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# Selection and Breeding of Lysine-accumulating *Saccharomyces cerevisiae* as a Stable Source of Lysine in the Rumen

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**The possibility of using lysine-accumulating yeast cells as a rumen-stable source of lysine for ruminants was investigated. The *Saccharomyces cerevisiae* strain AJ14599 accomulated free lysine amounting to 15% of the dry weight of the cells when cultured in a medium with the lysine precursor, L-α-aminoadipate (AAA). A mutant, LA-1, which was induced from AJ14599 and resistant to S-(β-aminoethyl)-cysteine (AEC), accumulated 4% free lysine in AAA-free medium. In both LA-1 and AJ14599 cells, more than 90% of the free lysine was in vacuoles. In an *in vitro* evaluation, the intracellular lysine was stably maintained and protected from microbial degradation during incubation in intact rumen juice, but it was immediately and completely released in a digestive enzyme (pepsin) solution. Lysine in LA-1 cells was also nutritionally available for weanling rats. Thus, lysine-accumulating yeast cells were effective for use as a rumen-stable source of lysine.**



L-Lysine is the most limiting amino acid in cereal grains and widely used as a supplement for formula feed for swine and chickens. However, little is known of the nutritional availability of amino acids in ruminants because physiological quantities of amino acids are rapidly degraded in the rumen by microorganisms.[1] Mixtures of amino acids, casein, and other proteins increase milk yield and/or milk protein production in dairy cows when they are infused postruminally.[2–4] These investigations have indicated that methionine and lysine are the most limiting amino acids in milk production. These infusion methods were useful for experimental purposes but they were not practical for routine feeding of protein or amino acids to the ruminants. Because of the commercial potential for providing amino acids to ruminants, the development of rumen-stable amino acids is needed. Such rumen-stable amino acids should be protected from degradation in the rumen but absorbed rapidly and nutritionally available in the postruminal digestive organs. Rumen-stable forms of methionine and lysine have been developed as their bioequivalent analogs or encapsulated products.[5–7] The most commonly used analogs or derivatives are methionine hydroxy analog and dihydroxymethyl-L-lysine calcium. The capsules, which consisted of an amino acid core coated with pH-sensitive synthetic polymer, were effective in delivering amino acids to postruminal absorption sites.[7,8] The pH-sensitive polymeric coatings were designed to resist the degradation at the pH in the rumen, but to break down in the lowr pH environment of the abomasum, and there release the amino acids for absorption.

Yeast cells have been widely used for many years as feed supplements for domestic animals. Recent studies have indicated that the use of yeast products improve feed intake, milk production and milk composition in dairy ruminants such as cattle and goats.[9] Because L-lysine as well as L-arginine is accumulated inside the vacuoles of yeast cells in large amounts,[10–12] it is expected that yeast cells might offer a non-chemical means of delivering lysine. We studied the possibility of using lysine-accumulating yeast cells as a rumen-stable source of lysine. This paper describes the screening and breeding of strains of the yeast *S. cerevisiae* that accumulate large amounts of lysine and their evaluation as a rumen-stable source of lysine.

## Materials and Methods

*Yeast strains.* The *S. cerevisiae* strains of our stock cultures were used.

*Culture media.* Nutrient (N), synthetic (S), and cell production (CP) media were used as routine basic media. The N medium was composed of 4% glucose, 1% Polypepton (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.5% yeast extracts (Difco Laboratories, Detroit, Mich., U.S.A.), 0.5% $KH_2PO_4$, and 0.2% $MgSO_4 \cdot 7H_2O$. The S medium was composed of 2% glucose and 0.67% Bacto-yeast nitrogen base (Difco Laboratories). The Bacto-yeast nitrogen base was added aseptically to the autoclaved glucose solution by preparing a 6.7% stock solution of Bacto-yeast nitrogen base. The CP medium was composed of 5% glucose, 0.38% $(NH_4)_2SO_4$, 0.1% $KH_2PO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, and 500 mg/liter (total-nitrogen) of corn steep liquor. The medium pH was adjusted to 5.3 with KOH. Solid media were prepared by adding 2% agar to these media.

