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Cloning the Gene for the Malolactic Fermentation of Wine from *Lactobacillus delbrueckii* in *Escherichia coli* and Yeasts

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The gene responsible for the malolactic fermentation of wine was cloned from the bacterium *Lactobacillus delbrueckii* into *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. This gene codes for the malolactic enzyme which catalyzes the conversion of L-malate to L-lactate. A genetically engineered yeast strain with this enzymatic capability would be of considerable value to winemakers. *L. delbrueckii* DNA was cloned in *E. coli* on the plasmid pBR322, and two *E. coli* clones able to convert L-malate to L-lactate were selected. Both clones contained the same 5-kilobase segment of *L. delbrueckii* DNA. The DNA segment was transferred to *E. coli*-yeast shuttle vectors, and gene expression was analyzed in both hosts by using enzymatic assays for L-lactate and L-malate. When grown nonaerobically for 5 days, *E. coli* cells harboring the malolactic gene converted about 10% of the L-malate in the medium to L-lactate. The best expression in *S. cerevisiae* was attained by transfer of the gene to a shuttle vector containing both a yeast 2-μm plasmid and yeast chromosomal origin of DNA replication. When yeast cells harboring this plasmid were grown nonaerobically for 5 days, ca. 1.0% of the L-malate present in the medium was converted to L-lactate. The *L. delbrueckii* controls grown under these same conditions converted about 25%. A laboratory yeast strain containing the cloned malolactic gene was used to make wine in a trial fermentation, and about 1.5% of the L-malate in the grape must was converted to L-lactate. Increased expression of the malolactic gene in wine yeast will be required for its use in winemaking. This will require an increased understanding of the factors governing the expression of this gene in yeasts.

The malolactic fermentation is a secondary fermentation that occurs in addition to the alcoholic fermentation in the production of many wines (14–16). This fermentation involves the NAD^+ and manganese-dependent decarboxylation of L-malate to L-lactate and CO_2 and is carried out by various species of lactic acid bacteria. These species all belong to one of three genera: *Lactobacillus, Leuconostoc*, or *Pediococcus* (15). Malic acid is a major organic acid found in grapes, and its decarboxylation to lactic acid results in a significant decrease in the acidity of the wine, a matter of importance in cool grape-growing regions where the grape must may be too acidic. In grape-growing regions with moderate climates, where acidity of the must is not a problem, the most important aspect of the fermentation is that its completion renders the wine bacteriologically stable for storage or aging.

Wine is not a medium conducive to rapid growth of lactic acid bacteria. If the malolactic fermentation is desired, the winemaker often tries to induce it by a variety of means which may also encourage microbiological spoilage. A fermentation that occurs after the wine is bottled is to be avoided because of the resulting turbidity and possible off flavors. Therefore, it is advantageous to have the malolactic fermentation occur during or shortly after the alcoholic fermentation so the wine can be adjusted for cellar storage without risk of becoming spoiled. In California, winemakers often wish to encourage a malolactic fermentation by inoculation with selected starter cultures of bacteria; but if large volumes are needed, the preparation is generally difficult or impractical (15). An effective alternative would be to transfer the genetic information necessary for the malolactic fermentation from a lactic acid bacterial species to a wine yeast, so that the genetically engineered yeast could perform both the alcoholic and malolactic fermentations simultaneously. Such a gene transfer would not only yield valuable information on the expression of a gene from a gram-positive procaryote in a yeast, but would also be expected to solve problems associated with the slow malolactic fermentation for the vintner.

