Isolation and Characterization of a Mutant of *Candida lipolytica* Which Excretes Long-chain Fatty Acids

Tokichi MIYAKAWA, Hiroshi NAKAJIMA, Kaoru HAMADA,
Eiko TSUCHIYA, Tatsuyuki KAMIRYO*
and Sakuzo FUKUI

Faculty of Engineering, Hiroshima University,
Saijo, Higashi-Hiroshima 724, Japan

*Faculty of Integrated Arts and Sciences, Hiroshima University,
Higashi-Senda, Hiroshima 730, Japan

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We isolated a mutant strain of *Candida lipolytica* which produces long-chain fatty acids in the culture medium. The procedure for the isolation of the mutant consisted of two steps: the first was the screening of strains which exhibited a low cellular density by Percoll density gradient centrifugation, and the second was the screening of strains which were capable of supporting the growth of fatty acid auxotrophic cells layered on the mutant colonies. One of the mutant strains produced more than 1 mg of fatty acids per ml of the medium under optimal conditions. The excreted fatty acids were mainly palmitic and stearic acids in the nonesterified form.

The production of long-chain fatty acids by microorganisms would be greatly facilitated by the use of mutant strains which produce fatty acids in the culture medium. From this point of view, we attempted to isolate such a mutant strain of *Candida lipolytica*. The parental strain used was defective in acyl-CoA synthetase I¹⁾ and incapable of degrading fatty acids via β -oxidation. These two defects caused a high ratio of nonesterified fatty acids to the total fatty acids (more than 65%). The procedure for the isolation of the mutant consisted of two steps: the first was the screening of strains which exhibited a low cellular density by Percoll density gradient centrifugation, and the second was the isolation of mutants which were capable of supporting the growth of a fatty acid auxotrophic yeast strain layered on the mutant colonies. In the present paper, we describe the details of the procedure and the characteristics of strain S-1, one of the mutants thus obtained.

MATERIALS AND METHODS

Yeast strains and growth conditions. Candida lipolytica

LB-742,²⁾ a double mutant strain defective in acyl-CoA synthetase I and in β -oxidation, was used as a parental strain to isolate mutants capable of producing long-chain fatty acids in the culture medium. A fatty acid auxotrophic strain A-1, a derivative of *C. lipolytica* NRRL Y-6795, was isolated by mutagenesis with ethyl methanesulfonate followed by a replica plating method. Cells were grown by reciprocal shaking at 28°C in 1.8 × 18 cm test tubes with 5 ml of YPG medium which contains 0.4% yeast extract, 0.5% polypeptone, 2% glucose, 0.1% KH_2PO_4 , 0.005% MgSO_4 and 50 $\mu\text{g/ml}$ of chloramphenicol. To grow strain A-1, 0.15% myristic acid, 0.15% oleic acid and 1% Brij 58 were added to YPG medium. YPG-agar plates contained 2% agar in addition to YPG medium.

Isolation of mutants. In all the experiments, cells were mutagenized by UV irradiation as described previously³⁾ at survival rates of 10^{-3} to 10^{-4} . The irradiated cells were grown for 24 hr for segregation of mutations.

Isolation of mutants with low cellular density. The mutagenized cells of LB-742 were suspended in YPG medium at a concentration of 10^9 cells/ml and an aliquot of 1 ml was applied on top of a discontinuous Percoll density gradient consisting of 0.8 ml each of 60, 50, 40, 30 and 20% (v/v) of Percoll diluted with 0.25 M sucrose containing 5 mM EDTA. After 2.5 hr of centrifugation at $4600 \times g$, cells located between the surface of the gradient and the major band ($\rho = 1.128 \text{ g/ml}$) were collected. The cells, washed with YPG medium, were suspended in 5 ml of YPG medium and then grown for two days. After repeating three cycles of the above procedure, the cells in the major

band fraction ($\rho = 1.120$ g/ml) were plated on a YPG agar plate.

Isolation of mutants which excrete long-chain fatty acids. Strain N-1 was one of the low-density mutants obtained. The mutagenized cells of N-1 were plated on YPG agar plates to yield about 100 colonies per plate. After 5 days of incubation at 28°C, the plates were exposed to UV light (15 W) for 15 min at a distance of 30 cm and then 5 ml of YPG medium containing 0.75% agar, 0.5% Brij 58 and 10^5 cells of strain A-1 was poured gently on the surface of the plates. The UV-treatment was necessary to prevent smears caused by the cells tailing from the colonies. After 2 days of incubation, surviving cells in the colonies which support the growth of the assay cell (strain A-1) were picked up with toothpicks and purified from contamination by the assay cells by single-colony isolation on YPG agar plates.