*Rumen juice.* Rumen fluid was taken from a 500-kg rumen-fistulated Holstein cow, which had been fed on a concentrate and rice straw or hay, before the morning feeding. The rumen fluid was strained through three layers of gauze. The effluent was kept anaerobically in a thermos bottle and used for experiments within two hours.

*Analysis of intracellular free amino acids.* Yeast cell extracts were prepared by resuspending the cells in distilled water in the original volume of cultures, following heating for 15 min at 100°C. This suspension was centrifuged, and the supernatant was used as the sample for the analysis of intracellular free lysine and other free amino acids. Amino acids were analyzed with a Hitachi 835 amino acid analyzer.

*In vitro evaluation of lysine-accumulating yeast cells as a rumen-stable lysine source.*

*(1) Cell preparation.* Freshly grown cells on a N agar slant were inoculated into 20 ml of CP medium with or without AAA (L-α-

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aminoadipate) in a 500-ml Erlenmeyer flask. After two days of shaking at 30°C, the cells were harvested by centrifugation and washed twice with 0.05 M phosphate buffer (pH 6.0).

(2) *Stability of intracellular free lysine in McDougall's buffer.* McDougall's buffer[13] ($NaHCO_3$, 7.43 g/liter; $Na_2HPO_4 \cdot 12H_2O$, 7 g/liter; NaCl, 0.34 g/liter; KCl, 0.43 g/liter; $MgCl_2 \cdot 6H_2O$, 0.1 g/liter; and $CaCl_2$, 0.05 g/liter) was used in the initial evaluation as a convenient substitute of natural rumen juice. The washed cells containing about 200 mg of free lysine were suspended in 100 ml of the buffer and incubated for 24 h at 39°C. During incubation, gaseous carbon dioxide was bubbled into the buffer to adjust the pH and maintain anaerobic conditions. Before and after incubation, intracellular and extracellular free lysines were assayed to estimate the stability of lysine in the cells and its excretion into the buffer. Authentic free lysine was also tested as a control.

(3) *Stability of intracellular free lysine in natural rumen juice.* Fifty ml of McDougall's buffer and an equal volume of intact rumen juice were mixed and used for an *in vitro* evaluation of the resistance of intracellular lysine to microbial degradation. These experiments were done as described above.

(4) *Digestion of yeast cells in pepsin solution.* A pepsin solution was used to simulate digestive juice for an *in vitro* evaluation of the release of intracellular free lysine in postruminal digestive organs. Washed cells containing about 200 mg free lysine were suspended in 100 ml of 0.075 N HCl containing 0.2% pepsin, and incubated for 16 h at 39°C with shaking. After incubation, the solution was passed through a 0.2-$\mu$m filter and free lysine in the filtrate was assayed to measure the amount of lysine released.

*In vivo evaluation of availability of lysine in yeast cells.* Cells of an AEC-resistant mutant, LA-1, were tested as a nutritional source of lysine for weanling rats. Three diets were prepared as follows. An L-lysine-limiting basal diet was composed of 30% sucrose, 12% wheat gluten, 8.14% amino acids mixture,[14] 5% corn oil, 4% cellulose powder, 4% mineral mixture,[15] 1% vitamin mixture,[15] 0.2% choline chloride, 0.01% vitamin E, and 40.65% corn starch. This basal diet was estimated to contain 0.3% L-lysine by calculation. For other two diets, L-lysine·HCl and LA-1 cells that contained 4% free lysine (8% total lysine) were added to the above basal diet at levels calculated to result in 0.4% total lysine content. Five weanling male SD rats (5 weeks of age) were assigned to each of the three diets for eight days' growth assay.

*Induction and isolation of AEC-resistant mutant. S. cerevisiae* AJ14599 cells grown in the S medium were washed twice with 0.05 M phosphate buffer and diluted to about $10^8$ cells per ml, of which one ml was spread on S agar plates containing 0.1 to 0.5% AEC. UV irradiated the AEC-plates for 40 s onto AEC-plates at a distance of 35 cm from a 15-W germicidal lamp ($\lambda = 253$ nm). After incubation for four days at 30°C, large colonies were isolated as AEC-resistant strains.

