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FATTY ACID DEPENDENT HYDROGEN PEROXIDE PRODUCTION IN LACTOBACILLUS

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Lactobacillus leichmanii growing in complex medium supplemented with decanoic acid accumulated high concentrations of hydrogen peroxide in the culture. The H_2O_2-generating system was especifically induced by one of the saturated fatty acids from 4:0 to 16:0 or oleic acid. The induction of this system was assocciated with the presence of a fatty acyl-CoA -dependent H_2O_2 -generating activity in the cell-free extracts. This activity is shown for the first time in a procaryote organism.

Hydrogen peroxide is produced in aerobic cultures of Lactobacilli due to the activity of oxidases which catalyse the direct reduction of O_2. Pyruvate, NADH and alfa-glycerophosphate oxidases were reported in Lactobacillus organisms (1-5). Here we report a study on H_2O_2 production by Lactobacillus leichmanii growing aerobically in complex medium in the presence of fatty acids. The accumulation of H_2O_2 was associated with the presence of a fatty acyl-Coenzyme A -dependent H_2O_2 -generating activity in the cell-free extracts.

MATERIALS AND METHODS: *Organism.* Lactobacillus leichmanii ATCC 4797 maintained by subculture in reconstituted sterile skim milk (10% solution wt/vol) were used. *Media and culture conditions.* Cultures were grown aerobically at 37°C in a New Brunswick gyratory shaker, Model G 25, in 500 ml-capacity elermeyers containing 100 ml of TYPS medium (tryptone 10 g, yeast extract 10 g, peptone 15 g, succinic acid 23 g, and water to 1000 ml), pH 6.4, plus 1 % lactose and when indicated 3.3 ug/ml (8.2 U/ml) catalase. The bacteria were unable to grow on TYPS in the absence of an additional carbon source. Cultures were inoculated with bacterial suspensions grown in the same media. Turbidity was monitored at 560 nm. When the exponential cuture reached DO_{560} = 0.500, it contained about 1x 10^8 cells/ml. Fatty acids (neutralized with KOH) were added at 1mM final concentrations. Fatty acids are designated by *n:k* where *n* is the number of carbon atoms and *k* is the number of double bonds.

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Preparation of cell suspensions. Cells were harvested, at the final exponential phase of growth, by centrifugation, washed several times to eliminate exogenous catalase by resuspension in 50 mM potassium phosphate buffer (pH 7.0) containing 0.12 M NaCl and 1 mM $MgSO_4$, and the pellet was stored on ice until used within the same day.

H_2O_2 production and consumption by nongrowing cells. Thick suspensions (about 1 x 10^9 cells per ml) of cells grown in the presence of exogeneous catalase were prepared immediately before the assay in the same above buffer containing 40 ug/ml chloramphenicol. Suspensions (0.5 ml) were incubated in 50ml-capacity elermeyers at 30°C for 60 min under initial velocity conditions with constant and vigorous shaking in the presence of adequated substrates as indicated in Tables and Figures The suspensions were clarified by centrifugation in Epperdorff tubes and 0.2 ml aliquots of the supernatants were assayed for H_2O_2.

Consumption of H_2O_2 by cells grown in the absence or the presence of exogenous catalase was determined in the same buffer. Five Epperdoff tubes containing 0.5 ml of an adequate dilution of the cell suspension and 160 nmoles of H_2O_2 were incubated at 30°C and sampled at regular intervals by rapid centrifugation. 0.2 ml aliquots of the supernatants were assayed for H_2O_2. The time required for decreasing the initial H_2O_2 concentration from 100% to 85% was evaluated using at least four time points within 15 min after the addition of H_2O_2. This time was used to calculate the H_2O_2-destroying activity which was expressed as nmoles of H_2O_2 consumed per 5 min and per $1x10^9$ cells.

Preparation of cell-free extracts. Cells (about 1 g wet weight) were disrupted in 0.02 M phosphate buffer (pH 7.0) by shaking 5 ml of suspension in two separate runs for 1 min/run at 4°C with 5 g of glass beads (75 to 150 um in diameter) in a B.Braun Melsungem A.G. disintegrator. Debris was removed by centrifugation at 35,000 x g for 10 min to give a cell-free extract. The cell-free extract was stored for not more than few hours in ice.

