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# Occurrence of Arginine Deiminase Pathway Enzymes in Arginine Catabolism by Wine Lactic Acid Bacteria

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**L-Arginine, an amino acid found in significant quantities in grape juice and wine, is known to be catabolized by some wine lactic acid bacteria. The correlation between the occurrence of arginine deiminase pathway enzymes and the ability to catabolize arginine was examined in this study. The activities of the three arginine deiminase pathway enzymes, arginine deiminase, ornithine transcarbamylase, and carbamate kinase, were measured in cell extracts of 35 strains of wine lactic acid bacteria. These enzymes were present in all heterofermentative lactobacilli and most leuconostocs but were absent in all the homofermentative lactobacilli and pediococci examined. There was a good correlation among arginine degradation, formation of ammonia and citrulline, and the occurrence of arginine deiminase pathway enzymes. Urea was not detected during arginine degradation, suggesting that the catabolism of arginine did not proceed via the arginase-catalyzed reaction, as has been suggested in some earlier studies. Detection of ammonia with Nessler's reagent was shown to be a simple, rapid test to assess the ability of wine lactic acid bacteria to degrade arginine, although in media containing relatively high concentrations (>0.5%) of fructose, ammonia formation is inhibited.**

Wine lactic acid bacteria (LAB), particularly strains of *Leuconostoc oenos*, are commonly used to induce malolactic fermentation in wine, a secondary fermentation important for deacidification of high-acid wine and for flavor complexity (6, 21, 44). Some wine LAB are known to degrade L-arginine, one of the major amino acids found in grape juice and wine, with the formation of ornithine and ammonia (13, 20, 22, 32, 39, 43). There are two different proposals concerning the pathway by which arginine is catabolized. It has been suggested that arginine catabolism involves the action of arginase with the formation of ornithine and urea (20, 38–40). Other reports suggest that the arginine deiminase (ADI) pathway, formerly referred to as the arginine dihydrolase pathway, is involved (22, 24, 32). There is no experimental evidence for the presence of the enzyme arginase in LAB. However, the ADI pathway has been found in several dairy and meat LAB (5, 25–28). Therefore, it is very likely that wine LAB may also catabolize arginine by means of the ADI pathway. This pathway involves three enzymes, arginine deiminase (EC 3.5.3.6) (ADI), ornithine transcarbamylase (EC 2.1.3.3) (OTC), and carbamate kinase (EC 2.7.2.2) (CK), which catalyze the following reactions:

$$\text{L-arginine} + H_2O \xrightarrow{\text{ADI}} \text{L-citrulline} + NH_3 \quad (1)$$

$$\text{L-citrulline} + P_i \xleftrightarrow{\text{OTC}} \text{L-ornithine} + \text{carbamyl-P} \quad (2)$$

$$\text{carbamyl-P} + ADP \xleftrightarrow{\text{CK}} ATP + CO_2 + NH_3 \quad (3)$$

In the wine industry, there is a concern that LAB may be involved in the formation of ethyl carbamate (urethane) (22, 38–41). Ethyl carbamate is a carcinogen found in many fermented foods and alcoholic beverages, including wine (30). Ethyl carbamate is formed from ethanol and a compound that contains a carbamyl group, such as citrulline, carbamyl phosphate, or urea (31). Our previous study (24) demonstrated the excretion of citrulline during arginine degradation by two different species of wine LAB and the corresponding formation of ethyl carbamate in wine. This suggested that wine LAB are potential producers of one of the ethyl carbamate precursors, citrulline, if arginine is catabolized. Therefore, information on the ability of wine LAB strains to degrade arginine is an important consideration in selecting starter cultures to be used for inducing malolactic fermentation in wine in order to minimize the formation of the ethyl carbamate precursors citrulline and possibly carbamyl phosphate.

This study was undertaken to demonstrate the presence of ADI pathway enzymes in wine LAB capable of degrading arginine and to survey the occurrence of these enzymes in a wide range of commercial and laboratory wine LAB strains. The correlation between the formation of ammonia and citrulline from arginine catabolism and the occurrence of ADI pathway enzymes was investigated with the aim of developing a simple procedure for screening wine LAB for the ability to degrade arginine.