*Extraction of cytosolic and vacuolar amino acid pools.* The procedures of Wiemken and Nurse[12] were used.

## Results

### *Selection of a parent strain and its properties*

Stock cultures of *Saccharomyces cerevisiae* were grown in the CP medium with the addition of 0.5% L-α-aminoadipate (AAA), a biosynthetic precursor of lysine in yeast, and intracellular and extracellular free lysine were assayed. Seven strains were selected as primary candidates because of their high conversion yields from AAA to lysine and their higher intracellular accumulation of lysine (Table I).

All of these strains produced lysine from AAA with the conversion yield of 40 to 65%, and 60 to 80% of the lysine produced was accumulated inside the cells. Free lysine amounted 10 to 15% of dry cell weight. On the other hand, no lysine production was detected for any of the strains cultured in AAA-free medium. AJ14599 was selected for further studies because this strain showed (1) the highest conversion yield to lysine, (2) the highest intracellular lysine accumulation, and (3) lower extracellular excretion of lysine.

**Table I.** Conversion of L-α-Aminoadipic Acid (AAA) to Lysine by Strains of *S. cerevisiae* and Their Ability to Retain the Lysine in Cells

Each strain was cultured in CP medium with 0.5% AAA. Intracellular free lysine was assayed as described in Materials and Methods.

| Strain | Growth (mg dry cell/ml) | Intracellular free lysine (mg/ml) | Extracellular free lysine (mg/ml) | Lysine/dry cell (%) | Yield of lysine[a] (%) |
|---|---|---|---|---|---|
| AJ14599 | 18.9 | 2.9 | 0.9 | 15.3 | 62 |
| AJ14598 | 19.7 | 2.8 | 0.8 | 14.2 | 59 |
| AJ14577 | 15.4 | 2.2 | 1.0 | 14.3 | 54 |
| AJ14600 | 17.3 | 2.2 | 1.0 | 12.7 | 54 |
| AJ14464 | 17.5 | 1.9 | 1.0 | 10.9 | 46 |
| AJ14545 | 14.5 | 1.6 | 1.1 | 11.0 | 44 |
| AJ14581 | 15.9 | 1.6 | 0.9 | 10.1 | 40 |

[a] Lysine produced/AAA.

**Table II.** Intracellular Distribution of Free Lysine in *S. cerevisiae* Strains AJ14599 and LA-1

AJ14599 and LA-1 were cultured in AAA-free and AAA-supplemented CP media. Cytosolic and vacuolar pools were extracted by the method of Wiemken and Nurse.[12]

| Strain | Addition of AAA to the medium (%) | Free L-lysine ($\mu$g/mg dry cell weight) | |
|---|---|---|---|
| | | Cytosolic extracts | Vacuolar extracts |
| AJ14599 | 0.0 | <0.2[a] | <0.2[a] |
| | 0.5 | 6.8 | 70.4 |
| LA-1 | 0.0 | 2.0 | 22.0 |
| | 0.5 | 2.5 | 39.2 |

[a] Not detected.

### *AEC-resistant mutants and their properties*

S-(β-aminoethyl)-cysteine (AEC)-resistant mutants were induced from AJ14599 to obtain lysine-accumulating strains in AAA-free medium. Of 190 AEC-resistant mutants isolated, 48 were lysine producers that accumulated lysine intracellularly without excretion. The mutant that showed the highest accumulation, LA-1, contained 4% free lysine and 8% total lysine (wt/dry cell wt), even when cultured in AAA-free CP medium. When LA-1 was cultured in the CP medium, the amount of free lysine rose to 6.5% of dry cell weight in the logarithmic phase, but then decreased to 4% by the starting period of the stationary phase. However, no further decrease occurred during the following 24 h. Free lysine accumulation was 7% of dry cell weight when the cells were cultured in the medium with AAA. This value was smaller than that of the parent strain, AJ14599 (15%).

