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Manganese and Defenses against Oxygen Toxicity in *Lactobacillus plantarum*

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Lactobacillus plantarum is aerotolerant during log-phase growth on glucose, but is an obligate aerobe on polyols. Respiration was cyanide resistant and under certain conditions was associated with the accumulation of millimolar concentrations of H_2O_2. On glucose, optimal growth was observed in the absence of O_2. Extracts of *L. plantarum* did not catalyze the reduction of paraquat by reduced nicotinamide adenine dinucleotide, but plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was readily reduced. Such extracts produced O_2^- in the presence of NADH plus plumbagin. Plumbagin caused a 10-fold increase in the rate of respiration of intact cells in the presence of glucose and also imposed a loss of viability which was dependent upon both glucose and O_2. Although extracts of *L. plantarum* were devoid of true superoxide dismutase activity, this organism was comparable to superoxide dismutase-containing species in its resistance toward hyperbaric O_2 and toward the oxygen-dependent lethality of plumbagin. *L. plantarum* required Mn-rich media and actively accumulated Mn(II). Soluble extracts were found to contain approximately 9 μg of Mn per mg of protein and 75 to 90% of this Mn was dialyzable. Such extracts exhibited a dialyzable and ethylenediaminetetraacetic acid-inhibitable ability to scavenge O_2^-. This O_2^--scavenging activity was due to the dialyzable Mn(II) present in these extracts and could be mimicked by $MnCl_2$. Cells grown in Mn-rich media were enriched in dialyzable Mn and were more resistant toward oxygen toxicity and toward the oxygen-dependent plumbagin toxicity than were cells grown in Mn-deficient media. *L. plantarum* exhibited no nutritional requirement for iron and little or no iron was present in these cells, even when they were grown in iron-rich media. *L. plantarum* thus appears to use millimolar levels of Mn(II) to scavenge O_2^-, much as most other organisms use micromolar levels of superoxide dismutases.

The superoxide theory of oxygen toxicity posits that the univalent reduction of dioxygen is a commonplace event and that the superoxide radical is an important agent of oxygen toxicity. Superoxide dismutases (SOD), which catalytically scavenge this radical, are thought to provide an essential defense. In one of the early tests of this theory (30), a variety of microorganisms were surveyed for their contents of SOD and catalase. The aerobes were found to contain both of these activities, the aerotolerant anaerobes contained SOD but no catalase, and the strict anaerobes contained neither SOD nor catalase. Among the organisms studied, *Lactobacillus plantarum* at first appeared to be anomalous in that it could grow in the presence of dioxygen, yet contained no SOD or catalase. An apparent explanation was found in the lack of respiration of log-phase cultures of *L. plantarum* growing on a medium containing glucose (16, 30). Thus, cells which do not reduce dioxygen do not produce O_2^- or H_2O_2, and so can dispense with enzymatic scavengers for these reactive intermediates. These results were confirmed in subsequent studies, which also demonstrated that *L. plantarum* was more resistant to the lethality of hyperbaric oxygen than was *Escherichia coli* (16).

Yet *L. plantarum* and related organisms have long been known to contain soluble oxidases based upon flavin adenine dinucleotide and to consume O_2 under some conditions (37, 38). The question of oxygen metabolism and toxicity in *L. plantarum* was reopened by Yousten et al. (41), who noted that respiration increased during the stationary phase in glucose-exhausted media and reported low levels of SOD in extracts of these cells. Iwamoto et al. (23, 24) described an inducible NADH oxidase in *L. acidophilus* and noted that respiration was a function of both carbon source and culture age. Götz et al. (13, 14) also confirmed Strittmatter's finding of an NADH oxidase and peroxidase in *L. plantarum* and reported the presence of low levels of SOD. A thorough reexamination of respiration, of oxygen toxicity, and of the scavenging of O_2^- in *L.*

plantarum was clearly needed to clarify the apparent conflicts in the literature, and such a study is described here. The results indicate that *L. plantarum* uses an unusual means of scavenging O_2^-.

MATERIALS AND METHODS

Organisms and culture conditions. *L. plantarum* strains 14917 and 8014 were obtained from the American Type Culture Collection (ATCC), Rockville, Md. *E. coli* B, *Bacillus megaterium*, *Streptococcus lactis*, and *S. faecalis* were all either direct ATCC type strains or derived from them. Lyophile cultures were subcultured onto glucose-APT agar slants, which were stored at −60°C until used. Each experiment used a fresh agar slant.

Cells were grown on glucose-APT (9) lacking Tween 80. Glucose was added after autoclaving, and iron or manganese was varied as dictated by experimental design. In some experiments 50 mM mannitol, 50 mM sorbitol, or 5 mM glucose plus 50 mM glycerol replaced the 50 mM glucose. MRS medium (8) was obtained from Difco Laboratories. In some experiments involving metal restriction, Chelex-100, Na^+ form, from BioRad Laboratories, was used to remove traces of metal from APT by stirring for 1 h at 25°C, followed by vacuum filtration through Whatman no. 1 filter disks. This filtration was repeated three times, through fresh filter disks, to ensure complete removal of the Chelex-100. Manganese, potassium, and a trace metal solution were then added. The final concentrations of Cu, Mo, Zn, and Co were 0.1 μg/ml. APT was found to contain 1.6 to 2.0 μM Mn before Chelex treatment. In contrast, the APT medium, as formulated (9), contained 710 μM Mn and 290 μM Fe.