Biological assay of nonesterified fatty acids. The amount of long-chain fatty acids was determined by a bioassay using a fatty acid auxotroph (A-1). The washed cells of A-1 were suspended in YPG medium at a concentration of 10^6 cells/ml. Aliquots (0.1 ml) of the suspension were added to 5 ml of YPG medium which contains fatty acids and were incubated at 28°C for 24 hr with shaking until the growth reached a plateau. Cell densities were determined by absorbance at 660 nm after an appropriate dilution with YPG medium. The amount of fatty acids in unknown samples were calculated from a standard curve made with palmitic acid. Thus, values of fatty acids are all expressed as palmitic acid-equivalent amounts.

Analytical methods. Cellular lipids were extracted as described.⁴⁾ Fatty acid species were determined by high performance liquid chromatography. Conditions for the chromatography were as follows: a column of ZOBAX-ODC, a solvent system of methanol-*ortho*-phosphoric acid (80%)-H₂O (60 : 0.2 : 39.8, v/v/v), a detector of Shodex RI SE-31, a flow rate of 0.6 ml/min, and room temperature. Protein was determined by the method of Lowry *et al.*⁵⁾ with bovine serum albumin as a standard. Density of Percoll was determined gravimetrically.

RESULTS

Isolation of mutants with an increased level of intracellular long-chain fatty acids

A double mutant strain of *C. lipolytica*, LB-742, was used as a parental strain for the isolation of the mutant, since the ratio of nonesterified fatty acids to the total fatty acids was high in this strain. To increase the intracellular level of fatty acids, we screened mutant strains with lower densities than the parental strain by Percoll density gradient

FIG. 1. Sedimentation of a Low-density Mutant (N-1) and the Parental Strain (LB-742) by Percoll Density Gradient Centrifugation.

Cells grown to the late logarithmic phase (3×10^8 cells/ml) were collected and resuspended at a concentration of 3×10^9 cells/ml in YPG medium. Aliquots (0.3 ml) of the cell suspension were layered on top of the Percoll gradient (see MATERIALS AND METHODS) and centrifuged at $4600 \times g$ for 2.5 hr.

centrifugation. The defect in β -oxidation in the strain LB-742 is expected to prevent degradation of fatty acids. After repeating three cycles of the enrichment procedure, the major band in the Percoll density gradient shifted from $\rho = 1.128$ g/ml to 1.120 g/ml. When analyzed by Percoll density gradient, four (N-1, -2, -3, and -4) out of the five strains obtained, were located at $\rho = 1.120$ g/ml. Figure 1 shows locations of a low density mutant (N-1) and the parental strain (LB-742) in Percoll density gradient.

The fatty acid contents of the mutant and parental strain were determined by the biological assay method (Table I). As expected, the low-density mutant strain contained free fatty acids at a significantly high level, corresponding to about 1.6 times of the parental strain. This mutant was used for the subsequent isolation of fatty-acid-excreting mutants.

Isolation of fatty-acid-excreting mutants

For the isolation of mutants which produce

TABLE I. FATTY ACID CONTENTS OF *C. lipolytica* STRAINS

The amounts of fatty acids were determined by the biological assay described in MATERIALS AND METHODS. Except for strain S-1, the values of free fatty acids and total fatty acids are the amounts of intracellular fatty acids before and after saponification of the samples, respectively. The values for strain S-1 were obtained similarly except that fatty acids produced in the culture filtrate of the equivalent number of cells (10^{10}) were determined.

Strains ^a	Free fatty acids (A) (mg/ 10^{10} cells)	Total fatty acids (B) (mg/ 10^{10} cells)	(A)/(B) (%)
LB-742	8.1	12.1	67
N-1	14.4	18.0	80
S-1	38.0	38.0	100

^a Strains LB-742, N-1 and S-1 are a series of mutants obtained by sequential mutageneses in this order, corresponding to the parental strain, the low-density strain and the strain which excretes long-chain fatty acid, respectively.

FIG. 2. A Mutant Which Excretes Long-chain Fatty Acids Detected by Fatty Acid Auxotrophic Cells as an Indicator.

Colonies of a fatty acid excreting mutant (S-1), a low-density mutant (N-1), and the parental strain (LB-742), on a YPG-agar plate were treated with soft agar containing the indicator cells as described in the text.

fatty acids in the medium, we needed a convenient screening method for the mutants. For this purpose, we designed a method to detect fatty acid excreted into the medium by the use of a strain auxotrophic for fatty acids, expecting that the mutant colonies would support the growth of the indicator cells layered with soft agar onto the colonies. From about 10,000 colonies of mutagenized N-1 strain screened, a mutant strain (S-1) with the desired phenotype was obtained (Fig. 2).

FIG. 3. Excretion of Long-chain Fatty Acids in Liquid Culture by a Fatty-acid-excreting Mutant, S-1.

Fatty acids excreted into the media by S-1 cells grown in the presence (●) or absence (○) of 1% Brij 58 were determined. Also shown in the figure are cell growth (▲) determined by counting total cell number in a hemocytometer, and glucose consumption (■) of the culture of S-1 strain in the presence of Brij 58.