### *Distribution of lysine pool in the cells*

The cellular distribution of the accumulated free lysine was investigated when AJ14599 and LA-1 were cultured in the CP medium with and without the addition of AAA. Cells were harvested after 48 h of cultivation, and cytosolic pools and vacuolar pools were extracted with the cytochrome treatment and the subsequent osmotic shock treatment, respectively. In both medium with and without the addition of AAA, for both strains, more than 90% of free lysine was found in vacuoles (Table II). Other basic amino acids such as arginine and glutathione were not detected in the vacuoles.

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**Table III.** The Effects of McDougall's Buffer, Rumen Juice and Pepsin Solution on the Retention of Intracellular Free Lysine by Two Strains of Lysine-accumulating Yeast, AJ14599 and LA-1

AJ14599 and LA-1 cells were cultured in CP medium in the presence of 0.5% AAA and in the absence of AAA, respectively. An *in vitro* evaluation for a rumen-stable lysine was performed as described in Materials and Methods.

| Strain | Incubation | | Free lysine (mg/ml) | | | |
|---|---|---|---|---|---|---|
| | | | McDougall's buffer | Rumen juice | | Pepsin solution |
| | | | Yeast cells | Yeast cells | Authentic free lysine | Yeast cells |
| AJ14599 (0.5% AAA) | Before | Intracellular | 211 | 211 | — | 211 |
| | | Extracellular | 0 | 0 | 202 | 0 |
| | After | Intracellular | 194 | 198 | — | 0 |
| | | Extracellular | 2 | 4 | 126 | 213 |
| LA-1 (AAA free) | Before | Intracellular | 150 | 150 | — | 150 |
| | | Extracellular | 0 | 0 | 203 | 0 |
| | After | Intracellular | 159 | 145 | — | 0 |
| | | Extracellular | 3 | 4 | 135 | 160 |

*Evaluation of lysine-accumulating AJ14599 and LA-1 cells as a rumen-stable lysine*

Table III shows the results of an *in vitro* evaluation of AJ14599 and LA-1 cells, which were cultured in the CP medium with and without AAA, respectively, as a rumen-stable lysine. In both cell types, neither significant leakage nor auto-digestion of intracellular free lysine was observed. Furthermore, most of the lysine was retained in the cells during incubation in McDougall's buffer. A similar result was observed during the incubation in rumen juice. However, authentic free lysine added to the rumen juice decreased by 60%. In contrast to its stability in the rumen juice, 100% of intracellular free lysine was released when incubated in a pepsin solution. These results show that lysine-accumulating yeast cells have the properties necessary for a rumen-stable source of lysine.

Table IV shows an *in vivo* evaluation of LA-1 cells as lysine source. The total lysine in the yeast cells was found to be as effective as L-lysine·HCl in meeting the lysine requirement of rats, as shown by nearly equivalent values in terms of gain in body weight, food intake, and supplemental lysine intake. This result suggests that lysine in yeast cells might be nutritionally effective in the digestive organs of ruminants.

**Table IV.** Body Weight Gain, Food Intake, and Supplemental Lysine Intake in Rats Fed Diets Containing LA-1 Cells

LA-1 cells cultured in CP medium were harvested, washed with 0.05 M phosphate buffer (pH 6.0) and lyophilized. The experiment for an *in vivo* evaluation of availability of lysine in yeast cells was performed as described in Materials and Methods.

| Diet | Total lysine content (%) | Food intake[a] (g/day) | Supplemental lysine[a] intake (mg/day) | Weight gain[a] (g/day) |
|---|---|---|---|---|
| Basal (B) | 0.30 | 17.4±1.2 | — | 3.0±0.5 |
| (B)+L-lysine·HCl | 0.40 | 20.9±1.8 | 20.9±1.8 | 6.2±1.0 |
| (B)+LA-1 | 0.40 | 21.3±2.0 | 19.6±1.8 | 5.9±1.1 |

[a] $n=5$.

