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Positive Selection for Uracil Auxotrophs of the Sulfur-Dependent Thermophilic Archaebacterium *Sulfolobus acidocaldarius* by Use of 5-Fluoroorotic Acid

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Uracil auxotrophs of *Sulfolobus acidocaldarius* were positively selected by using 5-fluoroorotic acid. The wild-type strain was unable to grow in medium containing 5-fluoroorotic acid, whereas the mutants grew normally. Positive selection could be done for the auxotrophs. Mutants deficient in orotidine-5′-monophosphate pyrophosphorylase activity were isolated.

Archaebacteria consist of three types of organisms: methanogens, halophiles, and sulfur-dependent thermophiles. Mutants have been reported for the former two groups (2, 11–13, 15, 17, 18), and transformation methods have been developed for several strains (2, 3, 6, 7, 10, 11, 13) in those groups. No mutant has been reported for thermophilic archaebacteria, which hinders the development of genetic manipulation methods for that group.

Uracil-requiring auxotrophs that lack either orotidine-5′-monophosphate pyrophosphorylase (OMPpase) or orotidine-5′-monophosphate decarboxylase have unique characteristics. These strains become resistant to 5-fluoroorotic acid (5- FOA), which inhibits the growth of wild-type strains. Thus, 5-FOA has been successfully used for positive isolation of uracil auxotrophs in yeasts (4, 9) and other microorganisms (8, 16, 20). We have successfully isolated uracil auxotrophs from *Sulfolobus acidocaldarius* by using 5-FOA.

S. acidocaldarius #7 (19) was cultured in tryptone medium, which is the basal salt medium of Brock et al. (5) supplemented with 0.1% glucose and 0.1% tryptone, pH 3.0. Cell density was microscopically determined. Uracil 50 (μg/ml) and 5-FOA (0.1 mg/ml) were added to the medium when necessary. Solid support was prepared by using Gelrite as the gelling agent. Samples were plated by the soft gel overlay method (14) or spread directly on the supporting gel. The final concentrations of Gelrite were 0.8 and 0.4% in the supporting gel and the soft gel, respectively; the gels were solidified by adding 6 mM $CaCl_2$ to the medium. Plates were incubated at 76°C. Colonies were counted after 10 days of incubation. The plating efficiency was 60 to 100% for the soft gel overlay method.

Spontaneous 5-FOA-resistant mutants were isolated by inoculating cells (about 10^8/ml) into tryptone medium containing 5-FOA and uracil. After several days of lag period, growth was observed in some cultures. Uracil auxotrophs were isolated from these cultures.

For UV mutagenesis, a log-phase culture (100 ml; 10^8 cell per ml) was centrifuged and resuspended in 1/10 volume of basal salt medium, which contains no organic compounds. It was transferred into a glass petri dish and irradiated with a 15-W germicidal lamp (Toshiba) at a distance of 56 cm. The cells were centrifuged, resuspended in 10 ml of tryptone medium supplemented with uracil (50 μg/ml), and incubated at 80°C for 2 days. Then 100 μl of irradiated cell culture was spread on a tryptone plate containing 5-FOA and uracil and incubated at 76°C for 10 days. 5-FOA-resistant clones were tested for uracil auxotrophy, and stable clones were selected.

For the preparation of cell extracts, a mid- to late-log-phase culture grown in tryptone medium containing uracil was harvested. The pellet was washed twice with distilled water. One gram of the cells was resuspended in 10 ml of 1 M Tris-HCl, pH 8.0, and disrupted by a Branson Sonifier (cell disrupter 200) at a 25% duty cycle for 10 min on ice. The lysate was centrifuged at 30,000 × g for 30 min at 4°C, and the supernatant was stored at −80°C. Protein concentration was determined by using Bio-Rad protein assay kit and bovine serum albumin as the standard.

OMPpase activity was measured by the method of Beck-

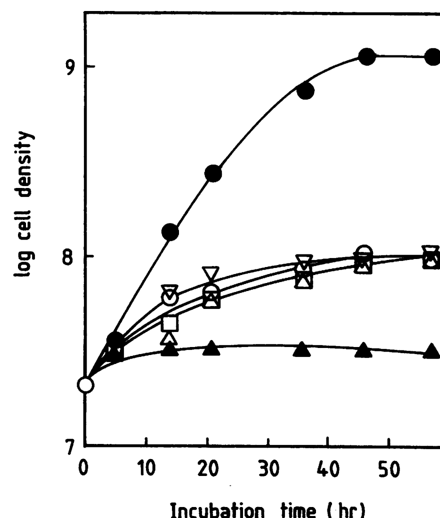

FIG. 1. Growth of *S. acidocaldarius* #7. Basal salt medium containing 0.1% glucose was supplemented with following compounds: yeast extract and Casamino Acids (●), Casamino Acids (▽), alanine and isoleucine (○), alanine (△), isoleucine (□), and none (▲). The final concentration of each amino acid was 0.1 mg/ml; those of other compounds were 1 mg/ml. Cultures were continuously shaken at 80°C.