The malolactic reaction was at first thought to result from the joint action of two enzyme activities: the decarboxylation of L-malate to pyruvate (with the concomitant reduction of NAD^+) catalyzed by the "malic" enzyme coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (13). It has since been shown that the reaction is due to a single enzyme (the malolactic enzyme) which is active in the presence of NAD^+ and Mn^{2+} (14, 16, 18, 25, 26). Caspritz and Radler recently purified the malolactic enzyme from *Lactobacillus plantarum* and found it to have a molecular weight of ca. 140,000, consisting of two apparently identical subunits each with a molecular weight of ca. 70,000 (4). The fact that a single enzyme consisting of multiple identical subunits appears to catalyze the malolactic activity suggests that the genetic information necessary for the reaction resides in a single gene. This report describes the cloning and expression of the malolactic gene from a wine strain of *Lactobacillus delbrueckii* in *Escherichia coli* and the yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Strains and plasmids. The wild-type strain of *L. delbrueckii*, UCD Enology Cuc-1, used in this study was obtained from the Department of Viticulture and Enology, University of California, Davis, and was originally isolated from wine (21). *E. coli* strains K-12 RR1 (F^- *pro leu thi lacY*

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hsdR hsdM ara-14 gal-2 xyl-5 mtl-1 supE44 endoI$^-$) and CSR603 (F$^-$ *thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44* λ^-) were obtained from R. L. Rodriguez (23). *S. cerevisiae* 2514-10c (*a trp1-289 leu2-3 leu2-112 thr4-1 ura3-52 his1-68*) was constructed by R. Snow. *E. coli* plasmids pBR322 (Tet^r Amp^r) and pBR327 (Tet^r Amp^r) were obtained from R. L. Rodriguez (23, 27). The *E. coli*-yeast shuttle plasmid pRC3 (*trp-1*$^+$ Tet^r Amp^r Kan^r) was obtained from Ferguson et al. (7).

Purification of *L. delbrueckii* DNA. *L. delbrueckii* DNA was isolated by the method of Garvie (8) with the following modifications. Cells were incubated for 4 h at 37°C with gentle shaking in the lysozyme-4-amino salicylate suspension without pronase; 0.1 ml of 5-mg/ml RNase A per g of wet-packed cells was then added, and incubation was continued for another 4 h. After this incubation, 0.05 ml of 25% sodium dodecyl sulfate was added per g of wet-packed cells, and incubation was continued for another 4 h. Finally, 0.25 ml of pronase at 40 mg/ml (preincubated 1 hour at 37°C) and 1.5 ml of chloroform were added per g of wet-packed cells, and incubation was continued for another 12 h. The lysed cells were not treated with 0.5% isopropyl naphthalene sulfonate. Two phenol extractions were used instead of one to deproteinize the viscous solution. After the chloroform-isoamyl alcohol extraction, a 1/30 volume of 5 M NaCl and two volumes of ice-cold 95% ethanol were added, and the mixture was placed on ice for 5 to 10 min. The DNA was then spooled out on a glass rod and rinsed by dipping in ice-cold 95% ethanol. The DNA was dissolved in 25 ml of fresh standard saline-citrate buffer, 50 ml of 95% ethanol was added, and the solution was kept on ice for 5 min. The DNA was again spooled on a glass rod, rinsed in ice-cold 95% ethanol, and then dissolved in 2.0 ml of 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA–5 mM NaCl. The DNA was dialyzed against 1 liter of this Tris-EDTA-NaCl buffer for 6 h at room temperature, changed to fresh buffer (1 liter) and dialyzed for another 6 h. It was stored at 4°C with 1 drop of chloroform added to maintain sterility.

Preparation of plasmid DNA. Large- and small-scale plasmid DNA preparations followed the methods of Rodriguez and Tait (23). DNA restriction fragments were isolated from preparative gels by using the procedure of Langridge et al. (17).

Transformation of *E. coli* and *S. cerevisiae* with plasmid DNA. *E. coli* was transformed with plasmid DNA by using the method described by Rodriguez and Tait (23). *S. cerevisiae* was transformed by following the procedure of Tschumper and Carbon (28).

Restriction endonuclease digestion, ligation of DNA fragments, and gel electrophoresis. Procedures used for restricting and ligating chromosomal and plasmid DNA molecules as well as for running agarose and polyacrylamide gels were those described by Rodriguez and Tait (23).