## Discussion

The results in this study showed the possibility that lysine-accumulating yeast cells are effective for use as a rumenstable source of lysine and offered a non-chemical means of delivering lysine to ruminants.

The α-aminoadipate pathway for the biosynthesis of lysine in *S. cerevisiae* is regulated by several different mechanisms.[16,17] Homocitrate synthase (EC 4.1.3.21), which catalyzes the first reaction of the pathway, is feedback-inhibited by lysine,[18] and saccharopine dehydrogenase (lysine-forming; EC 1.5.1.7), which catalyzes the final biosynthetic reaction, is inhibited by lysine and α-ketoglutarate.[19] Homocitrate synthase and saccharopine dehydrogenase (glutamate-forming; EC 1.5.1.10) are repressed by lysine.[20] The metabolic flux through the pathway is also regulated by compartmentalization of lysine in the vacuoles, where excess lysine in the cytoplasm is sequestered.[21]

*S. cerevisiae* AJ14599 accumulated a large amount of lysine (15% of dry cell weight) when cultured in the presence of the lysine precursor AAA, and more than 90% of the lysine was located in vacuoles, but lysine accumulation was not observed when the cells were cultured in the absence of AAA. These results suggest that, in this strain, the first part of the pathway before the α-aminoadipate step is rate-limiting and is under strong regulatory control in lysine biosynthesis. On the other hand, the compartmentalization of lysine in vacuoles, which decreases cytosolic lysine concentrations, seems to release the regulatory controls to some extent and to enhance the accmulation of a large amount of lysine in cells. This also suggests that the selection of strains having greater vacuole capacities could lead to the construction of a higher lysine-accumulating yeast.

AEC-resistant mutants of yeasts are known to accumulate lysine. One of such mutants, derived from *Candida pelliculosa*, overproduced lysine.[22] Because homocitrate synthase in this mutant was not inhibited by either lysine or AEC, it was speculated that the overproduction of lysine was due to the release of the feedback inhibition of this

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enzyme by lysine. This mutant accumulated lysine up to about 3.0% of dry cell weight during the logarithmic phase. However, during the stationary phase, the lysine was rapidly excreted into the medium, resulting in intracellular concentrations that were only about one-tenth of those in the log phase. Haidaris and Bhattacharjee derived AEC-resistant mutants from *S. cerevisiae*, one of which produced an extracellular lysine concentration of about 50 mg per liter of medium, but the intracellular lysine pool decreased during cultivation.[23,24]

In contrast to the above mutants, our mutant, LA-1, stably maintained its lysine pool during a long period of cultivation. As a preliminary experiment, we induced some AEC-resistant mutants from a variety of yeast species to measure their ability to accumulate lysine. Several mutants derived from *Candida pelliculosa*, *Kluyveromyces polysporus* and *Hansenula anomala* accumulated lysine, but most excreted lysine into the medium. Therefore, *S. cerevisiae*, in comparison with other yeast species, appears to have the ability to accumulate lysine in cells without excretion, and thus to be suitable for a rumen-stable lysine reservoir. Although the mechanism of overproduction of lysine by an AEC-resistant mutant, LA-1, has not been investigated, it is possible that homocitrate synthase in this strain has been released from feedback inhibition or repression by lysine. Strain LA-1 overproduced free lysine to some extent, but lysine accumulation by LA-1 in AAA-free medium (4%) was still low in comparison with the accumulation by the parent strain AJ14599 in medium with AAA (15%). This suggests that significant regulatory controls in lysine biosynthesis still remain in LA-1. On the other hand, free lysine accumulation of LA-1 (7%) was smaller than that of the parent strain AJ14599 (15%) when the cells were cultured in medium supplemented with AAA. The mechanism of lysine overproduction and regulatory controls of lysine biosynthesis in LA-1 remain to be investigated.

Although this study showed that lysine-accumulating yeast cells had the required properties for acting as a rumen-stable source of lysine, further studies will be needed to evaluate their effects on animal performance.

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