondria were prepared essentially by the method of Daum et al. (8). Spheroplasts were prepared from the yeast cells in the presence of Zymolyase-20T (Siekbagaku Kogyo). For enzyme assays, the mitochondrial pellet was broken by vigorously mixing with glass beads and centrifuging; the resulting supernatant was used for enzyme assays.

Enzyme assays. Fumarase (fumarate hydratase) was assayed by the method of Kanhrek and Hill at 250 nm with L-malic acid as substrate (12). NAD^+-isocitrate dehydrogenase was measured by following the formation of NADH at 340 nm (9). β-Galactosidase activity was assayed by the method of Miller (18).

Bioconversion of fumaric acid to L-malic acid. The conversion of fumaric acid to L-malic acid was carried out as described by Yamamoto et al. (27). Yeast cells were grown in SD medium containing galactose to stationary phase at 30°C. Cells were harvested by centrifugation at 3,000 × *g* and 4°C for 5 min and washed once with a sterile saline solution. One gram (wet weight) of cells was suspended in 30 ml of solution containing 1 M fumaric acid (adjusted to pH 7.5 with NaOH) and 0.2% (wt/vol) sodium deoxycholate. Cells were incubated at 37°C with agitation. Samples were taken at the times indicated and acidified with HCl to pH 2. The precipitates obtained after acidification (fumaric acid) and yeast cells were removed by centrifugation. Acids were determined in the clear supernatant fraction obtained.

Protein labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *S. cerevisiae* DMM1-15A cells containing the appropriate plasmid were grown on SD medium containing glucose to 1 to 1.5 optical density units at 600 nm. Cells were harvested and suspended in 5 ml of SD medium containing galactose and lacking methionine and leucine. After 4 h of growth at 30°C, cultures were labeled for 60 min with 10 μCi of $[^{35}S]$methionine per ml. The labeled cells were collected by centrifugation, washed once with 1 ml of distilled water, and suspended in 300 μl of TE buffer containing 1 mM phenylmethylsulfonyl fluoride. Cells were mixed vigorously with glass beads for 1 min and centrifuged to recover a clear supernatant fraction. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, equivalent amounts of cell extracts were loaded onto gels as described by Laemmli (13).

Analyses. Organic acids were determined by high-pressure liquid chromatography as described previously (10). L-Malic acid was also determined by using an enzymatic method with L-malate dehydrogenase. The reaction mixture contained hydrazine-glycine buffer (hydrazine, 0.4 M; glycine, 0.5 M; pH 9), 2.55 mM NAD^+, 10 U of L-malate dehydrogenase (cytoplasmic enzyme from porcine heart; Sigma Chemical Co.), and L-malic acid varying in concentration from 20 to 140 μM. The reaction mixtures were incubated for 30 min at 37°C, and the reaction was stopped by boiling samples for 2 min. L-Malic acid was determined by following the formation of NADH at 340 nm. Protein was determined by the method of Lowry et al. (15), with crystalline bovine serum albumin as a standard.

RESULTS

Cloning of *FUM1* downstream of the *GAL10* promoter. The aim of this study was to develop an efficient system for bioconversion of fumaric acid to L-malic acid. To achieve this aim, our approach was to overexpress the *FUM1* gene which encodes the enzyme fumarase from the yeast *S. cerevisiae*. A *GAL10* expression system (3) was chosen which allows strong and rapid induction of transcription of the cloned gene when galactose is the carbon source. The shuttle vector YEp51 (3), which contains the *GAL10* promoter, is a 2μm-derived, high-copy-number plasmid in *S. cerevisiae*. Two plasmids were constructed for expression in *S. cerevisiae*: one contains the complete *FUM1* gene, and the other lacks the region coding for the proposed amino-terminal mitochondrial import signal. The strategy used for cloning is shown in Fig. 1. To adjust the ends of the fragment to be cloned, the *FUM1* gene from vector pG5/T2 (26) was inserted into the multiple cloning site of plasmid pUC18. A 1.77-kilobase *Sal*I/*Hin*dIII fragment from the resulting plasmid containing the complete *FUM1* protein-coding sequence was inserted downstream of the *GAL10* promoter in YEp51. In a similar way, *FUM1* lacking the proposed mitochondrial import signal (C-*FUM1*) from vector pG5/ST1 (26) was cloned into YEp51 on a 2.9-kilobase *Sal*I/*Hin*dIII fragment. The final plasmids (pFT2 containing the complete *FUM1* gene and pFST1 containing the C-*FUM1* gene) were used to transform a leucine-requiring yeast strain.

