JOURNAL OF BACTERIOLOGY, July 1975, p. 242-247

Vol. 123, No. 1
Printed in U.S.A.

Oxygen Metabolism of Catalase-Negative and Catalase-Positive Strains of *Lactobacillus plantarum*

ALLAN A. YOUSTEN,* JOHN L. JOHNSON, AND MARVIN SALIN

Department of Biology and Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, and Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710*

Received for publication 7 March 1975

Two catalase-negative strains of *Lactobacillus plantarum* and a strain producing the atypical, nonheme catalase were studied to determine if the ability to produce the atypical catalase conferred any growth advantage upon the producing strain. Both catalase-negative strains grew more rapidly than the catalase-positive strain under aerobic or anaerobic conditions in a glucose-containing, complex medium. Upon exhaustion of glucose from the medium, all three strains continued growth under aerobic but not under anaerobic conditions. The continued aerobic growth was accompanied by production of acetic acid in addition to the lactic acid produced during growth on glucose. Oxygen was taken up by exponential phase-cell suspensions grown on glucose when glucose or glycerol were used as substrates. Cells harvested from glucose-exhausted medium oxidized glucose, glycerol, and pyruvate. Oxygen utilization by a catalase-negative strain increased as did the specific activity of reduced nicotinamide adenine dinucleotide peroxidase during late growth in the glucose-exhausted medium. The catalase-positive strain and the catalase-negative strain tested both possessed low but readily detectable levels of superoxide dismutase throughout growth. The growth responses are discussed in terms of the presence of enzymes which would allow the cells to remove potentially damaging reduction products of O_2.

Although the utilization of oxygen as an electron acceptor is normally advantageous to bacteria, this reaction may produce H_2O_2 or the superoxide free radical, O_2^-, both of which have the potential for causing cell damage. Most bacteria which utilize oxygen prevent such damage by producing catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) or NADH peroxidase (reduced nicotinamide adenine dinucleotide: H_2O_2 oxidoreductase, EC 1.11.1.1) to decompose H_2O_2 and by producing superoxide dismutase to eliminate O_2^- (10, 19).

Although several lactic acid bacteria have been shown to be capable of utilizing O_2 (6, 8, 11, 20, 23, 26), they characteristically do not form catalase and possess only a low level of superoxide dismutase (19). In contrast to the typical catalase-negative lactic acid bacteria, there are a number of isolates from various genera which possess an atypical, nonheme catalase (15, 26). It is questionable whether possession of the atypical catalase confers any advantage on the organisms forming it. Dobrogosz and Stone (7) found enhanced aerobic growth on glycerol among catalase-positive pediococci, but Jones et al. (17) found no growth advantage with two catalase-positive strains of *Streptococcus faecalis* grown on glucose or glycerol. The superoxide dismutase content of the pediococci and streptococci was not reported in those experiments because the existence of the enzyme was not known at that time. It was reported that *Lactobacillus plantarum* was the only one of several aerotolerant anaerobes that lacked superoxide dismutase (19). By using a strain of *L. plantarum* possessing the atypical catalase (16), we thought it possible to observe the effect of this enzyme on growth under conditions where superoxide dismutase was not involved in O_2 metabolism. We have found that *L. plantarum* does utilize small amounts of O_2. However, the strain which produces an atypical catalase does not grow better aerobically on glucose or glycerol than two strains which are catalase negative. Under the growth conditions used in these experiments, both catalase-positive and catalase-negative strains possessed a low level of superoxide dismutase.

MATERIALS AND METHODS

Bacteria. *L. plantarum* ATCC 14917, *L. casei* var. *casei* 64H, *L. casei* var. *casei* C1 28, and *L. casei* var. *rhamnosus* ATCC 7469 were obtained from the collection of Anaerobe Laboratory, Virginia Polytechnic Institute and State University. *L. plantarum* ATCC 8014 and *L. plantarum* T-1403-5 were obtained from E. A. Delwiche, Cornell University. The catalase produced by strain T-1403-5 has been described in detail (16). *Escherichia coli* was obtained from the Culture Collection of the Biology Department, Virginia Polytechnic Institute.