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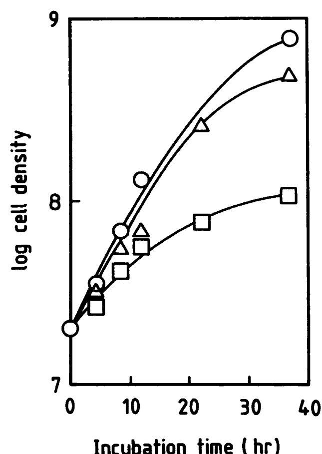

FIG. 2. Growth of *S. acidocaldarius* #7 on complex substrates. Conditions were the same as for Fig. 1. Symbols: ○, yeast extract; △, tryptone; □, peptone.

with et al. (1). The reaction mixture contained 20 μl of 1 M Tris (pH 8.0), 20 μl of 0.1 M $MgCl_2$, 15 μl of 20 mM sodium orotate, 10 μl of 10 mM 5-phosphoribosyl-1-pyrophosphate, and 50 μl of cell extract in a total volume of 1 ml. The reaction was carried out at 50°C, and absorbance was monitored at 295 nm.

Figure 1 and 2 show the growth curves of *S. acidocaldarius* #7 in different media. The strain cannot grow on glucose as a sole carbon source. Growth was observed in the presence of several amino acids, including alanine and isoleucine or Casamino Acids. The highest growth yield was obtained in the presence of yeast extract. Although peptone could not fully support growth, both tryptone and yeast extract could do so. Because of the relative simplicity of the tryptone ingredient, we used tryptone for selecting uracil auxotrophs.

Figure 3 shows the effect of 5-FOA on the growth of wild-type *S. acidocaldarius*. Growth was completely inhibited by 50 μg 5-FOA per ml, although some cultures showed growth after several days of incubation. Because the culture can grow in a fresh medium containing the same concentration of 5-FOA, the growth cannot be attributed to the degradation of 5-FOA. 5-FOA-resistant clones were selected from those cultures. We screened the mutants on solid medium containing 5-FOA and uracil. Of 50 clones that grew on this medium, 29 were found to be uracil auxotrophs. One clone was reserved from each original culture.

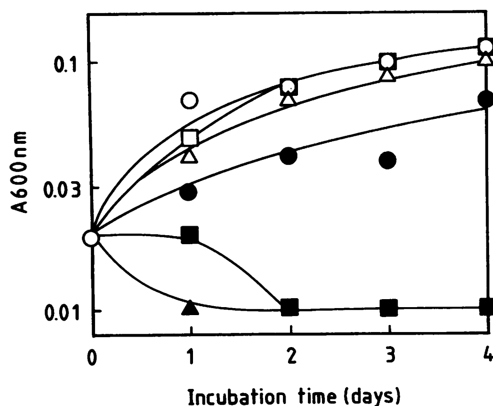

FIG. 3. Effect of 5-FOA on the growth of *S. acidocaldarius*. Cultures were incubated at 80°C in tryptone medium containing uracil (50 μg/ml) and 5-FOA at 0 (○), 0.001 (□), 0.005 (△), 0.01 (●), 0.05 (■), and 0.1 (▲) mg/ml. Turbidity was monitored at 600 nm.

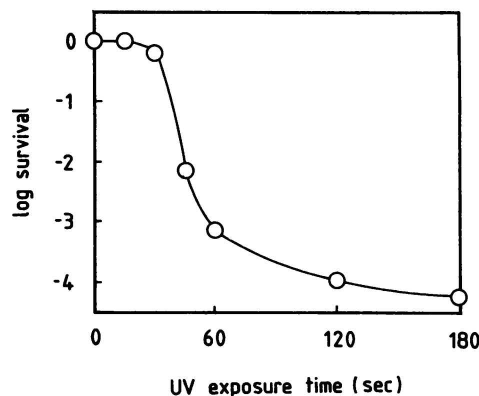

FIG. 4. UV dose-survival curve for *S. acidocaldarius*. The wild-type cells were irradiated in liquid medium and plated on tryptone plates, using the soft gel overlay method as described in the text.

Exposure of *S. acidocaldarius* to UV light led to a time-dependent decrease in the number of viable cells (Fig. 4). A similar UV dose curve has been reported for a methanogen (2). An exposure time of 40 s gave a survival rate of about 1% and was used for the mutagenesis of *S. acidocaldarius*. Of 5×10^7 cells, 40 colonies appeared on the medium containing 5-FOA and uracil. All the 40 clones were found to be uracil auxotrophs. Considering the survival rate, the mutation rate was about 10^{-4}.

OMPpase activities of cell extracts of several isolates were estimated and are summarized in Table 1. Four spontaneous 5-FOA-resistant strains showed markedly decreased OMPpase activity. Two UV-induced 5-FOA-resistant strains also showed reduced OMPpase activity, although the activity of strain KY1 was higher than that of the wild type.

Either one of the two possible types of 5-FOA-resistant uracil auxotrophs has been preferentially obtained in some microorganisms (16, 20). In our case, OMPpase-deficient mutants were preferentially obtained especially after the spontaneous isolation procedure. On the other hand, substantial OMPpase activity was detected in UV-induced auxotrophs. One of them, KY1, showed higher activity than did the wild type. Although our attempts to detect OMPdecase

TABLE 1. OMPpase activity in cell extracts of 5-FOA-resistant uracil auxotrophs

Strain	Genotype	OMPpase activity (U/mg of protein)
#7	Wild type	0.072
KY1[a]	*pyr-1*	0.36
KY2[a]	*pyrE2*	0.017
KY3[a]	*pyrE3*	0.024
KY103[b]	*pyrE4*	<0.005
KY310[b]	*pyrE5*	<0.006
KY402[b]	*pyrE6*	<0.004
KY504[b]	*pyrE7*	<0.006

[a] Isolated after UV illumination.
[b] Spontaneous mutant.

activity in both wild-type and mutant strains were unsuccessful, KY1 may have a mutation in orotidine-5′-monophosphate decarboxylase.

These uracil auxotrophs may be useful for developing gene manipulation systems for thermophilic archaebacteria.

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