Expression of the *FUM1* gene. To examine the activity of fumarase, yeast cells harboring plasmids containing the *FUM1* gene were grown in medium with either galactose or glucose as the carbon source. Fumarase activity was assayed spectrophotometrically in cell extracts, and the calculated specific activities are summarized in Table 1. A 12-fold increase in fumarase specific activity was observed due to the higher copy number of the wild-type gene on a 2μm yeast vector (pG5/T2) when compared with a control strain that included only one chromosomal copy of the gene (Table 1).

The highest fumarase specific activity was obtained for cells containing the *GAL10* expression vector pFT2, when grown on galactose. This activity was 240-fold higher than the activity of a strain harboring a plasmid containing no fumarase gene (YEp51). This fumarase activity was 19-fold higher than the activity in cells containing the fumarase gene (*FUM1*) under the control of its natural promoter on a multicopy plasmid (cf. pFT2 with pG5/T2 in Table 1). Of interest is that fumarase activities in cells grown on galactose and harboring plasmids pG5/ST1 and pFST1 that lack the proposed mitochondrial import signal, were lower by 7- and 72-fold than those measured for cells containing the com-

TABLE 1. Fumarase activities in yeast strains containing the *FUM1* gene[a]

Plasmid	Sp act (μmol/min per mg of protein)	
	Galactose medium	Glucose medium
YEp51	0.6	0.5
pG5/T2	7.6	5.9
pG5/ST1	1.1	0.7
pFT2	143.6	1.9
pFST1	2.0	0.5

[a] DMM1-15A cells harboring the respective plasmids were grown on SD medium containing either glucose or galactose to the stationary phase at 30°C. Fumarse specific activity was determined in cell extracts.