Growth conditions. Unless otherwise stated, the medium used for cell growth contained 1% tryptone, 0.5% yeast extract, 0.7% K_2HPO_4, and 2.78 mM glucose or 5.56 mM glycerol (the latter two added from separately autoclaved stock solutions). Inocula were grown without shaking for 14 h at 30 C. An aliquot of the inoculum equal to 5% (vol/vol) of the broth to be inoculated was centrifuged and the cell pellet was washed into the flask with 1 ml of uninoculated broth. Growth curves were performed at 32 C in 500-ml sidearm flasks containing 50 ml of broth shaken at 200 rpm. For anaerobic growth the sidearm flask was fitted with a two-hole rubber stopper and after inoculation, the flask was flushed with rapidly flowing N_2 for 5 min before sealing. Cells for use in measuring O_2 uptake, superoxide dismutase, and NADH peroxidase were grown at 32 C in 2-liter flasks containing 200 ml of broth shaken at 200 rpm. A New Brunswick model G25 shaker was used for the above experiments. Growth was followed turbidimetrically using a Klett-Summerson colorimeter with a number 54 filter. At the completion of each growth curve, a portion of each culture was centrifuged and the cell pellet was mixed with a drop of 3% H_2O_2 to verify the presence or absence of catalase.

NADH peroxidase. Cells were grown on the glucose-containing medium for 5 or 11.5 h, harvested by centrifugation at 2 C, and washed once with 1 mM morpholinopropane sulfonic acid buffer, pH 7.0. The cells were suspended in the same buffer and broken by 15 min of sonication with a Virtis Virsonic cell disrupter. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 C. The enzyme activity was immediately assayed by the method of Walker and Kilgour (25). The cuvette contained 0.3 μmol of NADH; 4.0 μmol of H_2O_2; 100 μmol of sodium acetate buffer, pH 5.4; 0.01 ml of enzyme preparation, and water to 3.0 ml. The reaction was started by addition of the enzyme and NADH oxidation was followed at 340 nm. The very small amount of NADH oxidase activity present was measured in the absence of H_2O_2 and was used to correct the NADH peroxidase value. One unit of NADH peroxidase activity was defined as that which catalyzes the oxidation of 1 μmol of NADH per min at 25 C. A value of 6.22×10^3 liter/mol per cm was used as the molar extinction coefficient of NADH at 340 nm. Specific activity was defined as units per milligram of protein. Protein was determined by the method of Lowry et al. (18).

Superoxide dismutase. Cells were grown on the glucose-containing medium for the indicated periods of time, harvested by centrifugation at 2 C, washed once with 50 mM potassium phosphate buffer, pH 7.0, resuspended in the phosphate buffer, and disrupted by 15 min of sonication with a Virtis Virsonic cell disrupter. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min and the extract was dialyzed against 5 mM potassium phosphate buffer, pH 7.0. The dialyzed extract was assayed in duplicate at pH 10.0 as described by Salin and McCord (22). One unit of activity is that amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% at pH 10. Specific activity is defined as units per milligram of protein.

Oxygen utilization. After growth on the glucose-containing medium, cells were harvested by centrifugation at 2 C, washed once with 50 mM potassium phosphate buffer, pH 7.0, and suspended in 5 mM potassium phosphate buffer, pH 7.0. Oxygen consumption was measured in a Clark oxygen electrode at 30 C. Substrates (18 mM glucose or glycerol, 30 mM pyruvate, final concentration) were added to the cell suspension in the stirred vessel after an average equilibration period of 15 min to allow endogenous O_2 uptake to reach a low level. Data reported in Table 1 are for the 2-min period after the first minute of reaction and are corrected for endogenous uptake. Dry weights were determined in triplicate on washed cells after heating at 110 C for 48 h.

Metabolite accumulation. Acetic and lactic acids were determined by gas chromatography. Acetic acid was determined by injecting acidified culture supernatant into a 4-foot (ca. 121.92 cm) column of 80/100 Chromosorb WAW having a liquid phase of 1% H_3PO_4 and 10% SP1200 (Supelco, Inc., Bellefonte, Pa.) run isothermally at 98 C on a Bendix model 2600 gas chromatograph equipped with flame-ionization detector. Lactic acid was determined after methylation and chloroform extraction (13) by injection of samples into a 6-foot (ca. 182.88 cm) column of Resoflex Lac 1-R-296 standard concentration (Burrell Corp., Pittsburgh, Pa.) programmed from 45 to 130 C at 5 C per min on a Hewlett-Packard model 700 dual-column gas chromatograph equipped with flame-ionization detector. Quantitive determinations were made by measur-

TABLE 1. *Percentage of homology between the DNA of several lactobacilli and that of L. plantarum ATCC 14917*

Unlabeled DNA	% Homology to *L. plantarum* ATCC 14917
Lactobacillus plantarum ATCC 14917	100
L. plantarum ATCC 8014	90
L. plantarum T-1403-5	84
L. casei: var. *casei* 64 H[a]	2
L. casei: var. *casei* cl 28[a]	3
L. casei: var. *rhamnosus* ATCC 7469	5

[a] Strain identification numbers are those of M. Rogosa, National Institute of Dental Research.

ing peak heights of standard acetic and lactic acid samples treated identically to culture supernatants.