Production of long-chain fatty acids in liquid culture

As shown in Fig. 3, the mutant S-1 produced a significant amount (about 1 mg/ml) of fatty acids in the liquid culture medium. The presence of a detergent such as Brij 58 (0.5%, v/v) in the culture medium was essential for fatty acid excretion (Fig. 3). No detectable amount

FIG. 4. Precipitation of Excreted Fatty Acids at Low Temperature.

Liquid cultures of strain S-1 and N-1, grown in YPG medium containing 1% Brij 58 for 5 days, were kept overnight at 4°C. The fresh medium was also treated as above (Cont.).

FIG. 5. The Effect of Addition of Glucose on Fatty Acid Production.

Cells of the strain S-1 were cultivated with (●▲) or without (○△) addition of 10 mg of glucose per ml of the medium at the time indicated by the arrow. Other conditions were as described in Fig. 3.

of fatty acids was excreted by N-1 under these conditions. The excretion started in the late logarithmic phase of growth and increased until glucose was exhausted, which was fol-

lowed by a rapid decrease of the amount of fatty acids in the medium. Since a β -oxidation-defective mutant was used for the parental strain, the decrease of fatty acid must be due to some other mechanism.

Fatty acids accumulated in the five-day culture of S-1 precipitated out from the medium when the culture was chilled to 4°C (Fig. 4). The composition was palmitic acid–stearic acid–oleic acid, 10 : 5 : 1, by weight. The composition was similar to that of the intracellular free fatty acids of N-1. The amount of fatty acids in the excreted lipid determined by the bioassay method was unchanged in the samples with or without saponification (Table I). Since the assay method detects only free fatty acids, the excreted fatty acids are suggested to be all in the nonesterified form.

As can be seen in Fig. 5, when glucose was added to the culture of N-1 prior to exhaustion of the initially added glucose, more fatty acids were produced, and again there was a later decrease.

DISCUSSION

Production of long-chain fatty acids by yeasts is a convenient means to convert sugar to compounds with higher chemical energy. Compared with “intracellular production,” “extracellular production” of long-chain fatty acids would facilitate recovery of the product from the culture and might also allow an increase in the amount produced. For these reasons, we developed procedures to isolate mutant strains of *C. lipolytica* that excrete long-chain fatty acids.

Unlike most other yeasts,⁶⁾ the ratio of free fatty acids to total fatty acids was high in *C. lipolytica* (Table I). For instance, yeasts such as *Saccharomyces cerevisiae* contained no detectable amount of free fatty acids by our assay method. To prevent degradation of fatty acids, a β -oxidation-defective strain (LB-742) was chosen for the parental strain. This mutant cell contained about 1.3 times the amount of long-chain free fatty acids of its parental strain, L-7.

To obtain a mutant with an increased level of fatty acids, we first isolated low-density mutants. Enrichment of low-density cells by Percoll density gradient centrifugation was effective for the isolation of the mutants. A mutant strain isolated (N-1) had a density of 1.120 mg/ml in contrast to 1.128 mg/ml of the parental strain, and contained free fatty acids at a level about 1.8 times of the parental strain (LB-742) (Table I). There were no distinguishable differences in cell size or in the amount of cellular proteins between the two strains.

The screening procedure for fatty-acid-excreting mutants using a strain auxotrophic for long-chain fatty acids as an indicator proved to be a convenient and efficient method. The growth of the fatty acid auxotrophic strain was restored by the addition of long-chain fatty acids with chain lengths between C_{14} and C_{18} , but shorter chain fatty acids ($<C_{12}$) or esters of long-chain fatty acids were not effective (data not shown). Thus, the assay is specific to unesterified, long-chain fatty acids.

The maximum amount of fatty acids excreted by the strain S-1 is about 2.6 times the amount of intracellular free fatty acids of the parental strain, N-1 (Table I). Although viability of the S-1 cells decreased to one-tenth of the total cells after five days of culture, excretion of fatty acids still increased in response to glucose added to the culture (Fig. 5). There were no indication of destruction of the general surface barrier of cells grown for five days, as examined by the increase in OD_{280} of the nondialyzable fraction of the culture medium. These results indicate that excretion of fatty acids is not due to simple leakage of an intracellular fatty acid deposits by the non-

viable cells through disrupted membranes, and that even the nonviable cells continue fatty acid production, consuming glucose.

The role of the detergent, Brij 58, for the excretion of fatty acids is obscure. Permeation of cellular fatty acids through the membrane may be promoted by the detergent specifically in the S-1 cells. However, no significant amount of fatty acids leaked from S-1 cells by incubation in buffer containing 0.5% Brij 58 for 12 hr at 28°C (data not shown). A metabolically active state of the cell may be required for excretion. The excreted fatty acids precipitated out during storage at 4°C (Fig. 4). This phenomenon may be useful for collecting fatty acids from the culture medium. If conditions to precipitate fatty acids at the cultivation temperature (28°C) are available, it would help to increase the amount of fatty acids produced. Studies are in progress to further increase fatty acid production by additional mutations and by changing the physiological conditions of growth.

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