## MATERIALS AND METHODS

**Organisms.** Wine LAB (Table 1) used were from the Food and Fermentation Laboratory Culture Collection of the Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

**Medium.** Two types of complex medium were used, tomato juice-arginine-glucose (TJAG) broth and heterofermentation-arginine (HFA) broth. A basal medium (pH 5.5) of TJAG broth consisted of the following components in deionized water (per liter): tryptone, 5 g; peptone, 5 g; yeast extract, 5 g; Tween 80 (5% aqueous solution), 1 ml; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.05 g; and clarified vegetable juice, 200 ml. The clarified juice was prepared from V8 vegetable juice (Campbell's Soups, Lemnos, Australia) with no added sugar. The juice was filtered through Whatman no. 1 filter paper and diatomaceous-earth filter aid (Diatomite Filteraid FW14; Eagle-Pricher Minerals, Inc., Reno, Nev.). This basal medium contained approximately 0.5 g of L-arginine per liter from

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TABLE 1. Wine LAB used in this research

| Strain | Source[a] | Reference(s) |
|---|---|---|
| Heterofermenters | | |
| *Leuconostoc oenos* | | |
| 2035 | LM | 3 |
| 2001 | LM | 3 |
| 2043 | LM | 3 |
| 2008 | LM | |
| Microenos B | LL | |
| Microenos B1 | LL | |
| Microenos B2 | LL | |
| Microenos B3 | LL | |
| DSIR-A | JP | |
| DSIR-B | JP | |
| DSIR-C | JP | |
| Er1a | MD | 15, 17 |
| Ey2d | MD | 15, 17 |
| 122 | CD | 7, 8 |
| 252 | CD | 7, 8 |
| MCW | MB | |
| OENO | QB | |
| INOBACTER | IO | |
| Viniflora Oenos | CP | |
| ML34 | RK | 33 |
| PSU-1 | RB | 2 |
| L181 | BR | 35 |
| *Lactobacillus hilgardii* | | |
| Microenos HP | LL | |
| *Lactobacillus buchneri* | | |
| CUC-3 | RK | 34 |
| *Lactobacillus brevis* | | |
| 250 | CD | 7, 8 |
| EQUILAIT | RK | |
| ML30 | RK | 34 |
| Homofermenters | | |
| *Lactobacillus plantarum* | | |
| Viniflora LP | CP | |
| 49 | CD | 7, 8 |
| *Lactobacillus delbrueckii* | | |
| CUC-1 | RK | 34 |
| *Pediococcus* spp. | | |
| 44.40 | PW | 37 |
| C5 | RK | 34 |
| CUC-4 | RK | 34 |
| *Pediococcus parvulus* | | |
| 93 | CD | 7, 8 |
| 272 | CD | 7, 8 |

[a] BR, B. Rankine, Australian Wine Research Institute, Glen Osmond, South Australia, Australia; CP, C. Prahl, Christian Hansen's Laboratorium A/S, Hørsholm, Denmark; CD, C. Davis, University of New South Wales; IO, Institut Oenologique de Champagne, Eperney, France; JP, J. Patel, Horticulture and Food Research Institute, Palmerston North, New Zealand; LL, Lacto-Labo, Dange, France; LM, Lallemand, Montreal, Canada; MB, M. Bannister, Vinquiry, Healdsburg, Calif.; MD, M. Daeschel, Dept. of Food Science and Technology, Oregon State University; PW, Pickering Winery Supplies, San Francisco, Calif.; QB, Quest Bioproducts Group, Sarasota, Fla.; RB, R. Beelman, Dept. of Food Science, Pennsylvania State University; RK, R. Kunkee, Dept. of Viticulture and Enology, University of California, Davis.

three possible sources: yeast extract, tryptone, and peptone. TJAG broth was prepared from the basal medium by adding L-arginine and D-glucose to a final concentration of about 5 g/liter each. A solution of arginine and glucose (pH 5.5; 10 g/liter [each]) was aseptically membrane filtered (0.45-μm pore size) and added to an equal volume of autoclaved (121°C and 15 min) twofold-concentrated basal medium. HFA broth was prepared as previously described (32). This medium is essentially the same as TJAG broth except that HFA broth contains 6 g of L-arginine per liter and 20 g of fructose per liter in addition to glucose as the carbohydrate source.

**Culturing conditions and procedures.** To survey the occurrence of ADI pathway enzymes, bacterial strains (Table 1) were cultured statically at 30°C in TJAG broth to the late-log/early stationary phase before being harvested. Preliminary trials (23) indicated that enzyme activity was optimal at this growth phase. A study of the correlation of the formation of ammonia and citrulline with the occurrence of ADI pathway enzymes was performed with HFA broth because this medium had been used in an earlier study to detect ammonia formation from arginine and to examine other biochemical characteristics of wine LAB (32). Strains were cultured statically at 30°C for 4 weeks, and then culture broths were used for analysis of ammonia, arginine, and citrulline concentrations.