Glucose was determined with glucose oxidase (glucostat reagent of Worthington Biochemicals, Freehold, N.J.).

DNA isolation and homology experiment. Cells for the isolation of unlabeled deoxyribonucleic acid (DNA) were grown in a medium containing mineral salts (21), 1.0% Trypticase, 0.5% yeast extract, 1.0% glucose, 0.05% cysteine, and 0.05 M sodium phosphate buffer, pH 7.0. For the isolation of labeled DNA, the concentration of Trypticase was lowered to 0.5% and that of yeast extract to 0.05%.

After disrupting the cells by shaking with glass beads, the lysates were treated with bovine pancreatic ribonuclease A (15 μg/ml) and then extracted two times with phenol (14). The DNA was isolated by adsorption onto hydroxyapatite (1, 2, 3). The S1 nuclease method was used for the DNA homology experiment (4). Labeled DNA (0.05 μg) and unlabeled DNA (30 μg) were incubated at 63 C in 0.11-ml volumes of 0.4 NaCl-10^{-3} M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.0. After incubation to a C_0t (moles/s per liter) of 50, 0.10 ml of the reaction mixture was removed and placed into 1.0 ml of pH 4.6 buffer containing 0.03 M sodium acetate, 1 mM $ZnSO_4$ and 5% glycerol (24). The S1 nuclease was then added and the mixture was incubated at 50 C for 20 min. The S1 resistant duplexes were precipitated with trichloroacetic acid, collected on membrane filters, dried, and counted in a liquid scintillation counter. The self renaturation of labeled DNA was determined by leaving out the unlabeled DNA and incubating the same volumes for the same length of time. The ratio of heterologous duplexes to homologous duplexes times 100 was taken as percentage of homology.

RESULTS

Relatedness of the isolates used in these experiments. A fundamental characteristic of bacteria of the genus *Lactobacillus* is the absence of catalase. Although the catalase-positive isolate, *L. plantarum* T-1403-5, was provided with assurances that it possessed the characteristics of *L. plantarum* (with the exception of catalase production), verification of the classification was obtained by the technique of DNA hybridization. The data in Table 1 clearly demonstrate the relatedness of *L. plantarum* T-1403-5 (catalase-positive) to the catalase-negative strains.

Growth rates. The aerobic and anaerobic (under N_2) growth of the lactobacilli in glucose-containing broth is presented in Fig. 1. The growth rates of the two catalase-negative strains (8014 and 14917) were similar for the first 7 h and were not affected by the presence or absence of O_2. In contrast, the catalase-positive strain (T-1403-5) grew at a somewhat lower rate although its growth for the first 7 h was also not affected by the presence or absence of O_2. The rate of glucose utilization by strain T-1403-5 was also slightly lower than that of the catalase-negative strains. After 7 h the presence of O_2 made a decided difference in the growth rates in all three strains. In the absence of O_2 growth ceased, while in its presence, growth continued for 2 or more h. The increased cell mass at 12 h in aerobic flasks was confirmed by dry weight determinations. Phase contrast microscopy at 12 h showed no indication of cell lysis in the anaerobic flasks that might account for the decreased cell yield compared to the aerobic flasks.

Replacement of glucose in the medium with glycerol at a molar concentration two times that of glucose resulted in the growth pattern dis-

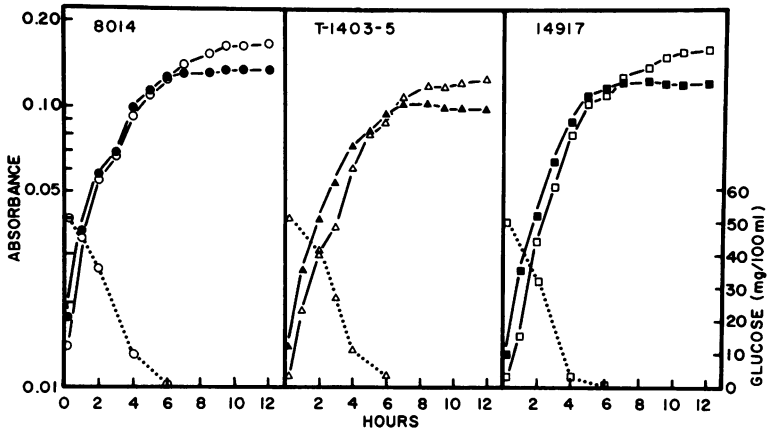

FIG. 1. *Growth of catalase-negative and catalase-positive strains of L. plantarum on glucose-containing complex medium. Open symbols with solid lines represent growth in air; closed symbols with solid lines represent growth in N_2; open symbols connected by dotted lines represent glucose utilization in air.*