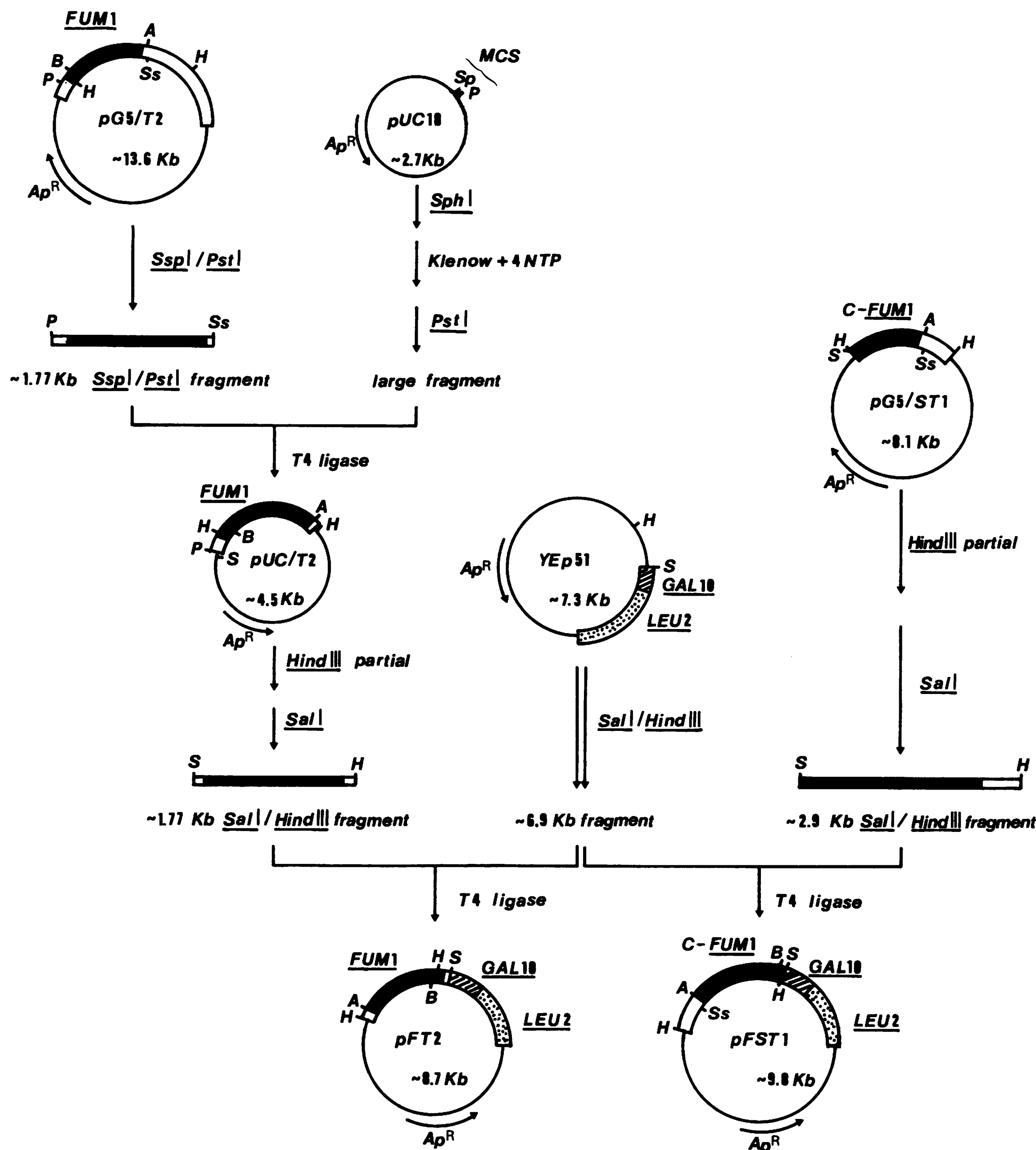

FIG. 1. Schematic representation of pFT2 and pFST1 construction (not to scale). Protein-coding sequences of the *FUM1* gene were inserted between the *GAL10* promoter and the transcription termination site of YEp51. Ap^R, β-Lactamase gene; *FUM1*, fumarase gene including the complete translation sequence; C-*FUM1*, fumarase gene lacking the 5′ 51 base pairs encoding the fumarase amino terminus; MCS, multiple cloning site; A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; Ss, *Ssp*I. Boxed areas represent sequences of yeast origin; solid boxes represent the protein-encoding sequence of *FUM1*. kb, Kilobases.

plete *FUM1* gene on plasmids pG5/T2 and pFT2, respectively. A reasonable explanation for this observation is the existence of stop codons immediately upstream of the first ATG in pG5/ST1 and pFST1. As expected from a galactose-inducible promoter, fumarase specific activities, measured for *GAL10-FUM1* transformants grown on galactose, were higher than the activities measured in cells grown on glucose (Table 1).

