

Lactose Hydrolysis by Lactose Transport System Defective ($lacS^-$) *Streptococcus thermophilus*

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Mutant strains of *Streptococcus thermophilus* with a defective lactose transport system were isolated at a frequency of 10^{-2} per surviving colony forming unit following treatment with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. Mutant cultures failed to ferment lactose and glucose but grew normally in a sucrose medium. The $lacS^-$ cells synthesized 25% less β -galactosidase (16 U/mg protein) than untreated $lacS^+$ cells (21 U/mg protein). Data on lactose hydrolysis in aqueous solutions indicated that $lacS^-$ *S. thermophilus* cultures may be valuable as microcarriers of food-grade β -galactosidase with potential utility in food and commercial applications where reduction of lactose content is sought. © 1990 Academic Press, Inc.

General interest in the commercial use of β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) is sustained by three major considerations: (i) lactose is poorly digested by a large segment of the world's adult population leading to the avoidance of milk in the diet as a source of essential nutrients (1, 2); (ii) lactose has limited solubility in water (18% at 20°C) resulting in undesirable crystallization in concentrated dairy foods (3); and (iii) lactose has only one-fifth the degree of sweetness of sucrose, thus limiting the use of whey concentrates or whey powder in processed foods (3). Many of these problems may be alleviated by the enzymatic hydrolysis of lactose to its hexose components, glucose and galactose.

β -Galactosidases for food applications are available from various yeast and fungal sources. Enzymes that are used most extensively are the β -galactosidases of *Kluyveromyces fragilis* (4, 5) and *K. lactis* (6, 7) which have the preferred pH optimum of near neutrality. However, the yeast enzymes do not tolerate the high temperatures used under industrial conditions.

The properties of β -galactosidase from *Streptococcus thermophilus*, an important microorganism in dairy food fermentations, have been studied in various laboratories (8-11). The relatively high thermostability (55°C), pH optimum (8.0), and stability under prolonged storage (1 year) impart definite advantages to this bacterial β -galactosidase over the yeast enzymes currently in industrial use. The cytoplasmic β -galactosidase of *S. thermophilus* may be conveniently studied with acetone:toluene-treated ("decryptified") cells as the enzyme source (8). However, purified or large-scale production of β -galactosidase requires sonic disintegration of the cells (8-11). To circumvent this problem, the use of rapidly autolyzing strains of *S. thermophilus* in β -galac-

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tosidase production was described (11). Autolysis was regulated by medium pH (5.2–6.0) and occurred at the end of the growth phase with the concomitant release of β -galactosidase (12, 13).

In our laboratory, we investigated the characteristics of a lactose transport system defective strain of *S. thermophilus* and its potential use as a microcarrier of β -galactosidase in the enzymatic hydrolysis of lactose.

MATERIALS AND METHODS

Organism and Culture Conditions

S. thermophilus ST128 was from our laboratory culture collection. On the basis of its carbohydrate utilization pattern, it had been previously classified as a galactose-negative (Gal^-) strain (14). The culture was maintained in basal broth medium (BB)² consisting of tryptone (Difco,³ 30 g/liter), yeast extract (Difco, 10 g/liter), beef extract (Difco, 2 g/liter), and KH_2PO_4 (5 g/liter) with a pH 6.5 before sterilization. The basal medium was supplemented with lactose (BBL), sucrose (BBS), glucose (BBG), or fructose (BBF), as required, at 5 g/liter concentration. Incubation was at 37°C for 24 h. Between weekly transfers, cultures were stored at 4°C.

Isolation of Lactose Transport System Defective (lacS^-) Mutants

An overnight culture of *S. thermophilus* ST128 was transferred to fresh BBL medium and incubated at 37°C until $\text{OD}_{660} = 0.4$ was attained. Cells were pelleted by centrifugation at 10,000g for 10 min at 4°C and resuspended in 50 mM K_2HPO_4 – KH_2PO_4 (pH 7.0) buffer (PO buffer) with 500 $\mu\text{g/ml}$ *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (NTG). After 60 min at room temperature, cells were washed twice with PO buffer and resuspended in peptone (Difco, 0.1 g/liter) water. Aliquots of serially diluted samples were plated in BBS medium with agar (Difco, 15 g/liter). After 3 days at 37°C, 250 randomly selected colonies were patched on BBL and fresh BBS agar plates. The lacS^- mutants were scored after incubation for 48 h at 37°C. Mutation frequency was expressed as the number of lacS^- colonies per surviving colony forming unit (CFU).

Growth Studies

The lacS^- phenotype of lactose nonfermenting colonies that grew on BBS plates was verified by growth studies in BBL and BBS media. Isolates failing to show growth in BBL after 5 days were classified as having the lacS^- phenotype. Possibility of reverse mutations to the lacS^+ phenotype was checked routinely by plating BBS-grown cells on BBL agar.