Assays for L-lactate and L-malate. L-Lactate and L-malate assays were performed on *E. coli* or *S. cerevisiae* cultures by the enzymatic method of Hohorst (10, 11). In screening *E. coli* clones for conversion of L-malate to L-lactate, cells were grown in M9-A medium (M9 medium plus [per liter] 1.0 mg of thiamine, 20 mg of L-proline, 20 mg of L-leucine, 20 mg of L-aspartic acid, 20 mg of ampicillin) plus 0.1, 0.3, 0.6, or 1.0% L-malate. For aerobic growth conditions, *E. coli* cells were grown in tubes containing 3.0 ml of M9-A medium at 37°C with shaking for 17 to 48 h. For nonaerobic growth conditions, *E. coli* cells were grown in tubes containing 10 ml of M9-A medium at 37°C with no shaking for 5 days.

Yeast clones tested for conversion of L-malate to L-lactate were grown in SM-A medium (Difco yeast nitrogen base [without amino acids] supplemented with [per liter] 20 mg of arginine, 20 mg of histidine, 30 mg of isoleucine, 30 mg of leucine, 30 mg of lysine, 20 mg of methionine, 200 mg of threonine, 20 mg of adenine sulfate, 10 mg of uracil) plus 0.1, 0.3, 0.6, or 1.0% L-malate. This medium contains only 2.0% glucose, probably not enough to cause glucose repression of aerobiosis. For aerobic growth conditions, *S. cerevisiae* cultures were grown in tubes containing 3.0 ml of SM-A medium at 30°C with shaking for 17 to 48 h. For nonaerobic growth conditions, *S. cerevisiae* cultures were grown in tubes containing 10 ml of SM-A medium at 30°C with no shaking for 5 days. *L. delbrueckii* Cuc-1 controls were assayed for conversion of L-malate to L-lactate in the same way as *E. coli* or yeast cultures.

RESULTS

Cloning the malolactic gene in *E. coli*. Chromosomal DNA isolated from *L. delbrueckii* was cleaved with the restriction endonuclease *Sal*I and ligated into the *Sal*I site in the *tet* gene of plasmid pBR322. The ligated DNA was used to transform *E. coli* K-12 RR1. Ampicillin-resistant tetracycline-sensitive transformants were selected by the fusaric acid method of Bochner et al. (3). Greater than 90% of the transformants selected in this way contained DNA inserts at the *Sal*I site in the *tet* gene. Transformants were tested for their ability to carry out the malolactic fermentation by growing them aerobically in M9-A medium containing 0.3% L-malate and then assayed for the presence of L-lactate. Control experiments showed that neither *E. coli* K-12 RR1 nor *S. cerevisiae* 2514-10c produced L-lactate under these conditions. From over 4,000 *E. coli* transformants screened, 2 were identified that produced L-lactate. The two clones were designated RR1(pSW1) and RR1(pSW2). Since detailed restriction mapping indicated that the clones were indistinguishable, only RR1(pSW1) was used in further work.

Results of L-lactate assays on RR1(pSW1) and controls demonstrated that RR1(pSW1) produced about 30% as much L-lactate as *L. delbrueckii* controls grown under similar conditions (Table 1). Plasmid DNA isolated from RR1(pSW1) and analyzed by restriction digests indicated that the plasmid was pBR322 with a 5-kilobase (kb) *Sal*I restriction fragment inserted at the *Sal*I site. The new plasmid containing the 5-kb *Sal*I insert was designated pSW1. RR1(pSW2) contained the same plasmid with the same 5-kb insert.

Characterization of the malolactic fragment. Plasmid DNA isolated from RR1(pSW1) was used to retransform RR1 to demonstrate that the lactate-producing character was carried on the plasmid. Seven of the eight ampicillin-resistant, tetracycline-sensitive transformants tested produced L-lactate (Table 2). Much of the variability observed in L-lactate production between the various transformants could be due

TABLE 1. L-Lactate assays on *E. coli* RR1(pSW1)

Bacterial strain[a]	% Malate in growth medium	L-Lactate produced (μmol/ml)
E. coli RR1(pSW1)	0	0.02
	0.3	0.36
E. coli RR1(pBR322) (control)	0	<0.02
	0.3	<0.02
L. delbrueckii Cuc-1 (control)	0.3	1.19

[a] All cultures were incubated for 48 h with aeration.