**Cell harvesting and preparation of cell extracts.** Cells were harvested by centrifugation at 7,000 × *g* for 10 min at 5°C in a Sorvall RC-5B refrigerated centrifuge. Harvested cells were divided into two or three portions, each containing approximately 300 mg of wet cells. Wet cells were washed three times by resuspension in 0.01 M cold potassium phosphate buffer (pH 7.0) and centrifugation as described above; then washed cell pellets were stored frozen (−70°C) until used. Preliminary experiments showed no loss of enzyme activity during storage of frozen cells (23). To prepare cell extracts, cell pellets were first resuspended in 5 ml of cold potassium phosphate buffer as described above and then passed twice through a French pressure cell (Aminco, Silver Spring, Md.) at a pressure of 7,600 lb/in$^2$. Cell debris was removed by centrifugation at 14,500 × *g* for 10 min at 5°C, and the supernatant extract was used to assay for the activities of ADI pathway enzymes.

**Enzyme assays.** The assay methods described below are based on a study (23) that defined the appropriate conditions for measuring the activities of ADI pathway enzymes of wine LAB.

The assay of ADI activity was based on the method of Oginsky (29) with modifications. The standard assay mixture (6.1 ml) consisted of 131 mM potassium phosphate buffer (pH 5.8), 52 mM L-arginine (Sigma) (pH 5.8), and the appropriate amount of extract. The enzymatic reaction (in duplicate) was started by the addition of enzyme extract to a preincubated (ca. 10-min) reaction mixture that contained phosphate buffer and arginine at 37°C. At 10- or 15-min intervals, a 1.4-ml aliquot of reaction mixture was removed and mixed into 0.1 ml of ice-cold 70% perchloric acid to stop the reaction. One milliliter of supernatant was then analyzed for citrulline concentration by the method of Archibald (1). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of citrulline per min.

OTC activity was assayed according to the method of Jones (18) with modifications. The standard assay mixture (5.0 ml) consisted of 460 mM Tris-HCl (pH 8.2), 48 mM L-ornithine-HCl (pH 8.2) (Sigma), 40 mM carbamyl phosphate (Sigma), and the appropriate amount of diluted extract. The reaction was initiated by adding carbamyl phosphate and then enzyme extract to the preincubated (37°C) mixture of Tris-HCl and L-ornithine-HCl. At 3-min intervals, a 1.2-ml aliquot of reaction mixture was removed and mixed into 0.1 ml of ice-cold 70% perchloric acid to stop the reaction. One milliliter of supernatant was then analyzed for citrulline. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of citrulline per min. Because small amounts of citrulline can be synthesized nonenzymatically from ornithine and carbamyl phosphate at 37°C (18), control assays without enzyme extracts were routinely carried out. The amount of citrulline synthesized nonenzymatically was subtracted from the values obtained with complete reaction mixtures.

CK activity was measured by a coupled enzyme assay. The rate of ATP production in the CK-catalyzed reaction was determined by coupling ATP via hexokinase to glucose-6-phosphate production, which was in turn coupled to reduction of $NADP^+$ in the presence of excess glucose-6-phosphate dehydrogenase. This method is based on that described by Crow and Thomas (5) with modifications. The standard assay mixture (2.73 ml) consisted of 147 mM Tris-HCl (pH 7.9), 14.7 mM carbamyl phosphate (Sigma), 7.3 mM ADP (Boehringer Mannheim), 7.3 mM $MgCl_2$, 37 mM glucose, 0.73 mM $NADP^+$, 900 U of hexokinase, 450 U of glucose-6-phosphate dehydrogenase, and the appropriate amount of diluted extract. The assay mixture (without carbamyl phosphate and enzyme extract) was equilibrated at room temperature for at least 10 min and further equilibrated (10 min) in the cuvette compartment of the spectrophotometer at 37°C after the addition of carbamyl phosphate. The enzymatic reaction was initiated by the addition of 0.1 ml of extract. The change in $A_{340}$ was monitored continuously at 37°C with a Gilford 260 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of ATP per min since the amount of ATP produced is stoichiometrically equal to the amount of NADPH formed. Interference by myokinase (adenylate kinase) activity was significant only at high concentrations of cell extracts. Therefore, when undiluted extracts or low dilutions of extracts were used, CK activity was calculated by subtracting the values for controls (without carbamyl phosphate) from the values obtained for complete reaction mixtures.