played in Fig. 2. For strains 8014 and 14917, growth rates were less than those obtained in glucose and the final absorbance was 10 to 15% less. The catalase-positive strain, T-1403-5, also grew more slowly in glycerol than in glucose. Both catalase-negative and catalase-positive strains were able to grow in the medium without added glucose or glycerol. The catalase-negative strains grew more rapidly and achieved a higher final absorbance than the catalase-positive strain.

Oxygen utilization. The observation that during growth with glucose there was a late O_2-dependent increment of growth suggested that the lactobacilli were using O_2 as an electron acceptor. Table 2 shows the O_2 consumption of glucose-grown cells assayed in buffer containing glucose, glycerol, or pyruvate. The cells had a very low endogenous rate of O_2 uptake after 15 min of incubation without added substrate, never more than 1 or 2 nmol of O_2/min per mg (dry weight). Young cells of either the catalase-positive or the one catalase-negative strain selected for the experiment had a low but detectable level of O_2 uptake with either glucose or glycerol. Older cells of strain 8014 had a markedly increased ability to oxidize either glucose or glycerol and also oxidized pyruvate. In contrast, older cells of T-1403-5 retained only a low level of oxidation on glucose or glycerol but had a definite ability to oxidize pyruvate.

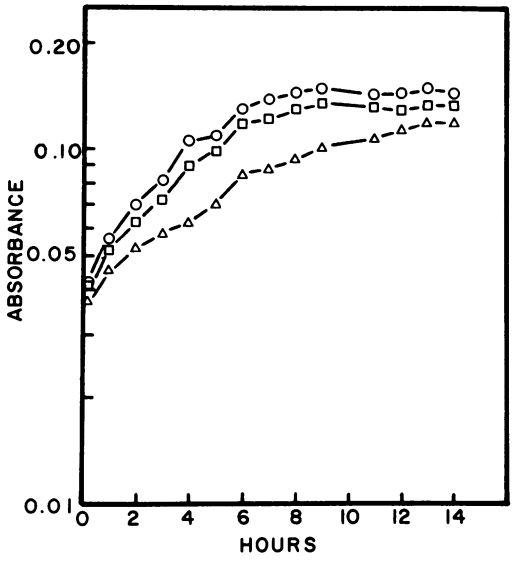

FIG. 2. *Aerobic growth of catalase-negative and catalase-positive strains of L. plantarum on glycerol-containing, complex medium. Symbols:* (○) *8014;* (△) *T-1403-5;* (□) *14917.*

TABLE 2. *Oxygen uptake by cells of L. plantarum*

Culture age (h)	ATCC 8014 (catalase-negative)			T-1403-5 (catalase-positive)		
	Glucose	Pyruvate	Glycerol	Glucose	Pyruvate	Glycerol
5	2.4[a]	ND[b]	7.1	5.9	ND	5.9
13	26.4	17.4	23.6	3.1	11.0	6.1

[a] Nanomoles of O_2 per minute per milligram (dry weight).

[b] ND, Not determined.

Metabolites produced during growth with glucose. The late O_2-related increment of growth found with all three lactobacilli grown on glucose and the increase in O_2 consumption by older cells suggested an O_2-related, energy-yielding shift in metabolism in older cells after glucose exhaustion. Analysis of culture supernatants obtained at 9 and 13 h revealed continued lactic acid accumulation during late stages of growth but an even greater percentage of increase in acetic acid (Table 3). The slower growing, catalase-positive strain, T-1403-5, accumulated less of each metabolite than the catalase-negative strains.

Superoxide dismutase. When grown aerobically on the glucose-containing medium, both the catalase-positive (T-1403-5) and a catalase-negative strain (8014) possessed low but readily detectable levels of superoxide dismutase (Table 4). In contrast, an extract prepared from stationary phase *E. coli* had a specific activity of 47.4.