TABLE 2. L-Lactate assays on *E. coli* RR1 transformed with pSW1 and pSW3

Bacterial strain[a]	L-Lactate produced (μmol/ml)
E. coli RR1(pSW1) transformant 1.....	0.03
E. coli RR1(pSW1) transformants 2 to 8....0.22–0.40 (mean, 0.31)	
E. coli RR1(pSW3) transformants 1 to 5....0.43–0.64 (mean, 0.55)	
E. coli RR1(pBR322) (control)	<0.02
L. delbrueckii Cuc-1 (control).....	1.20

[a] All cultures were incubated for 48 h with aeration in medium containing 0.3% malate.

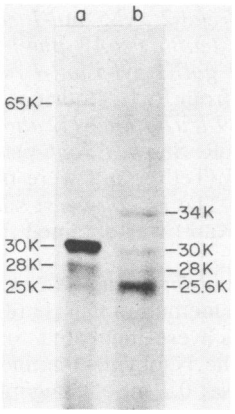

FIG. 1. Plasmid-coded proteins labeled with [^{35}S]methionine prepared by the maxicell procedure of Sancar et al. (24). Cell extracts of *E. coli* CSR603(pSW1) (lane a) and CSR603(pBR328) (lane b) were electrophoresed into a denaturing sodium dodecyl sulfate-polyacrylamide gel, and proteins were visualized by fluorography. The proteins encoded by the control plasmid pBR328 can be seen in lane b. The darkest band is the 25,600 (25.6K) protein product of the *cam* gene. The two bands immediately above the 25.6K band are the 30K and 28K protein products of the *amp* gene. The 25K protein product of the *amp* gene is obscured by the 25.6K protein. The band immediately above the 30K band is the 34K product of the *tet* gene. In lane a, the 30K, 28K, and 25K protein products of the *amp* gene are all visible and align with the corresponding bands in lane b. There is no 25.6K protein because pSW1 does not possess the *cam* gene. There is also no 34K protein present because the *tet* gene has been inactivated by the insertion of the 5-kb malolactic fragment. The band at the top of lane a is estimated to be of a molecular weight of 65K (protein size markers not shown) and is presumably coded by the 5-kb malolactic fragment cloned from *L. delbrueckii*.

to plasmid instability. RR1(pSW1) was then cured of its plasmid by serial transfer of the strain through several rounds of growth to stationary phase in liquid (LB) medium (19). After the third round, a sample of culture was spread on LB plates and ampicillin-sensitive, tetracycline-sensitive colonies that had lost the plasmid were selected. Ten such colonies assayed for L-lactate production in M9-A medium containing 0.3% L-malate were all L-lactate negative. Thus, most transformants containing pSW1 were L-lactate positive, whereas transformants cured of pSW1 were always L-lactate negative.

Further evidence that the 5-kb fragment was responsible for the ability of *E. coli* cells to convert L-malate to L-lactate came from experiments in which the fragment was purified from preparative agarose gels and inserted into the *Sal*I site of the *E. coli* vector pBR327. The new plasmid pSW3 was transformed into RR1 and gave greater L-lactate production than did pSW1 in RR1 (Table 2). To determine whether a new protein of the appropriate molecular weight was encoded by pSW1, *E. coli* CSR603 maxicells transformed with pSW1 were prepared, and protein extracts were isolated by the method of Sancar et al. (24). Proteins radioactively labeled with [^{35}S]methionine encoded by the pSW1 plasmid were run on acrylamide gels and visualized by using autofluorography. Banding patterns indicated that pSW1 coded for the appropriate *amp* gene products (24). However, the band representing the protein encoded by the *tet* gene was missing, as expected, due to insertional inactivation of the gene by the 5-kb fragment. A new, relatively faint, protein band with a molecular weight of ca. 65,000 was observed that did not appear in the controls (Fig. 1). We concluded that this new protein was probably encoded by a gene located on the 5-kb fragment.