Enzyme Assays

The β -galactosidase activity of lacS^+ (wild type) and lacS^- *S. thermophilus* was measured in decriptified cells treated with an acetone–toluene (AT) mixture as de-

² Abbreviations used: BB, basal broth; BBL, BB supplemented with lactose; BBS, BB supplemented with sucrose; BBG, BB supplemented with glucose; BBF, BB supplemented with fructose; NTG, *N*-methyl-*N*-nitroso-*N'*-nitroguanidine; AT, acetone–toluene.

³ Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

scribed previously (8). Activity was determined by measuring the amount of glucose released from 5% lactose in 50 mM PO buffer (pH 7.0) containing 1 mM MgCl₂. Reactions were carried out at 50°C for 10 min. Glucose was determined spectrophotometrically with a Glucose (HK) Kit (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's recommendations. A unit of β -galactosidase activity was defined as the amount of enzyme that released 1 μ mol of glucose/min. Fructose was determined by enzymatic analysis (15). Protein was estimated by the method of Lowry (16) with bovine serum albumin as the standard.

Lactose Hydrolysis by Entrapped lacS⁻ Cells

The lacS⁻ phenotype cells from 21 of BBS medium following overnight growth were centrifuged (10,000g, 15 min, 4°C), washed twice with PO buffer, and finally resuspended in PO buffer for AT treatment. AT-treated cells were again washed twice before resuspension in PO buffer. Dry cell concentration of the suspension was 24 mg/ml (determined from a dry weight vs OD₆₆₀ standard curve). Equal volumes (12 ml) of cell suspension and 3% agarose (FMC Bioproducts, Rockland, ME) in PO buffer were mixed at 45°C and poured on a 150 × 65-mm strip of GelBond film (FMC Bioproducts).

RESULTS AND DISCUSSION

Mutation of S. thermophilus to lacS⁻ Phenotype

Treatment of *S. thermophilus* ST128 with NTG resulted in a 53% kill of the cell population under experimental conditions. Preliminary evaluation of clones on BBL and BBS agar plates indicated an apparent frequency of 10⁻¹ for the lacS⁻ phenotype per surviving CFU. However, more exhaustive growth experiments with putative lacS⁻ mutants showed that several isolates grew in BBL very slowly, showing visible turbidity only after 96 h or longer at 37°C. Since these strains appeared only partially impaired in lactose transport, they were discarded.

Mutation affecting the lactose transport system led to inability to ferment lactose. However, in lacS⁻ mutants high levels of cytoplasmic β -galactosidase may be synthesized (β -gal⁺), and other carbohydrate transport systems (e.g., sucrose, sucS⁺) may also be unimpaired. This appeared to be the case in several stringently selected (no growth in BBL) mutants of *S. thermophilus* ST128 which were lacS⁻, sucS⁺, and β -gal⁺. The frequency of such mutants was 10⁻²/surviving CFU following NTG treatment. The wild-type ST128 grew well in BBL and BBS, poorly in BBG, and not at all in BBF. The lacS⁻ ST1281 grew exclusively in BBS (Fig. 1). These findings were anticipated since most wild-type strains of *S. thermophilus* ferment lactose and sucrose preferentially to their constituent monosaccharides which are fermented slowly or not at all (17).

Recently, the gene responsible for lactose transport in *S. thermophilus* was characterized following its cloning in *Escherichia coli* (18). It was postulated that lactose metabolism genes (lacS and β -gal) in *S. thermophilus* are organized in an operon similar to that in the lac genes of *E. coli*. In our work, the exact nature of the lesion(s) impairing lacS in *S. thermophilus* was not studied at the gene level.

Enzyme analysis showed that β -galactosidase activity in ST1281(lacS⁻) (16 U/mg protein) was about 25% lower than that in BBS-grown wild-type ST128 (21 U/mg

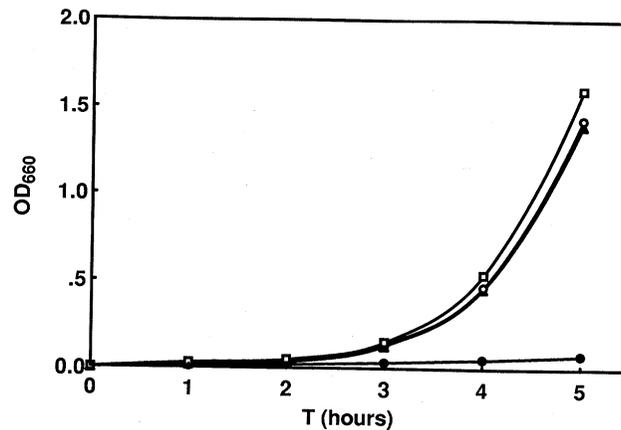


FIG. 1. Growth of wild-type *S. thermophilus* ST128 in glucose (●), lactose (○), and sucrose (▲) media, and growth of ST1281 (*lacS*⁻) in sucrose (□) medium. Incubation was at 37°C.

protein). According to earlier data, β -galactosidase in *S. thermophilus* may be inducible (8) or in some strains constitutively expressed (11). Results of this study indicated the presence of a constitutive β -galactosidase system both in wild-type ST128 and in mutant ST1281. The mutant strain appeared to be an excellent source of the enzyme.