Fatty acyl-CoA-dependent H_2O_2 generation was assayed with freshly prepared cell-free extracts (0.4-0.6 mg protein) in a medium (final volume: 0.2 ml) containing 50 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, and either 0.12 mM fatty acyl-CoA or fatty acid (1 mM decanoic acid or 0.12 mM palmitic acid) plus 0.1 mM CoA and 2.5 mM ATP according to Inestrosa et al (6). Incubations were at 30°C with shaking for 20 min.The reaction was stopped by the addition of 0.8 ml of a solution containing the reactives for H_2O_2 determination. No turbidity was noticiable.

Other procedures. Protein was estimated by the method of Lowry et al (7). Hydrogen peroxide was determined by modification of the o-dianisidine/ horseradish peroxidase method (8) adding 0.8 ml of 10 mM phosphate buffer (pH 7.4) containing 0.16 mM o-dianisidine, 1.2 ug per ml peroxidase and 0.02% Triton X-100 to 0.2 ml of sample.

RESULTS: *Accumulation of H_2O_2 in the culture medium in the presence of decanoic acid.* The generation of H_2O_2 during the growth of L. leichmanii on TPYS-Lactose medium or TPYS-Lactose plus decanoic acid was examined. Fig. 1 shows that both cultures accumulated H_2O_2, but the presence of the fatty acid increased five-fold the concentration of H_2O_2 compared to the culture medium without fatty acid. The maximun cell yield was lower in TPYS-Lactose plus decanoic acid than in TPYS-Lactose cultures. When exogenous catalase was added to both culture media, the cell yield increased only in TPYS-Lactose plus fatty acid (Fig. 1), indicating that the accumulation of H_2O_2 at high levels (about 7 mM) has an inhibitory effect on these cells.

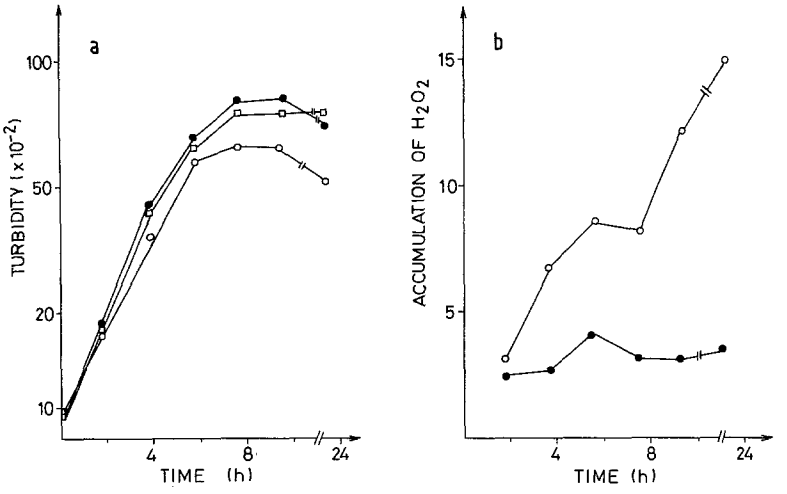

Fig 1. A: Growth of L. Leischmanii ATCC 4797 in TPYS - Lactose or TPYS-lactose plus catalase (●); TPYS-Lactose plus decanoic acid (○); and TPYS-lactose plus decanoic acid plus catalase (□). B:Accumulation of H_2O_2 in the culture media from cells grown on TPYS-lactose (●) and TPYS-lactose plus decanoic acid (○). Accumulation of H_2O_2 is expressed as the ratio mM of H_2O_2 accumulated per turbidity of culture.

As will be shown below, development of H_2O_2 in the culture medium induced a H_2O_2-detroying activity in the cells. Addition of exogenous catalase avoided the accumulation of H_2O_2 in the medium and the presence of H_2O_2-destroying activity in the cell. Since the H_2O_2-destroying activity difficulted the measurement of H_2O_2 generation by cells and cell-free extracts, these measurements were performed using cells grown in the presence of exogenous catalase.

Production of H_2O_2 by resting cells grown on TPYS-Lactose plus decanoid acid. To measure exactly the amount of H_2O_2 generated from fatty acids, the cells harvested from culture medium containing exogenous catalase, were washed several times to eliminated this catalase activity, and concentrated ten-fold before assaying H_2O_2 production in buffer containing chloramphenicol to block further enzymatic induction (both H_2O_2-detroying or H_2O_2-generating systems).