A DNA/DNA hybridization experiment demonstrated that the 5-kb fragment was indeed from the *L. delbrueckii* genome. The purified fragment was radioactively labeled by nick translation (22) and then hybridized to *Sal*I-cleaved *L. delbrueckii* chromosomal DNA in dry agarose gels (S. Tsao, C. Brunk, and R. E. Pearlman, manuscript in preparation). The fragment hybridized to a single 5-kb band of *L. delbrueckii* DNA but not to control λ DNA.

Thus, based on several lines of evidence, we concluded that the malolactic gene of *L. delbrueckii* had been cloned in pBR322. On the basis of the L-lactate assays and maxicell labeling of plasmid-coded proteins, we also concluded that the malolactic gene is expressed in *E. coli*, although weakly as compared with its expression in *L. delbrueckii*.

Transfer of the malolactic fragment to an *E. coli*-yeast vector. The 5-kb fragment which had been purified by using preparative agarose gels was ligated into the *E. coli*-yeast shuttle vector pRC3 at the *Sal*I site in the *tet* gene, creating a new plasmid designated pHW2 (Fig. 2). The *Sal*I site in pRC3 is located downstream from the promoter of the *tet* gene, as in pBR322 and pBR327. pRC3 possesses both yeast chromosomal and 2-μm plasmid origins of DNA replication that enable the plasmid to replicate when transformed into yeasts. It also has the pBR322 origin of DNA replication, allowing it to replicate in *E. coli* also. *E. coli* RR1 transformed with pHW2 was found to produce L-lactate (Table 3).

In addition to production of L-lactate, RR1(pHW2) was also assayed for utilization of L-malate. Assays indicated that after only 17 h of aerobic growth, much of the L-malate had been used both by RR1 cells containing the control plasmid (pBR327) and by RR1 cells containing pHW2, although RR1(pHW2) used more of it in the same period of time (Table 4). Since growth under nonaerobic conditions (no shaking and no aeration) should inhibit the tricarboxylic acid cycle, thereby slowing the usual catabolism of L-malate, it was reasoned that nonaerobic growth would increase the amount of L-malate available for conversion to L-lactate by the malolactic enzyme. Growth of the control strain of *E. coli* under nonaerobic conditions did result in a much-reduced utilization of L-malate in the growth medium (Table 4). As in aerobic growth, RR1(pHW2) possessing the 5-kb fragment used up more of the L-malate in nonaerobic growth than RR1 without the 5-kb fragment (Table 4). Since nonaerobic growth results in a reduced utilization of L-malate by *E. coli*, more L-malate should be present for conversion to L-lactate by strains carrying the malolactic gene. In fact, when grown nonaerobically, RR1(pHW2) did produce more L-lactate than when grown aerobically (compare Tables 3 and 5).

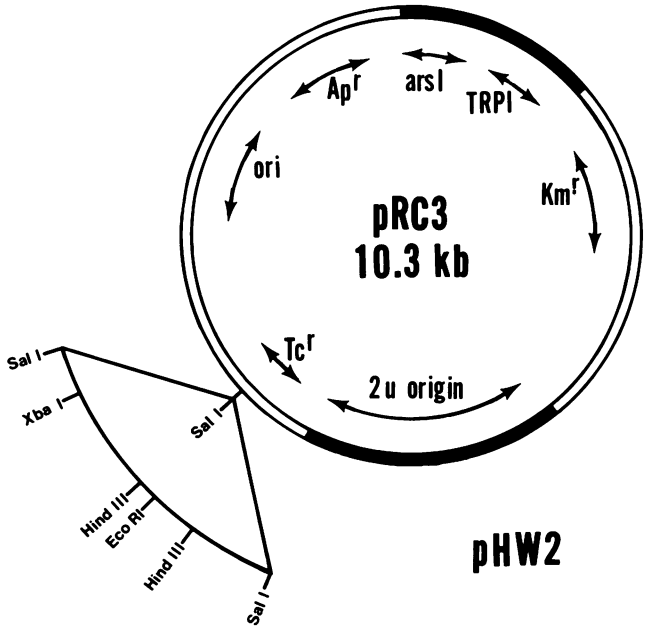

FIG. 2. Plasmid map of pRC3 showing the 5-kb malolactic fragment cloned from *L. delbrueckii* inserted into the *Sal*I site of the *tet* gene. This chimeric plasmid is designated pHW2. The dark lines indicate yeast DNA sequences. This plasmid contains the yeast *TRP1* gene and the chromosomal replicator *ars1* as well as the 2-μm plasmid origin of replication.