TABLE 3. *Production of lactic and acetic acids by three strains of L. plantarum grown aerobically in glucose-containing broth*

Lactobacillus plantarum	Lactic acid (mM)[a]		Acetic acid (mM)[a]	
	9 h	13 h	9 h	13 h
ATCC 8014	7.8	11.2	0.40	1.60
ATCC 14917	8.2	12.0	0.25	1.80
T-1403-5	6.8	10.5	0.05	0.70

[a] Values corrected for lactic and acetic acid present in uninoculated broth.

TABLE 4. *Superoxide dismutase activity in cells of L. plantarum*

Culture age	Sp act of superoxide dismutase	
	ATCC 8014 (catalase-negative)	T-1403-5 (catalase-positive)
5	5.8	5.8
9	3.1	2.3
13	5.8	6.3

NADH peroxidase. An enzyme catalyzing the H_2O_2-dependent oxidation of NADH was detected in crude extracts of strain 8014. The specific activity in 5-h cells was 1.45, and in 11.5-h cells it was 3.31. It was not possible to assay the peroxidase in strain T-1403-5 because of the catalase content of cell extract.

DISCUSSION

Bacteria which reduce O_2 may produce a variety of potentially damaging end products from the reaction. These include H_2O_2, O_2^-, and $OH\cdot$, the latter a product of reaction between H_2O_2 and O_2^- (9, 12, 27). Gregory and Fridovich (9) reported that *L. plantarum* ATCC 8014 grown aerobically in APT broth (Baltimore Biologicals, Inc.) did not react with O_2 and therefore would not require enzymes to detoxify products of O_2 reduction. They did not detect superoxide dismutase in the organism, the organism was killed by an exogenous flux of O_2^-, and it was partially protected from O_2^- by the addition of superoxide dismutase, catalase, or mannitol (an $OH\cdot$ scavenger). The growth medium used in their studies differed from that used in this study, the method used to obtain aerobic growth was not specified, and the cells were harvested during the logarithmic stage. Gregory and Fridovich (9) specifically stated that their findings could not be generalized to the same organism grown under different conditions.

Under different growth conditions we found that both *L. plantarum* ATCC 8014 and a catalase-positive strain, T-1403-5, were capable of utilizing O_2, particularly during the late stages of growth. This suggested the need for enzymes which would protect the lactobacilli from H_2O_2 and O_2^- which might be formed. Superoxide dismutase was present at low but constant levels in both catalase-positive and catalase-negative strains. NADH peroxidase was present in strain 8014 and this enzyme increased in specific activity as the cells aged and entered the period of increased O_2 uptake. The presence of this enzyme should protect the organism against toxic effects of H_2O_2 in the absence of catalase production. In strain T-1403-5 this role can be performed by the very active atypical catalase, although this strain may also possess the peroxidase.

When growth rates of the catalase-negative and catalase-positive strains were compared, the rates of the catalase-negative strains were greater than that of the catalase-positive strain. Therefore, it did not appear that the possession of catalase conferred any advantage on strain T-1403-5.

The lower growth rate and final absorbance observed with T-1403-5 may be a manifestation of both a slightly lower rate of glucose utilization and a lesser ability to utilize other ingredients of the medium for growth. In the one report (8) in which catalase-positive pediococci were shown to grow more rapidly than catalase-negative strains, the superoxide dismutase and NADH peroxidase contents of the cells were not reported. It would be interesting to examine these strains of pediococci to see if the differences in growth rates can be further explained by differences in their H_2O_2- or O_2^--decomposing enzymes.

Though peripheral to the main goal of this investigation, the shift to an O_2-utilizing, acetate-producing metabolism late in the growth of all three strains was interesting. Aerobic and anaerobic growth rates were similar during the early stages of growth when glucose was still present in the medium. Upon exhaustion of glucose, anaerobic growth ceased and aerobic growth continued with the production of both lactate and acetate. The specific compounds used for late lactate and acetate production are not known. However, total lactate production was greater than allowed by the amount of glucose supplied in the medium. It is apparent, therefore, that other components of the complex medium, tryptone and yeast extract, contributed to the end products. Dirar and Collins (5, 6) have proposed a flavin-mediated (coenzyme A and lipoic acid-independent) path to O_2 for acetate production by *L. plantarum*. The two to three times increase in the specific activity of NADH peroxidase suggests that H_2O_2 may be a product of this pathway. Adenosine triphosphate produced by substrate level phosphorylation during acetate production could account for the late growth increment under aerobic conditions. Since most of the acetate was produced after glucose was exhausted from the medium, this path may be regulated by catabolite repression.

ACKNOWLEDGMENTS

We thank E. A. Delwiche for helpful discussions during the course of this work.

This work was supported in part by Public Health Service grant GM 14604, from the General Medical Science Division.

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