Hydrolysis of Lactose by ST1281(lacS⁻)

Lactose hydrolysis was studied with cells of ST1281(*lacS*⁻) grown in BBS for 16 h at 37°C. Pelleted (10,000g) and twice washed cells were resuspended in PO buffer at $OD_{660} = 5.0$ for AT treatment at room temperature. After AT treatment cells were washed and resuspended at a final $OD_{660} = 2.5$ in lactose solutions prepared at 5, 10, 15, and 20% (w/v) concentrations in PO buffer. Reaction mixtures (5 ml) were held at 50°C. Aliquots were taken at appropriate intervals and cleared by centrifugation, dilutions were made if needed, and multiples of 10- μ l samples were assayed for glucose content. The results of these experiments are shown in Fig. 2. At a low (5%)

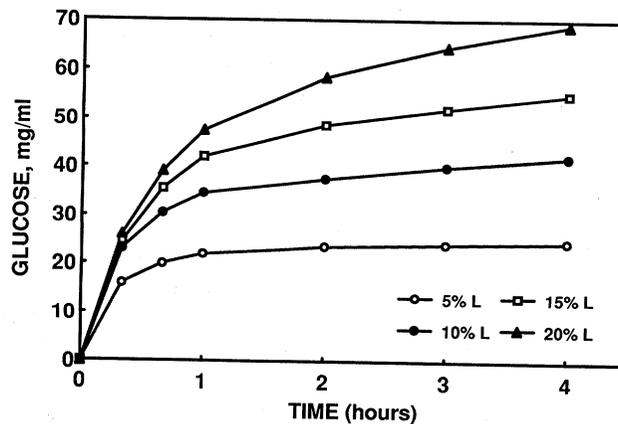


FIG. 2. Hydrolysis of lactose by ST1281 (*lacS*⁻) mutant cells in PO buffer (pH 7.0) at 50°C. L, lactose. Data plotted represent averages of three replicate determinations.

TABLE I
Lactose Hydrolysis by Immobilized ST1281 (lacS⁻) Cells

Time (min)	Glucose (mg/ml)	% Lactose hydrolysis
0	0.008	0.1
30	2.18	41.4
60	3.26	62.0
90	3.83	72.7
120	4.43	84.2
150	4.68	88.9
180	4.86	92.2

concentration which approximated the lactose content of cheese whey permeate from ultrafiltration, 83% of lactose was already hydrolyzed after 60 min and hydrolysis was nearly complete after 2 h of incubation. At higher concentrations (10, 15, and 20%), the percentage of lactose hydrolyzed decreased progressively. This may have been caused by the increasing level of galactose, also accumulating in equimolar amounts in the reaction medium, which had been shown to be a weak competitive inhibitor of *S. thermophilus* β -galactosidase during lactose hydrolysis (13).

The ST1281(lacS⁻) cells as microcarriers of β -galactosidase had excellent storage qualities. After decryptified cells were stored in PO buffer for 4 months at 4°C, there was no detectable loss of β -galactosidase activity. The lacS⁻ phenotype appeared to be conserved in mutant cultures, and reversal to lacS⁺ resulting in growth on lactose was not observed after at least 100 transfers.

ST1281(lacS⁻) cells immobilized in 2.5-mm-thick slabs of 1.5% agarose gel were also suitable for hydrolyzing lactose in aqueous solutions. The agarose gel strip was removed from the GelBond support and placed in the shape of a cylinder in 200 ml 1% lactose (in PO buffer) in a beaker and stirred magnetically at 45°C. As shown in Table I, glucose accumulated rapidly in the reaction mixture and after 3 h, 92% of the lactose was hydrolyzed. Clearly, ST1281(lacS⁻) cells immobilized in agarose and possibly other suitable entrapping materials (alginates, κ -carrageenan, locust bean gum) also have potential applications in lactose hydrolysis.

The use of lactose transport defective (lacS⁻) mutants of *S. thermophilus* has distinct advantages in commercial lactose hydrolysis. These food-grade enzyme microcarriers with high levels of constitutive β -galactosidase activity may be grown in inexpensive sucrose-based media. Since they cannot ferment lactose or glucose to lactic acid, they do not contribute to the acidification of foods, making the isolation of β -galactosidase from the cells unnecessary. Lactose nonfermenting cultures treated appropriately to express maximum enzyme activity may be added directly to food systems to reduce lactose content or used in large-scale lactose hydrolysis.

Research continues in our laboratory on the application of lacS⁻ mutant cultures to the reduction of lactose content of milk and whey products.

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