

Selecting Lysine-Excreting Mutants of Lactobacilli for Use in Food and Feed Enrichment

D. C. SANDS AND LESTER HANKIN

Departments of Plant Pathology and Biochemistry, The Connecticut Agricultural Experimental Station,
New Haven, Connecticut 06504

Received for publication 24 May 1974

Lysine analogues are used to select for lysine-excreting mutants of *Lactobacillus plantarum*. The use of lactobacilli that excrete lysine for the enrichment of foods and feedstuffs by fermentation is discussed. The increase in lysine content of soybean milk by a mutant of *L. bulgaricus* and in silage by *L. plantarum* is shown.

Lysine, a limiting amino acid in both human and animal nutrition, is added to many foods and feedstuffs. If lysine could be incorporated into foods and feedstuffs in the course of natural fermentation by lactic acid bacteria, or yeasts (e.g., fermented dairy products, beer, or silage), considerable nutritional advantage would accrue.

Bacteria regulate their amino acid biosynthesis so that they produce no higher concentrations of amino acids than they need for growth (3). However, mutants have been reported (3) which overproduce amino acids, generally because of defective repressor systems or inoperative feedback inhibition. We report a method to select and assay for spontaneous mutants that overproduce lysine. *Lactobacillus plantarum*, ATCC 8014, a common lactic acid producer, excreted less than 1 μg of lysine per ml when grown in a lysine-free broth (lysine assay medium, Difco Laboratories, Detroit, Mich.). Cultures were incubated in shake culture for 24 h at 30 C, and cell number increased from 10^6 to 6×10^7 . Growth was completely inhibited by the lysine analogue S-2-aminoethyl-L-cysteine (2; Sigma Chemical Co., St. Louis, Mo.) at concentrations as low as 5 $\mu\text{g}/\text{ml}$. This inhibition was eliminated by L-lysine at about one-fifth the molar concentration of the analogue.

To select spontaneous lysine-excreting mutants, paper disks impregnated with 2 μg of the analogue were placed on the surface of lysine assay medium solidified with 1.1% Ionagar (Colab Laboratories, Inc., Glenwood, Ill.) that had been seeded on the surface with *L. plantarum*. After 4 days of incubation at 30 C, spontaneous mutants of *L. plantarum* appeared within the zone of inhibition.

The ability of these spontaneous mutants to

excrete lysine was tested by streaking them on the surface of plates of solid lysine assay medium seeded with *Leuconostoc mesenteroides* ATCC 8042, a strain that requires lysine and is used in standard assays. We tested DL- α,ϵ -diaminopimelic acid (Sigma Chemical Co., St. Louis, Mo.) of which the L form is the immediate precursor of lysine and found it would not support growth of *L. mesenteroides*. Colonies that provided maximal growth of the assay organism were isolated and subjected to mutation selection cycles with solid media. That is, each mutant was retested against a higher level (10 $\mu\text{g}/\text{disk}$) of S-2-aminoethyl-L-cysteine, reisolated, and tested against a still higher level of the analogue. The cycle of testing and reisolation was repeated at least four times, until *L. plantarum* isolates resistant to a level of 5 mg of the analogue per disk were obtained. Lysine excretion of selected spontaneous mutants of *L. plantarum* and the increase with higher doses of S-2-aminoethyl-L-cysteine is shown in Table 1. The highest level of lysine excretion obtained was 72 $\mu\text{g}/\text{ml}$, compared with less than 1 $\mu\text{g}/\text{ml}$ for the wild type.

Silage isolates of *L. plantarum* (obtained from M. K. Woolford, Grassland Research Institute, Hurley, Maidenhead, England) were grown on lysine assay medium and excreted less than 1 μg of lysine per ml, as was found with *L. plantarum* ATCC 8014. These silage isolates were also tested with S-2-aminoethyl-L-cysteine, as previously described, and lysine-excreting mutants were selected. Mutants were also tested against two other lysine analogues, L-lysine hydroxamate and β -hydroxylysine, mixed DL and DL-allo (Sigma), and both were as effective as S-2-aminoethyl-L-cysteine. Mutants resistant to S-2-aminoethyl-L-cysteine

TABLE 1. Lysine excreted by mutants of *Lactobacillus plantarum* selected by mutation selection cycles in the presence of increasing concentrations of *S*-2-aminoethyl-*L*-cysteine

Mutation selection cycle	<i>S</i> -2-Aminoethyl- <i>L</i> -cysteine (mg/disk)	Growth after 24 h, ([cells/mg] × 10 ⁷)	Lysine excreted, (μg/ml of culture, filtrate)
Wild type	0.00	6.1	<1
1	0.02	6.4	9
3	1.00	6.1	57
4	5.00	5.6	72

were not necessarily resistant to other analogues, and vice versa.

Inoculation of chopped fodder is successful in improving the final product (5). We inoculated lysine-excreting mutants of *L. plantarum* into small samples of freshly chopped maize plants. After 3 days of anaerobic incubation the samples of silage inoculated with wild-type *L. plantarum* contained 0.45% *L*-lysine (dry weight basis), whereas the mutant strain yielded 0.63% *L*-lysine. The method for selection of spontaneous lysine-excreting mutants might be used for lactic acid bacteria used in yoghurt, sour cream, buttermilk, fermented soybean milk, and sauerkraut, as well as silage.

We selected lysine-excreting mutants of *L. bulgaricus* (wild type obtained from C. W. Hesseltine, Northern Regional Research Laboratory, Peoria, Ill.). The mutants and the wild type were inoculated into soybean milk (4). The mutants increased the lysine content of the fermented soybean milk from 4 to 14%.

Mutant yeast might also be employed in brewing beer, and in fact a lysine-excreting yeast, grown on hydrocarbon, has been described (1).

We thank Margaret Finkbeiner for technical assistance.

LITERATURE CITED

- Leavitt, R. I., and F. X. Ryan. 1974. *L*-Lysine decarboxylase as a specific inhibitor of the growth of a species of *Pichia*. *J. Gen. Microbiol.* **80**:311-313.
- Shiota, T., J. E. Folk, and F. Tietze. 1958. Inhibition of lysine utilization in bacteria by *S*-(β-aminoethyl) cysteine and its reversal by lysine peptides. *Arch. Biochem. Biophys.* **77**:372-377.
- Stadtman, E. R. 1963. Symposium on multiple forms of enzymes and control mechanisms. II. Enzyme multiplicity and function in the regulation of divergent metabolic pathways. *Bacteriol. Rev.* **27**:170-181.
- Wang, H. L., L. Kraidej, and C. W. Hesseltine. 1974. Lactic acid fermentation of soybean milk. *J. Milk Food Technol.* **37**:71-73.
- Woolford, M. K. 1972. Some aspects of the microbiology and biochemistry of silage making. *Herb. Abstr.* **42**:105-111.