Transformation of the malolactic gene into *S. cerevisiae*. Because pHW2 carries the yeast *TRP1* gene, it was used to transform a haploid laboratory yeast strain *S. cerevisiae* 2514-10c carrying the *trp1* mutation. Trp^+ transformants containing pHW2 were selected. In winemaking, the yeast is grown under nonaerobic conditions; for this reason pHW2 transformants were tested for L-lactate production after nonaerobic growth. Yeast cells grown aerobically catabolize L-malate via the tricarboxylic acid cycle so, as with *E. coli*, nonaerobic growth should increase the amount of L-malate available for conversion by the malolactic enzyme. When *S. cerevisiae* containing pRC3 (no 5-kb fragment) and *S. cerevisiae* containing pHW2 (with the fragment) were grown nonaerobically, yeast carrying the fragment produced about 3.5 times more L-lactate (Table 5).

Strain 2514-10c containing the malolactic gene was used to make wine in a trial fermentation as described by Amerine et al. (1). As is typical of laboratory yeast strains, this strain carried out an incomplete alcoholic fermentation, that is, vinification ceased before the sugar in the grape must was completely utilized. Assays showed that about 1.5% of the L-malate present in the must was converted to L-lactate. In a usual commercial malolactic fermentation essentially all of the L-malate would have been converted.

TABLE 3. L-Lactate assays on *E. coli* RR1 transformed with pHW2

Bacterial strain[a]	L-Lactate produced (μmol/ml) when grown in:	
	0% Malate	1.0% Malate
E. coli RR1(pHW2)	<0.02	1.01
E. coli RR1(pBR327) (control)	<0.02	0.06

[a] Cultures were incubated for 17 h with aeration.

DISCUSSION

The results of this study demonstrate that the gene coding for the malolactic enzyme from *L. delbrueckii* has been cloned into pBR322 and confers on the host *E. coli* cells the ability to convert L-malate to L-lactate. The gene lies on a 5-kb *Sal*I fragment which hybridizes to a single 5-kb band in *Sal*I-digested *L. delbrueckii* DNA. The newly constructed plasmid pSW1 was isolated from the original transformants and used to retransform *E. coli* competent cells which were then able to convert L-malate to L-lactate. The *Sal*I fragment was purified and transferred to other *E. coli* and *E. coli*-yeast shuttle vectors. With these vectors, *E. coli* transformants capable of converting L-malate to L-lactate were also obtained. Yeast cells capable of converting a small amount of L-malate to L-lactate were also obtained when transformed with an *E. coli*-yeast shuttle vector carrying the *Sal*I fragment. Results of *E. coli* maxicell experiments designed to identify plasmid-coded proteins revealed that pSW1 encodes a new polypeptide not encoded by the control plasmid pBR322. This polypeptide has a molecular weight of ca. 65,000 which agrees very well with the molecular weight estimate of 70,000 reported by Caspritz and Radler for the malolactic enzyme they have purified from *L. plantarum* (4).

Studies of *E. coli* and *S. cerevisiae* transformed with plasmids carrying the 5-kb malolactic fragment indicate that expression of the malolactic gene is much stronger in *E. coli* than in *S. cerevisiae*. *E. coli* carrying the malolactic gene on a multicopy plasmid and grown aerobically for 48 h converted about 5% of the L-malate in the growth medium to L-lactate, and when grown under nonaerobic conditions for 5 days, it converted about 10% of the L-malate. *S. cerevisiae* transformed with an *E. coli*-yeast shuttle plasmid carrying the malolactic gene (pHW2) and grown nonaerobically for 5 days converted about 1% of the L-malate. Under aerobic or nonaerobic conditions, *L. delbrueckii* control cultures converted about 25% of the L-malate. As expected, *E. coli* transformed with the plasmids pBR322, pBR327, or PRC3 did not convert L-malate under aerobic or nonaerobic conditions, nor did yeast transformed with pRC3 grown under nonaerobic conditions. A laboratory yeast strain carrying the cloned malolactic gene was used to ferment wine in a trial vinification, and ca. 1.5% of the L-malate in the grape must was converted to L-lactate.