Under this experimental condition, with cells previously grown on TPYS-lactose plus decanoic acid, the level of H_2O_2 in the assay medium appreciably increased in the presence of decanoic acid but not in its absence. Omittion of lactose did not impede the H_2O_2 production (Fig 2). On the other hand, cells grown in the absence of decanoic acid did not generated H_2O_2, within the sensitivity of the assay, when incubated with decanoic acid under the above conditions. Levels of H_2O_2 lower than 7 nmol can not be detected.

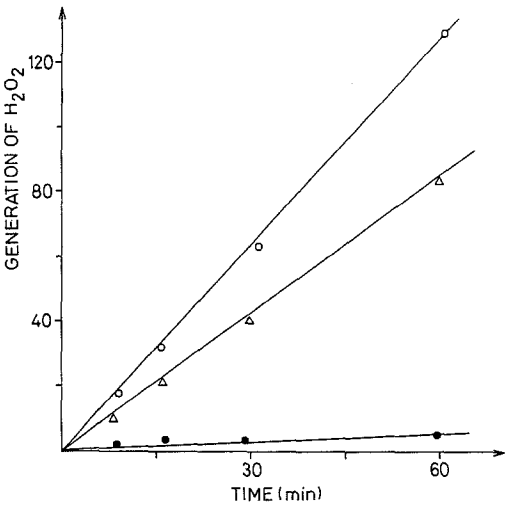

Fig 2. Generation of H_2O_2 by resting cells evaluated in the presence of 10 mM lactose (●), 1 mM decanoic acid (△) and 10 mM lactose plus 1 mM decanoic acid (○). The cells were grown on TPYS lactose plus decanoic acid, washed and resuspended as indicated in Materials and Methods. Generation of H_2O_2 is expressed as nmol of H_2O_2 per 1 x 10^9 cells.

Effect of different fatty acids on the production of H_2O_2 by resting cells. When other fatty acids (saturated fatty acids from 4:0 to 16:0 and oleic acid), instead of decanoic acid, were added to resting cells which had been grown on TPYS-Lactose plus decanoic acid, the level of H_2O_2 generated was similar to that observed with decanoic acid. This fact indicated that the H_2O_2-generating system induced by decanoic acid was also able to utilize several other fatty acids for the production of H_2O_2 (Table 1). None of these fatty acids generated H_2O_2 in resting cells which had been grown in the absence of a fatty acid (not shown).

Table 1. Production of H_2O_2 by resting cells previously grown on TPYS-lactose plus decanoic acid

Addition to H_2O_2-assay medium*	Generation of H_2O_2 (nmole of H_2O_2 per 60 min per 10^9 cells)
Lactose	0
plus 4:0	100
plus 6:0	115
plus 7:0	110
plus 8:0	110
plus 10:0	130
plus 12:0	65
plus 16:0	130
plus oleic acid	150

* H_2O_2-generating cell activity was evaluated as in Fig 2 in the presence of lactose and fatty acids at concentrations of 10 mM and 1 mM, respectively.