To identify the fumarase species produced, cells carrying the *FUM1* genes were grown and induced in galactose medium. Cultures were then labeled with [^{35}S]methionine, and cell extracts were prepared and analyzed by sodium dodecyl sulfate-polyacylamide gel electrophoresis. Two protein bands appear to be specifically induced by galactose in pFT2-containing cells (Fig. 2). The apparent molecular weights of these proteins species coincide with reported estimated values of mitochondrial and cytosolic fumarase previously purified from yeast (2). In contrast, in the strain harboring the shorter C-*FUM1* gene (on pFST1) it was not possible to identify any additional or more intensified bands upon comparison with the pattern of the control strain. Thus, production of high fumarase activity in strains harbor-

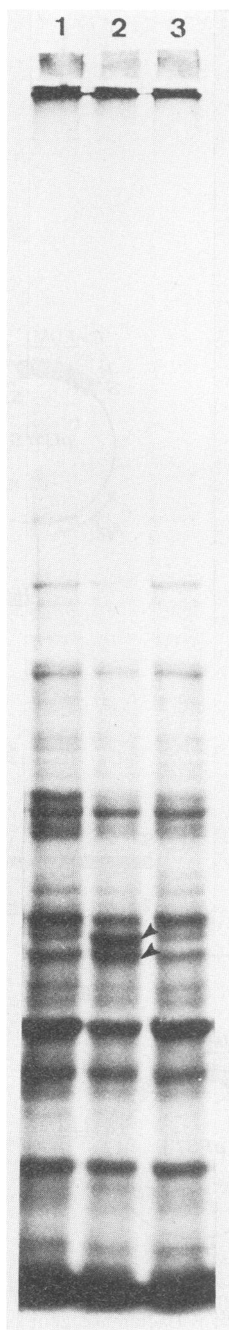

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total cellular proteins. Exponentially growing cultures in galactose medium were labeled with [^{35}S]methionine, and cell extracts were prepared as described in Materials and Methods. Equivalent amounts of cell extracts (lane 1, yeast with plasmid pFST1; lane 2, yeast with plasmid pFT2; lane 3, yeast with plasmid YEp51) were applied to each lane. Arrowheads show positions of fumarase protein species.

ing pFT2 is due to inducible overproduction of the protein, which is low in strains harboring plasmid pFST1.

Conversion of fumaric to L-malic acid. The conversion of fumaric acid to L-malic acid measured with a yeast strain which contains plasmid pFT2 and shows high fumarase activity was compared with cells containing the YEp51 control plasmid which contains no *FUM1* sequences (Table 2). The conversion kinetics of fumaric acid to L-malic acid shows that about 80% of the fumaric acid was converted to L-malic acid by yeast cells containing plasmid pFT2 within 15 min of incubation, and maximal conversion was observed after 30 min (0.88 mol of L-malic acid formed per mol of fumaric acid utilized; calculated from Table 2). The maximal conversion rate for cells harboring the pFT2 plasmid was 80.4 mmol of L-malic acid formed/h per g of cell wet weight compared with a value of 2.13 mmol/h per g for cells harboring control plasmid YEp51 (calculated from Table 2).

L-Malic acid was the only product of the conversion reaction detected by high-pressure liquid chromatography (not shown). This result was confirmed by a ^{3}H-nuclear magnetic resonance analysis (not shown). A good correlation for the L-malic acid measured was obtained by both the high-pressure liquid chromatography analysis and enzymatic assay with NAD^+–L-malate dehydrogenase (Table 2). It should be noted that the acids were determined in the supernatant fraction obtained after acidification of samples to pH 2 with HCl and removal of the fumaric acid precipitant and cell pellet by centrifugation. The maximal soluble concentration of fumaric acid is about 9 g/liter at pH 2 (25°C), and this was the concentration of fumaric acid detected in the supernatant fraction together with 118 g of L-malic acid per liter (Table 2).