It is clear that barriers to high levels of expression of the malolactic gene exist in *E. coli* and especially in *S. cerevisiae*. Such barriers to gene expression in heterologous hosts could exist at the level of transcription or translation. Reduced expression could also be due to instability of the plasmid, mRNA transcript, or protein product in the host cell. Frequent occurrence of codons in the malolactic gene

TABLE 4. L-Malate assays on *E. coli* RR1 transformed with pHW2

Bacterial strain[a]	L-Malate remaining in the medium (μmol/ml) when grown in:	
	0% Malate	0.3% Malate
M9 assay medium (no inoculum)	0.4	18.9
E. coli RR1(pBR327) (control, aerobic)	0.4	4.3
E. coli RR1(pHW2) (aerobic)	0.2	1.3
E. coli RR1(pRC3) (control, nonaerobic)	0.3	13.3
E. coli RR1(pHW2) (nonaerobic)	0.2	8.9

[a] Aerobic cultures were incubated for 17 h with shaking; nonaerobic cultures were incubated for 5 days without shaking.

TABLE 5. L-Lactate assays on *E. coli* RR1 and *S. cerevisiae* 2514-10c transformed with pHW2

Strain[a]	L-Lactate produced (μmol/ml) when grown in:		
	0% Malate	0.3% Malate	0.6% Malate
Bacteria			
E. coli RR1(pBR327) (control, nonaerobic)	<0.02	0.10	
E. coli RR1(pHW2) (nonaerobic)	<0.02	1.96	
Yeast			
S. cerevisiae 2514-10c(pRC3) (control, nonaerobic)	<0.02		0.08
S. cerevisiae 2514-10c(pHW2) (nonaerobic)	<0.02		0.28

[a] Aerobic cultures were incubated for 17 h with shaking; nonaerobic cultures were incubated for 5 days without shaking.

transcript that are used only rarely by yeasts could reduce translation efficiency (2, 12). Further research must be done to discern where expression of the malolactic gene is being limited, especially in yeasts. If expression is limited at either the transcriptional or translational level, then precise fusion of the malolactic gene coding sequence to a strong yeast promoter-leader sequence may provide the key to increasing expression in yeasts. Such a construction would provide the bacterial malolactic gene with both yeast RNA polymerase and ribosome binding sites. A successful example of this approach to increasing levels of expression of a foreign gene cloned in yeast is the fusion of the human gene coding for leukocyte interferon D to the yeast *ADHI* promoter-leader sequence (9).

Once a substantial increase in expression of the malolactic gene in laboratory yeast strains has been obtained, the gene will be transferred to a wine yeast strain by transformation. If plasmid instability is limiting expression in the wine yeast strain, the gene could be transferred to a plasmid carrying a cloned yeast centromere (5, 6). A yeast centromere sequence confers increased stability on an *E. coli*-yeast vector through both mitotic and meiotic divisions. Another strategy available for stabilizing cloned sequences in yeasts would be to integrate a plasmid carrying the malolactic gene into a yeast chromosome. A method for targeting plasmid integration to specific chromosomal sites in yeasts has been described by Orr-Weaver et al. (20). Such an integration could result in stable inheritance of the malolactic gene as part of the wine yeast genome.

Increased expression in yeasts of the cloned malolactic gene is essential if this recombinant is to be of practical use to vintners in overcoming problems associated with the slow malolactic fermentation. This will require an increased understanding of the factors governing expression of the malolactic gene in yeasts. Such efforts with wine yeast should provide information applicable to the genetic engineering of other organisms of economic importance into which genes could be inserted from procaryotes to expand their biochemical capabilities.

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