Acetate kinase (AK) was assayed under essentially the same conditions used for CK, except for the replacement of carbamyl phosphate with 14.7 mM acetyl phosphate. One unit of AK activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of ATP per min.

**Analyses.** Protein concentrations were determined by the dye binding method (36). Arginine concentrations were determined colorimetrically on the basis of the Sakaguchi reaction (14). Citrulline concentrations were also measured colorimetrically (1). In some experiments, citrulline concentrations were determined with an amino acid analyzer (Pharmacia LKB Alpha Plus). Analyses of ammonia and urea were performed enzymatically with Boehringer Mannheim enzyme test kits. Ammonia also was detected qualitatively with Nessler's reagent (32).

TABLE 2. Specific activities of ADI pathway enzymes in cell extracts of wine LAB

| Strain | Sp act (units/mg of protein) | | | | | | $n^a$ |
|---|---|---|---|---|---|---|---|
| | ADI | | OTC | | CK | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| Heterofermenters | | | | | | | |
| *Leuconostoc oenos* | | | | | | | |
| 2035 | 0.132 | 0.010 | 51.2 | 4.9 | 19.3 | 1.7 | 4 |
| 2001 | 0.137 | 0.004 | 42.5 | 6.0 | 22.6 | 1.5 | 6 |
| Microenos B1 | 0.126 | 0.013 | 35.3 | 2.4 | 27.3 | 1.2 | 6 |
| 2008 | 0.120 | 0.002 | 40.5 | 2.9 | 18.6 | 3.2 | 4 |
| 2043 | 0.117 | 0.026 | 47.0 | 8.1 | 15.4 | 3.7 | 6 |
| Microenos B2 | 0.125 | 0.015 | 32.3 | 2.7 | 18.3 | 2.3 | 4 |
| DSIR-A | 0.112 | 0.010 | 35.5 | 2.8 | 21.5 | 2.1 | 4 |
| 122 | 0.105 | 0.007 | 34.9 | 0.5 | 19.2 | 0.4 | 4 |
| MCW | 0.100 | 0.012 | 30.1 | 4.1 | 17.1 | 0.7 | 4 |
| Ey2d | 0.106 | 0.005 | 33.2 | 2.4 | 15.6 | 2.9 | 4 |
| OENO | 0.088 | 0.004 | 32.2 | 3.1 | 18.1 | 1.4 | 6 |
| DSIR-B | 0.084 | 0.002 | 31.0 | 1.0 | 20.4 | 3.4 | 4 |
| DSIR-C | 0.089 | 0.004 | 30.7 | 1.3 | 18.9 | 2.1 | 4 |
| Er1a | 0.088 | 0.007 | 30.2 | 3.3 | 18.1 | 1.6 | 6 |
| 252 | 0.091 | 0.005 | 28.8 | 1.2 | 15.0 | 2.3 | 6 |
| Vinoflora Oenos | 0.044 | 0.012 | 29.0 | 2.2 | 16.1 | 2.2 | 4 |
| INOBACTER | 0.024 | 0.005 | 5.1 | 0.8 | 4.6 | 0.6 | 4 |
| Microenos B3 | ND[b] | | 0.2 | 0.09 | ND | | 4 |
| ML34 | ND | | ND | | 0.8 | 0.11 | 4 |
| PSU-1 | ND | | ND | | 0.2 | 0.07 | 4 |
| L181 | ND | | ND | | ND | | 4 |
| Microenos B | ND | | ND | | ND | | 4 |
| *Lactobacillus* spp. | | | | | | | |
| *L. hilgardii* Microenos HP | 0.92 | 0.160 | 177 | 7 | 27.6 | 1.4 | 6 |
| *L. buchneri* CUC-3 | 0.67 | 0.120 | 217 | 15 | 41.5 | 2.4 | 6 |
| *L. brevis* EQUILAIT | 0.89 | 0.054 | 215 | 14 | 30.8 | 1.8 | 4 |
| *L. brevis* 250 | 0.76 | 0.076 | 229 | 18 | 20.8 | 2.9 | 6 |
| *L. brevis* ML30 | ND | | 2.2 | 0.3 | 3.4 | 0.2 | 6 |
| Homofermenters | | | | | | | |
| *Lactobacillus* spp. | | | | | | | |
| *L. plantarum* Viniflora LP | ND | | ND | | ND | | 4 |
| *L. plantarum* 49 | ND | | ND | | ND | | 4 |
| *L. delbrueckii* CUC-1 | ND | | ND | | ND | | 4 |
| *Pediococcus* spp. | | | | | | | |
| 44.40 | ND | | ND | | ND | | 4 |
| C5 | ND | | ND | | ND | | 4 |
| CUC-4 | ND | | ND | | ND | | 4 |
| 93 | ND | | ND | | ND | | 4 |
| 272 | ND | | ND | | ND | | 4 |