Fumarase activity in subcellular fractions. To examine the cellular localization of the plasmid-encoded fumarase, mitochondria were prepared from cells harboring pFST1 and pFT2 plasmids and from a strain harboring the control plasmid, YEp51. The activities of fumarase (Table 3) and NAD^+-isocitrate dehydrogenase, a mitochondrial marker enzyme (17), were measured in the mitochondrial and postmitochondrial supernatant (cytosolic) fractions. The highest fumarase specific activity was obtained in the mitochondrial

TABLE 2. Conversion kinetics of fumaric acid to L-malic acid by *S. cerevisiae*[a]

Plasmid	L-Malic acid produced (g/liter)					
	15 min		30 min		60 min	
	HPLC[b]	MDH[c]	HPLC	MDH	HPLC	MDH
YEp51	3	2	5	5	10	9
pFT2	90	98	116	124	118	119

[a] Bioconversion was carried out with intact yeast cells as described in Materials and Methods.
[b] Calculated from high-pressure liquid chromatography (HPLC) analysis.
[c] Calculated from enzymatic analysis with L-malate dehydrogenase (MDH).

TABLE 3. Subcellular distribution of fumarase activity in *S. cerevisiae*[a]

Plasmid	Mitochondrial fraction		Postmitochondrial fraction	
	Sp act (μmol of fumaric acid formed/min per mg of protein)	% Total activity	Sp act (μmol of fumaric acid formed/min per mg of protein)	% Total activity
YEp51	3.9	9.9	0.2	90.1
pFST1	5.1	2.2	1.7	97.8
pFT2	227.8	8.1	24.6	91.9

[a] DMM1-15A cells transformed with various plasmids and were grown in SD medium containing galactose as described in Materials and Methods. Preparation of the mitochondrial and postmitochondrial cellular fractions and the fumarase assay are described in Materials and Methods.

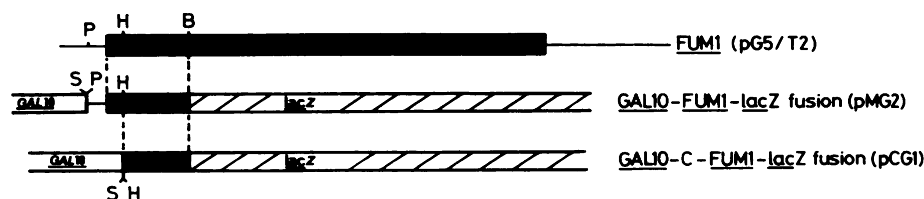

FIG. 3. Schematic representation of the *GAL10-FUM1-lacZ* fusion regions in pMG2 and pCG1 aligned with the *FUM1* gene. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. Solid boxes represent *FUM1* protein-encoding sequences.

fraction of pFT2 transformants, and this activity was 58- and 45-fold higher than the mitochondrial activities of YEp51 and pFST1 transformants, respectively. The postmitochondrial fumarase specific activity obtained for cells containing pFT2 was about 123- and 15-fold higher than the postmitochondrial activities of cells harboring YEp51 and pFST1, respectively (Table 3). The mitochondrial fumarase activity in transformants harboring pFST1, which lacks the proposed mitochondrial import signal, represented about 2% of the activity recovered in the combined fractions, whereas for YEp51 and pFT2 transformants mitochondrial fumarase represented 8 to 10% of the total enzyme activity (Table 3). One can conclude that the increase in fumarase activity measured in cell extracts (Table 1) of pFT2 transformants was due to an increase in the activity of both the mitochondrial and postmitochondrial fractions, whereas the increase in the activity measured for pFST1 transformants was, mainly, due to an increase in the postmitochondrial fumarase activity.

Activity of NAD^+-isocitrate dehydrogenase (not shown) showed that >95% of the total enzyme activity was found in the mitochondrial fraction of the cell, indicating a 5% leakage of soluble enzymes from the mitochondrial matrix to the cytosol during the fractionation procedures.