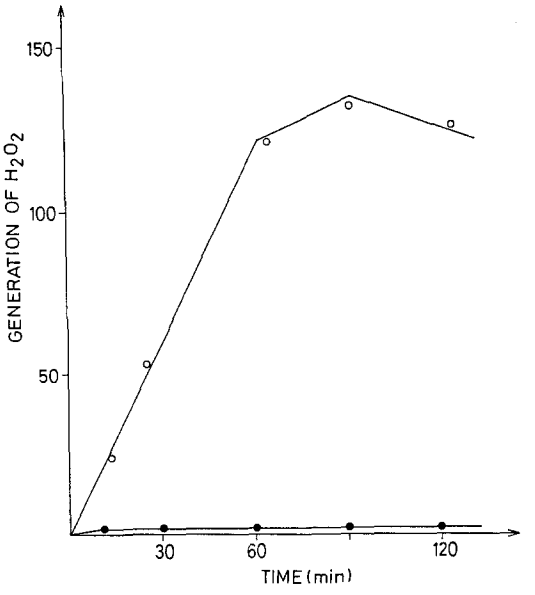

Fig 3. Induction of the H_2O_2-generating system by decanoic acid: L. Leischmanii ATCC 4797 was grown in TPYS-lactose medium containing catalase to final of logaritmic phase (DO_{600} = 800). Aliquots of 10 ml of this culture were added to several 250 ml elermeyers containing 10 ml of same fresh medium with or without 2 mM decanoic acid and incubated under vigorous shaking. At different times the cells from one elermeyer were harvested by centrifugation, washed several times to elimined exogenous catalase and resuspendend in 2 ml buffer. The production of H_2O_2 by these cellular suspensions was evaluated in the presence of 10 mM lactose plus 1 mM decanoic acid as indicated in Materials and Methods. Symbols: induced culture (○); noninduced culture (●). Generation of H_2O_2 is expressed as nmol of H_2O_2 per 60 min per 1 x 10^7 cells.

Induction of H_2O_2 production by decanoic acid. Fig. 3 shows the time dependent induction of the H_2O_2-generating system after the addition of decanoic acid to cells growing on TPYS-Lactose. Full induction was obtained after 60 min. This induction was prevented by the presence of chloramphenicol in the culture medium. Parallele experiments indicated that the presence of decanoic acid during 120 min in culture media without exogenous catalase also induced the H_2O_2-destroying system to a level about 30-fold higher than that in the absence of the fatty acid (40 and 1,200 nmol of H_2O_2 per 5 min per 1 x 10^7 cell for lactose and lactose plus decanoic cultures, respectivily). A similar increment was found when the H_2O_2 destroying activity was determined in the cell-free extracts.

Presence of fatty acyl-CoA -dependent H_2O_2 -generating activity in cell-free extracts. Table 2 shows that cell-free extracts from cells grown on TPYS-Lactose plus decanoic acid were able to generated H_2O_2. Supplementation with either decanoic or palmitic acid plus CoA and ATP led to H_2O_2 production. When CoA or ATP

Table 2. Cofactor requirements for production of H_2O_2 in cell-free extracts from cells grown on TPYS-lactose plus decanoic acid

Incubation mixture	Generation of H_2O_2 (nmol of H_2O_2 per 20 min per mg protein)
Basal	0
plus decanoic acid	0
plus palmitic acid	0
plus decanoic acid + ATP	0
plus palmitic acid + ATP	0
plus decanoic acid + CoA	0
plus palmitic acid + CoA	0
ATP + CoA	0
plus decanoic acid + ATP + CoA	66
plus decanoyl-CoA	90
plus palmitic acid + ATP + CoA	88
plus palmitoyl-CoA	80
plus hexanoyl-CoA	96
plus lauroyl-CoA	48
plus oleyl-CoA	100

was omitted in the incubation medium no H_2O_2 was detected. This result was considered to be evidence that fatty acids must be activated to the acyl-CoA derivative, by endogenous acyl-CoA synthetase, before to reach the enzyme system able to produce H_2O_2. Thus, as shown in Table 2, the corresponding acyl-CoA were the true substrates for the production of H_2O_2. Table 2 also shows that several different fatty acyl-CoA were able to support directly the H_2O_2 production by cell-free extracts. When the extract was filtrated on Sephadex G 50, this activity remained in the same level, indicating that endogenous cofactors of low molecular weight (eg NAD) were not necessary. This is an important indication that NADH oxidase does not participate in the above H_2O_2 production by the cell-free extract. NADH might have been generated by fatty acid metabolism through the β-oxidation system (6).Table 3 shows that the induction of the H_2O_2-generating system in whole cells by different fatty acids was always associates with the presence of a fatty acyl-CoA -dependent H_2O_2-generating activity in the cell-free extract. This activity was absent in the extracts of cells grown in media without a fatty acid supplement.