[a] Number of measurements for each of the enzymes from at least two separate cell extracts.
[b] ND, none detected.

## RESULTS

**Occurrence of ADI pathway enzymes.** Table 2 shows the specific activities of ADI pathway enzymes in cell extracts of a large number of strains of homofermentative and heterofermentative wine LAB cultured in TJAG broth that contained 5 g each of arginine and glucose per liter. Cells were harvested in the late-log/early stationary phase so that the activities of the ADI pathway enzymes were measured when they were maximal, as determined in preliminary experiments (23).

As shown in Table 2, the activities of ADI pathway enzymes varied considerably between species and strains of wine LAB. A high proportion (77%) of the leuconostocs and almost all of the heterofermentative lactobacilli tested possessed all three enzymes of the ADI pathway. No activity for any of these enzymes was found in any of the homofermentative lactobacilli and pediococci tested. Among heterofermentative wine LAB, some strains, such as *Leuconostoc oenos* Microenos B3, ML34, and PSU-1 and *Lactobacillus brevis* ML30, were deficient in one or two of these enzymes. In all of these cases, the specific activities of the enzymes that were detected were very low relative to the levels found in other strains. Some strains, such as L181 and Microenos B, did not show detectable activities for any of the three enzymes.

For leuconostocs and heterofermentative lactobacilli which possess the complete enzyme system, the specific activities of the three enzymes from lactobacilli were generally higher than those from leuconostocs, especially for ADI and OTC. Nevertheless, for those LAB with all three enzymes, the enzyme activities were generally similar within heterofermentative lactobacilli, as were the enzyme activities within leuconostocs. There were some exceptions, however, such as strain INOBACTER, in which the activities of the three enzymes were significantly lower, and strain Viniflora Oenos, in which

the ADI activity was considerably lower than those in other leuconostoc strains.

**Distinction between AK and CK activities.** It has been reported that the enzyme AK from *Escherichia coli* (42, 45) and *Serratia marcescens* (4) can catalyze the reaction between ADP and carbamyl phosphate to produce ATP. Since CK activity was assayed in this study by monitoring the rate of ATP formation, it is possible that some of the CK activity was due to the presence of AK in crude extracts. Therefore, the relative activities of CK and AK in crude extracts of several different wine LAB were determined. No correlation between the activities of AK and CK in the six strains of wine LAB examined was found. *Leuconostoc oenos* Microenos B had relatively high AK activity but no detectable CK activity, indicating that in this strain AK is unable to catalyze the reaction between carbamyl phosphate and ADP. It seems very unlikely that the specificity of CK differs substantially between strains of *Leuconostoc oenos*. Therefore, the result with the Microenos B strain suggests that CK activities measured in extracts of *Leuconostoc oenos* and probably other wine LAB are not due to AK. In any case, the affinity of AK from other bacterial sources for carbamyl phosphate is low (4, 42), and thus, interference due to AK in the CK assays in this study is unlikely to be significant.

**Correlation of ammonia formation with the occurrence of ADI pathway enzymes.** The operation of the ADI pathway should result in the production of ammonia as an end product of arginine catabolism. Therefore, ammonia production should provide a simple basis for the detection of ADI pathway activity in strains of wine LAB. Table 3 shows the data for arginine consumption and formation of ammonia by wine LAB cultured in the complex medium HFA broth (containing 5 g of glucose per liter, 20 g of fructose per liter, and 6 g of arginine per liter). Ammonia concentration was determined enzymatically and also detected qualitatively with Nessler's reagent to assess the usefulness of this simple test in screening strains for the ability to degrade arginine. Uninoculated HFA broth contains a basal level of 4 mM ammonia, but this low level of ammonia did not give a positive reaction with Nessler's reagent under the conditions used. The enzymatically determined values of ammonia formed were corrected for this basal level. On the basis of the results presented in Tables 2 and 3, wine LAB can be categorized into three groups, depending on the extent of arginine consumption, the amount of ammonia formed, and the activities of ADI pathway enzymes.