Construction and expression of *GAL10-FUM1-lacZ* fusions. The β-galactosidase structural gene *lacZ* can be fused to the protein-coding region of other genes as a way to provide an enzymatic marker for following gene expression and regulation (7). Plasmids pFT2 and pFST1 were used for construction of *GAL10* promoter-controlled *FUM1-lacZ* protein fusions in which the transcription and translation signals are located within the *GAL10-FUM1* region. To fuse the *E. coli lacZ* gene to the *GAL10-FUM1* region in plasmids pFT2 and pFST1, a 3.1-kilobase *Bam*HI-*Dra*I fragment of plasmid pMC1403 (6) containing the *lacZ* gene without the first seven codons was ligated in frame to the *Bam*HI site of pFT2 and pFST1, which contain the 5′ portion of the respective fumarase-encoding gene (Fig. 3). The resulting plasmids derived from pFT2 and pFST1 were designated pMG2 and pCG1, respectively. Plasmid pMG2 contains a 273-base-pair fragment of the fumarase-encoding gene, including the proposed mitochondrial import signal, whereas in pCG1 this signal is absent (Fig. 3).

β-Galactosidase activity in yeast strains harboring the plasmid constructions described above was determined in both cell extracts and subcellular fractions. A similar distribution of β-galactosidase and fumarase activity in subcellular fractions was obtained for cells harboring plasmids pMG2 and pFT2 containing the proposed mitochondrial import signal (cf. Tables 3 and 4). A small but significant portion of the enzyme activity was localized to the mitochondria: with fumarase, 8 to 10% for pFT2 transformants and for the YEp51 control strain; and with β-galactosidase, 5% for cells harboring the *GAL10-FUM1-lacZ* fusion (pMG2). In contrast, for cells harboring plasmids lacking the proposed mitochondrial import signal (pFST1 and pCG1), the vast majority of the fumarase and β-galactosidase activity was cytosolic (97.8 and 99.9%, respectively).

TABLE 4. β-Galactosidase activities in cell extracts and cell fractions of *S. cerevisiae*[a]

Plasmid	Total cell extract (units of β-galactosidase)[b]	Mitochondrial fraction		Postmitochondrial fraction	
		Sp act (μmol of *o*-nitrophenol formed/min per mg of protein)	% Total activity	Sp act (μmol of *o*-nitrophenol formed/min per mg of protein)	% Total activity
YEp51	ND	ND		ND	
pCG1	84	0.02	0.1	0.16	99.9
pMG2	2,038	7.00	4.9	1.40	95.1

[a] Growth conditions, enzyme assay, and preparation of cell extracts and cell fractions were as described in Materials and Methods.

[b] Calculated by the method of Miller (18) and corrected for equivalent cell densities in the reaction mixtures. ND, No activity detected.

DISCUSSION

In this study, we have developed a yeast strain for efficient bioconversion of fumaric acid to L-malic acid. By situating the *FUM1* gene downstream of the strong *GAL10* promoter of yeast, inducible overexpression was obtained. The strain harboring the fumarase expression vector pFT2 exhibited a 240-fold increase in fumarase activity when compared with the control strain (Table 1). The increase in fumarase activity is accompanied by an increase in synthesis of the protein as shown by (i) the labeled protein on polyacrylamide gels and (ii) activity of the *FUM1-lacZ* fusions.

The conversion rate of fumaric acid to L-malic acid was measured in cell suspensions and was about 38 times higher in the constructed strain with plasmid pFT2 than in the control yeast (80.4 versus 2.13 mmol/h per g of cell wet weight, respectively, as calculated from Table 2). When compared with *B. flavum* (24) and *B. ammoniagenes* (27), the maximal conversion rate of fumaric acid of these strains was four to eight times lower than for our high producing yeast strain.

An equilibrium constant, K_{eq} [L-malate]/[fumarate], can be calculated from the standard free energies of the reaction (1 M aqueous solutions at pH 7.0 and 25°C [14]): fumaric acid + $H_2O \leftrightharpoons$ L-malic acid. The K_{eq} value thus calculated (4.71) indicated that 0.825 mol of L-malic acid can be produced from 1 mol of fumaric acid utilized under these conditions. From the results shown in Table 2 (at pH 7.5 and 37°C), the calculation shows that 0.88 mol of L-malic acid was formed from 1 mol of fumaric acid by the constructed *S. cerevisiae* strain (harboring plasmid pFT2). This value is higher than the theoretical value and the values obtained for *B. flavum*

(0.833 at pH 7.0 and 37°C) and *B. ammoniagenes* (0.828 at pH 7.0 and 37°C) (see references 24 and 27).