DISCUSSION: In eucaryotic cells β-oxidation occurs in mitochondrial and peroxisomal organelles. Unlike the mitochondrial system, the first dehydrogenation step in the B-oxidation of fatty acids by peroxisomes involves the reduction of O_2

Table 3 Induction of the H_2O_2-generating system by different fatty acids[a]

Fatty acid added to the culture	Generation of H_2O_2[b] (nmol of H_2O_2 per 60 min per 10^7 cells)		Acyl-CoA oxidase[c] (nmol per 20 min per mg protein)		
	Lactose	Lactose plus decanoic acid	Hexanoyl -CoA	Lauroyl -CoA	Palmitoyl -CoA
None	0	2	0	0	0
4:0	6	110	40	42	70
6:0	6	80	90	90	70
7:0	5	80	ND	ND	72
8:0	4	96	90	88	ND
10:0	7	100	80	80	66
12:0	0	50	ND	ND	45
16:0	ND[d]	ND	ND	ND	85
oleic acid	7	96	ND	80	68

[a] The induction was performed as indicated in Fig 3 and the cells harvested 1h after fatty acid addition.
[b] H_2O_2-generating cell activity was evaluated as in Fig 2 in the presence of 10 mM lactose or 10 mM lactose plus 1 mM decanoic acid.
[c] H_2O_2-generating cell-free extract activity was evaluated as indicated in Materials and Methods.
[d] ND: not determined

to H_2O_2, first suggested by Cooper and Beevers (9), who found that β-oxidation in peroxisomes of caster bean endosperm (called glyoxisomes) was accompanied by stoichiometric formation of H_2O_2. Since the discovery that fatty acid β-oxidation in liver occurs not only in the mitochondria but also in peroxisomes (10), the existence of peroxisomal β-oxidation has also been reported in other mammalian tissues (11-16), Tetrahymena (17), yeast (18) and fungi (19). Although the question of the relative importance of mitochondrial and peroxisomal B-oxidation is still controversial, it has been suggested that the two systems each with its own substrate specificity (20-21) and mode of regulation (10,22-25) are complementary. A previous paper of this laboratory (26) showed the presence in Lactobacillus leichmanii of a fatty acid oxidation system involving β-hydroxyacyl-CoA dehidrogenase and thiolase activities, two enzymes of the fatty acid B-oxidation cycle. The present work suggests the presence of a peroxisomal-like B-oxidation system in this lactic bacteria. The existence of an additional B-oxidation system, similar to that operating in mitochondria, in whir' he first step of dehidrogenation does not generate H_2O_2 is also possible. two systems might operate simultaneously or be induced selectively under specific conditions. This point is under investigation. This paper indicates that the additional level

of H_2O_2 in cultures containing a fatty acid (Fig. 1) was produced by the presence of a fatty acyl-CoA -dependent H_2O_2-generating activity reported for the first time in a procaryote organism. Whether one or several enzymes are involved in this activity, as well as the characterization of fatty acid oxidation product, etc, are facts that deserve further investigation.

The appearence of conspicuous numbers of peroxisomes in yeast cells grown on alkanes or higher fatty acids is accompanied by a remarkable increase in the cellular catalase activity,(18). It was shown that peroxisomes contain catalase activity which is utilized to destroy H_2O_2. As show in this paper, in the case of Lactobacillus leichmanii the induction of H_2O_2 -generating system is also accompanied by a paralelle increase in the of H_2O_2-destroying activity. It is an important experimental point since the induction of the H_2O_2-destroying activity in cultures without exogenous catalase strongly interfered with the detection of H_2O_2 in both whole cell systems and cell-free extracts.

The ability to produce H_2O_2 is widespread among lactic bacteria (27). The concentration of H_2O_2 in aerated cultures is sometimes autoinhibitory (28-30) and sometimes inhibitory to other bacteria in the same environment (31-34). The potential antibacterial action of H_2O_2 produced by lactic bacteria due to fatty acid metabolism in a natural environment as milk must be considered. The level of free fatty acids in milk is low but increases by milk lipolysis in several conditions e.g. mechanical agitation, nutrition (poor quality feed), mastitis etc (35). In underfeeding, the free fatty acid content of milk increases from 1 mM to 6 mM (36). Preservation of milk and other lactic products by H_2O_2 generated "in situ" from natural substrates (free fatty acids) through the activity of Lactic bacteria described here appears as an attractive prospect in the field of food technology. In this context, it is important to note that two strains of L. bulgaricus CRL 420 and CRL 424 isolated in our laboratory from commercial yoghurt showed fatty acid-dependent H_2O_2-generating activities in both whole cells and cell-free extracts.

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