Strains in group I (Table 3) possess all three enzymes of the ADI pathway. These strains degraded all or nearly all of the initial arginine. Substantial amounts of ammonia were produced and released into media by group I strains. In this group of strains, there is good correlation among ammonia detection with Nessler's reagent, arginine degradation (Table 3), and the activities of the ADI pathway enzymes (Table 2).

The leuconostocs in group II (Table 3) catabolized arginine only to a very limited extent, even though their enzyme activities were mostly similar to those of group I strains (Table 2). In some fermentations, arginine consumption was too small to be measured, although ammonia formation was still detected by the enzymatic method. In contrast, for *Lactobacillus brevis* ML30, which also produced very little ammonia from arginine, the activities of OTC and CK were much lower than those for leuconostoc strains and there was no detectable ADI activity. Although ammonia formation by the strains in group II was detected by the enzymatic method, it was not detectable by the less sensitive Nessler's reagent test.

The heterofermentative leuconostocs in group III (Table 3) lacked detectable levels of ADI pathway enzymes or contained very low levels of OTC activity and CK activity. Homofermenters do not possess any of these enzymes (Table 2). Therefore, the strains in group III would not be expected to degrade arginine. This agrees with the results for arginine consumption (Table 3). The very small positive arginine consumptions reported in some fermentations were probably not significant. Although arginine was not catabolized by group III strains, very small amounts of ammonia were consistently formed and detected by enzymatic assay in fermentation broths, except for fermentations with strain Viniflora LP. This ammonia may

TABLE 3. Ammonia formation from arginine by wine LAB grown in HFA broth

| Strain | Arginine consumed (mM) | Ammonia formed[a] | |
|---|---|---|---|
| | | Enzyme assay (mM) | Nessler's test |
| Group I | | | |
| *Leuconostoc oenos* (heterofermentative) | | | |
| OENO | 32.7 | 55.5 | + |
| 2008 | 23.7 | 38.2 | + |
| 2043 | 32.7 | 47.5 | + |
| 2035 | 32.7 | 49.5 | + |
| DSIR-C | 32.7 | 54.9 | + |
| Ey2d | 25.9 | 44.3 | + |
| *Lactobacillus* spp. (heterofermentative) | | | |
| CUC-3 | 32.7 | 56.2 | + |
| EQUILAIT | 32.7 | 56.6 | + |
| Microenos HP | 32.7 | 50.7 | + |
| 250 | 32.7 | 53.5 | + |
| Group II | | | |
| *Leuconostoc oenos* | | | |
| 122 | 1.5 | 1.7 | − |
| 252 | 2.3 | 1.4 | − |
| MCW | 1.8 | 1.7 | − |
| Er1a | 0.6 | 1.4 | − |
| Microenos B1 | 1.2 | 1.2 | − |
| Microenos B2 | 0 | 1.5 | − |
| 2001 | 0.9 | 1.5 | − |
| DSIR-A | 1.8 | 1.7 | − |
| DSIR-B | 0.7 | 1.5 | − |
| INOBACTER | 2.1 | 1.7 | − |
| *Lactobacillus* spp. (heterofermentative) | | | |
| ML30 | 0.9 | 1.8 | − |
| Group III | | | |
| *Leuconostoc oenos* | | | |
| ML34 | 0.3 | 0.6 | − |
| PSU-1 | 0 | 0.7 | − |
| L181 | 0 | 0.4 | − |
| Microenos B | 0 | 0.9 | − |
| Microenos B3 | 0 | 0.6 | − |
| *Lactobacillus* spp. (homofermentative) | | | |
| CUC-1 | 0.3 | 0.6 | − |
| Viniflora LP | 0.3 | 0 | − |
| 49 | 0 | 0.6 | − |
| *Pediococcus* spp. (homofermentative) | | | |
| 44.40 | 0.3 | 0.6 | − |
| 272 | 0.3 | 0.6 | − |
| 93 | 0.3 | 0.6 | − |
| C5 | 0.6 | 0.4 | − |
| CUC-4 | 0.6 | 0.6 | − |

[a] Initial arginine and ammonia, 32.7 and 4 mM, respectively. Values of ammonia formed have been corrected for the initial ammonia concentration in the medium. +, positive reaction; −, negative reaction.