One of the major problems in the commercial process using bacterial strains (*B. flavum* or *B. ammoniagenes*) is the formation of an unwanted by-product, succinic acid, during the conversion process (24, 27). Since it is difficult to separate succinic acid from L-malic acid, it is desirable to suppress succinic acid synthesis. The results indicate that our yeast overproducing strain does not produce significant amounts of succinic acid.

The distribution of identical enzymatic activities between different subcellular compartments can be achieved by a number of routes. The existence of two or more genes is often used as a solution; however, a single gene, if specifically adapted, can also allow distribution between two cellular locations. For example, the localizations of invertase and histidyl-tRNA synthetase (5, 20, 22) are determined at the level of transcription for which, in both cases, two different mRNAs are produced from the same gene. In each case one of the mRNAs encodes a cleavable signal peptide, whereas a shorter mRNA species lacks this sequence and therefore encodes a cytoplasmically located protein. Such a mechanism was recently suggested by Wu and Tzagoloff (26) to control the localization of *FUM1* products in *S. cerevisiae*. These authors proposed that mitochondrial fumarase is translated from a longer mRNA encompassing the first in-frame ATG of the *FUM1* gene and that the cytoplasmic isoenzyme is translated from a shorter transcript lacking the amino-terminal signal sequence. This model predicts that the two *FUM1* mature proteins are of similar size or that the mitochondrial isoenzyme is even larger when the signal sequence is not removed (26). Contrary to this prediction is the recent finding that the *S. cerevisiae* cytosolic fumarase isoenzyme is substantially larger than the mitochondrial protein, i.e., 53,000 versus 48,000 molecular weight (2).

Subcloning of the *FUM1* gene in pFT2 is expected to inactivate the natural promoter of this gene since the major transcription initiation site (26) and sequences upstream from it are removed. In fact, in strains harboring pFT2, activity of fumarase with glucose as the carbon source is about three-fold lower than in the strain harboring pG5/T2, which has the original *FUM1* promoter (Table 1). In the pFT2-harboring strain repressed for *GAL10* expression by glucose, the low fumarase activity observed can be attributed to leakiness of the *GAL10* promoter and to background activity from the chromosomal copy of fumarase.

In our plasmid constructions, transcription from the *GAL10* promoter initiates upstream of the cloned *FUM1* sequences (3). Thus, the *GAL10* controlled transcript of pFT2 should include all *FUM1* sequences with the proposed signal sequence, and translation would be expected to initiate at the first ATG. In our study, we show that the *GAL10* promoter situated upstream of a promoterless *FUM1* gene (pFT2) leads to production and correct distribution of the two fumarase isoenzyme activities. In addition, we observe two fumarase species produced by pFT2 whose sizes coincide with those observed by Boonyarat and Doonan (2).

The explanation that we suggest for these data is that translation of both mitochondrial and cytosolic fumarases initiates at the first ATG; however, the mitochondrial targeted protein is processed by removal of the signal peptide to a smaller protein. This would explain a larger cytoplasmic isoenzyme (2) and the ability to produce both isoenzymes from a *GAL10* promoter in pFT2. The possibility of a single translation product differs from the model of Wu and Tzagoloff (26), who proposed the existence of two differentially translated mRNA species. Consistent, however, with their proposed signal sequence is our finding that 92 of 463 amino acid residues from the amino terminus of fumarase are sufficient to localize β-galactosidase activity to mitochondria.

The precise mechanism of fumarase distribution between subcellular compartments in *S. cerevisiae* remains to be elucidated.

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