TABLE 4. Correlation of citrulline excretion with arginine degradation by wine LAB cultured in HFA broth

| Strain | Arginine consumed[a] (mM) | Citrulline formed[b] (mM) |
|---|---|---|
| Group I | | |
| *Leuconostoc oenos* | | |
| OENO | 32.7 | 1.3 |
| 2008 | 32.7 | 1.9 |
| Ey2d | 32.7 | 2.2 |
| *Lactobacillus brevis* EQUILAIT | 32.7 | 2.6 |
| *Lactobacillus buchneri* CUC-3 | 32.7 | 0 |
| Group II | | |
| *Lactobacillus brevis* ML30 | 1.2 | 0 |
| Group III | | |
| *Leuconostoc oenos* | | |
| ML34 | 0 | 0 |
| PSU-1 | 0 | 0 |
| *Lactobacillus plantarum* Viniflora LP | 0 | 0 |

[a] Arginine concentration in uninoculated HFA broth was 32.7 mM.
[b] Citrulline was not detectable in uninoculated HFA broth.

TABLE 5. Ammonia formation from arginine degradation by *Leuconostoc oenos* strains cultured in HFA broth with and without the addition of fructose

| Strain | Ammonia formed[a] | | | |
|---|---|---|---|---|
| | − Fructose | | + Fructose | |
| | Nessler's test[b] | Enzyme assay (mM) | Nessler's test | Enzyme assay (mM) |
| Group I | | | | |
| OENO | + | 53 | + | 54 |
| Ey2d | + | 52 | + | 55 |
| 2035 | + | 54 | + | 60 |
| Group II | | | | |
| MCW | + | 42 | − | 1.4 |
| Er1a | + | 56 | − | 1.3 |
| 2001 | + | 60 | + | 38 |
| 122 | + | 55 | − | 1.1 |
| 252 | + | 48 | − | 1.2 |

[a] Based on two separate fermentations incubated at 30°C for 4 weeks.
[b] +, positive reaction; −, negative reaction.

come from other sources, such as deamination of amino acids other than arginine or citrulline.

**Correlation of citrulline formation with the occurrence of ADI pathway enzymes.** Citrulline concentrations were determined in samples of strains cultured in HFA broth with an amino acid analyzer because colorimetric analysis was precluded by color interference from the growth medium. The strains selected for analysis (Table 4) were representative of all three groups, classified on the basis of the ability to degrade arginine (Table 3) and the activities of ADI pathway enzymes (Table 2). Citrulline was excreted into broth by the heterofermentative strains OENO, 2008, Ey2d, and EQUILAIT from group I (Table 4). These strains also completely degraded arginine. However, citrulline was not detectable in broth inoculated with strain CUC-3, also from group I even though arginine was totally catabolized by this strain. (Citrulline has been shown to be excreted and reassimilated by this strain [24].) As expected, strains ML34, PSU-1, and Viniflora LP, which are representative of group III, did not degrade arginine or excrete citrulline since they lacked some or all of the enzymes of the ADI pathway (Table 2). Strain ML30 of group II degraded a small amount of arginine, but no detectable citrulline was found in broth.

**Ammonia formation from arginine in HFA broth with and without addition of fructose.** The finding that the *Leuconostoc* strains in group II (Table 3) degraded only a very small proportion of the arginine in media while the activities of ADI pathway enzymes were comparable to those of group I strains suggests that some factor(s) must be inhibiting ADI pathway enzyme activities in these strains. The enzyme activity data presented in Table 2 are for cultures grown in TJAG broth. However, the measurements of arginine degradation and ammonia formation are for cultures grown in HFA broth. A comparison of the compositions of these two broths shows that HFA broth contains 20 g of added fructose per liter, whereas fructose is not added to TJAG broth. Other components in the two media are similar. The presence of fructose may inhibit arginine degradation in some way. Therefore, an experiment was conducted to examine ammonia formation from arginine in HFA broth with and without the addition of fructose.

As shown in Table 5, large amounts of ammonia (i.e., cultured broth tested strongly positive with Nessler's reagent) were formed from arginine by both group I and group II strains when fructose was omitted. However, ammonia formation from arginine by group II strains was considerably lower when fructose (20 g/liter) was present. These results clearly demonstrate that the presence of fructose does indeed inhibit the formation of ammonia from arginine. The response of *Leuconostoc oenos* 2001 was inconsistent since it produced little ammonia in the original comparative study (Table 3) but formed relatively large amounts of ammonia in the subsequent experiment (Table 5).

## DISCUSSION

This study has investigated the occurrence of ADI pathway enzymes in homofermentative lactobacilli and pediococci and heterofermentative lactobacilli and leuconostocs isolated from the wine environment. The ADI pathway enzymes, ADI, OTC, and CK, were active in the majority of leuconostoc strains and heterofermentative lactobacilli tested, but no activity was detected in the homofermentative lactobacilli or pediococci examined.

The ability of the members of the genus *Leuconostoc* to hydrolyze arginine has been a matter of controversy. In *Bergey's Manual of Systematic Bacteriology*, Garvie (12) states that the species of genus *Leuconostoc* do not hydrolyze arginine. However, several reports have demonstrated the degradation of arginine by strains of *Leuconostoc oenos* (13, 20, 22, 32, 43), although other strains, such as ML34, are unable to hydrolyze arginine (33). The hydrolysis of arginine is usually demonstrated by detecting the production of ammonia with Nessler's reagent (11). However, this test has been shown to be relatively insensitive (32), and this may partially explain the discrepancies in the reported ability of leuconostocs to hydrolyze arginine. It is also clear from this survey that *Leuconostoc oenos* strains differ with respect to the activities of ADI pathway enzymes. Strains capable of hydrolyzing arginine and producing significant levels of ammonia detectable by Nessler's reagent have been shown to have high activities of ADI pathway enzymes (although the converse is not necessarily true, as in the case of group II strains). On the other hand, other strains (group III [Table 3]) lack detectable activity for all or some of the ADI pathway enzymes and do not significantly degrade arginine or produce ammonia.

The composition of the growth medium is another important factor to take into consideration in screening strains for the

ability to degrade arginine. HFA broth, used in this survey and in earlier studies (32), is not the ideal medium since it clearly contains some component that suppresses the ability of certain strains to degrade arginine even though these strains have high activities of ADI pathway enzymes. The omission of fructose from HFA broth resulted in significant production of ammonia from arginine by these strains, suggesting that fructose may be the particular component responsible. The difference between group I and group II strains of *Leuconostoc oenos* may lie in the rate of fructose metabolism. It is possible that group I strains may metabolize fructose at a faster rate than group II strains; therefore, suppression of arginine catabolism by fructose is relieved earlier. The mechanism whereby fructose prevents arginine catabolism merits further investigation. Fructose has been shown to reduce the activities of ADI pathway enzymes in *Streptococcus mitis* (16).

The absence of detectable activity of ADI pathway enzymes in the homofermentative wine lactobacilli and pediococci examined is consistent with previous reports of the inability of some homofermentative wine LAB to catabolize arginine (20, 22, 32). In contrast, arginine degradation in homofermentative LAB from other sources is well documented (5, 9, 10, 19, 28).

The findings of this research are important and applicable to the wine industry. The wine LAB strains examined degraded arginine and formed ammonia not only at 30°C, the temperature used in this study, but also at 18°C (data not presented), a common wine storage temperature. Citrulline, a known precursor of the carcinogenic compound ethyl carbamate (31), may be excreted during arginine degradation, as shown for several strains of wine LAB in this study (Table 4). A previous study (24) demonstrated a correlation between citrulline excretion and ethyl carbamate formation in wine. The screening of wine LAB for the ability to degrade arginine is therefore of paramount importance in selecting LAB starter cultures to conduct malolactic fermentation in wine in view of the potential for formation of the ethyl carbamate precursor citrulline. From the standpoint of citrulline formation, strains in group III should be safe to use for conducting malolactic fermentation in wine since they do not degrade arginine and, therefore, are not expected to excrete citrulline.

The traditional Nessler's reagent test for ammonia is simple and quick to perform. The good correlation between ammonia formation from arginine and the occurrence of ADI pathway enzymes suggests that this test is useful in the screening of potential wine LAB strains for use as starter cultures to conduct malolactic fermentation in wine. Heterofermentative wine LAB strains that produce a positive reaction with Nessler's reagent after growth in HFA broth (no added fructose) should be avoided since they possess the ADI pathway enzymes, degrade arginine, and have the potential to excrete the ethyl carbamate precursor citrulline. Heterofermentative wine LAB strains that produce a negative reaction probably require further testing by a more sensitive method such as enzymatic assay as some of these strains may degrade less arginine and thus produce less ammonia.

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