Aerobic cultures were shaken at 116 rpm at 37°C. Anaerobic growth was performed in a Coy chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). Exposure to hyperbaric oxygen was performed at 37°C in stainless-steel pressure vessels. All liquid cultures were grown in Erlenmeyer flasks with a ratio of vessel volume to fluid volume of 5:1. Culture growth was followed turbidimetrically in 50-ml Ryan flasks. This was validated by enumerating cells under the microscope with a Neubauer hemacytometer and by relating such direct counts to turbidity at 600 nm. *L. plantarum* cultures were seen to contain numerous pairs and groups of cells, with up to five cells per group, in addition to single cells. Since the average colony-forming unit was two cells, cell enumeration by plating and counting colonies would underestimate cell number by a factor of two.

Cells were harvested by centrifugation. They were washed once in APT-salts at pH 6.7 and then suspended in 50 mM potassium phosphate, pH 7.8, if they were to be used for assays of O_2^- scavenging or in APT-salts if they were to be used for measurements of respiration or viability. Cells in the phosphate buffer were disrupted by double passage through a French pressure cell (Aminco) at 20,000 lb/in^2, and the resultant extract was then clarified for 10 min at 8,700 × *g*.

Assays. A Perkin-Elmer model 107 atomic absorption spectrophotometer equipped with an HGA-2000 graphite furnace was used to measure metal contents. O_2^--scavenging activity was measured by the xanthine oxidase-cytochrome *c* method (29) with the omission of EDTA. Where noted, the cytochrome *c* was replaced by Nitro Blue Tetrazolium. Two photochemical assays for the scavenging of O_2^- were also used. One of these used Nitro Blue Tetrazolium (3), and the other used dianisidine (32). In all cases, 1.0 U of O_2^--scavenging activity was taken to be that amount causing either a 50% inhibition of the base-line rate (3, 29) or a doubling of the base-line rate (32). These assays were standardized with bovine erythrocyte SOD.

Electrophoresis on 10% polyacrylamide was performed according to Davis (7). O_2^--scavenging activity, on gel electropherograms, was stained as previously described (3). Oxygen consumption was measured polarographically at 37°C with a Clark electrode. H_2O_2 was measured in terms of the O_2 evolved upon addition of catalase.

Reagents. Acetaldehyde was from Eastman Organic Chemicals, glycerol was from Mallinkrodt, and catalase was from Worthington Biochemicals Corp. SOD was isolated from bovine erythrocytes (29); xanthine oxidase was isolated from unpasteurized cream (40). Ethylenediamine di(*o*-hydroxyphenyl acetate) (EDDA) was purified according to Rogers (34). Culture media were from Difco, with the exception of Trypticase peptone, which was from BBL Microbiology Systems. APT-salts contained, per liter, 5.0 g of K_2HPO_4, 5.0 g of NaCl, 1.25 g of Na_2CO_3, and 0.8 g of $MgSO_4$; the pH was 6.7. Miscellaneous organic reagents were from Sigma Chemical Co.

RESULTS

Respiration and oxygen tolerance. *L. plantarum* grown on glucose-APT showed low but measurable respiration, increasing in late log phase. However, when mannitol or sorbitol was used in place of glucose, rapid respiration was evident throughout the growth cycle (Table 1). Glycerol per se would not support growth, but cells placed in 5 mM glucose–50 mM glycerol-APT induced glycerol oxidation and commenced rapid respiration after the glucose was exhausted. In agreement with previous reports (16, 30), the respiration of *L. plantarum* on glucose-APT was ≤1% of that shown by *E. coli* on a comparable medium, and in all cases was unaffected by 1 mM KCN (Table 1).

L. plantarum grew most rapidly in anaerobic glucose-APT medium, and raising the pO_2 progressively slowed the growth rate (Fig. 1). Likewise, overnight growth of both strains of *L. plantarum* on MRS and glucose-APT agar plates, aerobically and anaerobically, showed the absence of O_2 to produce the best cell yield. The O_2 sensitivity of the *L. plantarum* was also tested and compared with that of several SOD-containing bacteria. *E. coli*, a facultative anaerobe, *B. megaterium*, an obligate aerobe, and *S. faecalis*, an aerotolerant organism, were compared with the two *L. plantarum* strains for

TABLE 1. *Oxygen consumption and H_2O_2 production by L. plantarum 14917 and E. coli B*

Organism and growth medium	Mid-log cells[a]			Late-log cells[b]			Stationary-phase cells[d] mM H_2O_2
	Oxygen consumption[c]		mM H_2O_2[f]	Oxygen consumption		mM H_2O_2	
	$-CN^-$	$+CN^-$[e]		$-CN^-$	$+CN^-$		
L. plantarum on glucose-APT	0.29	0.38	0	0.69	1.01	0–0.7	2.0
L. plantarum on 5 mM glucose–50 mM glycerol-APT	0.22	0.27	0	14.7	14.9	0.3	0.3[g]
L. plantarum on mannitol-APT	17.7	17.7	0	15.7	15.7	0.08	4.8
L. plantarum on sorbitol-APT	13.8	14.6	0	15.4	15.2	0	7.2
E. coli on nutrient broth	43.5	0.9	0	64.5	0.10	0	

[a] Two to eight hours postinoculation with $\lesssim 4 \times 10^8$ cells per ml of culture medium.
[b] Late log cells = 8 to 14 h postinoculation with $\approx 2 \times 10^9$ cells per ml of culture medium.
[c] Expressed as nanomoles of O_2 consumed per minute per 10^8 cells at 37°C in fresh medium.
[d] Twenty to twenty-six hours postinoculation.
[e] Where indicated, 1.0 mM cyanide was present during measurement of respiration.
[f] Concentration that had accumulated in the culture medium up to the time of cell harvest.
[g] Cell density reached a plateau at 1.5×10^8 to 2.0×10^8/ml. This was close to the yield seen on 5 mM glucose alone and was achieved after 6 to 8 h of growth. It thus appears that growth occurred chiefly on glucose, although the cells did oxidize the glycerol by an inducible enzyme once the glucose was exhausted.

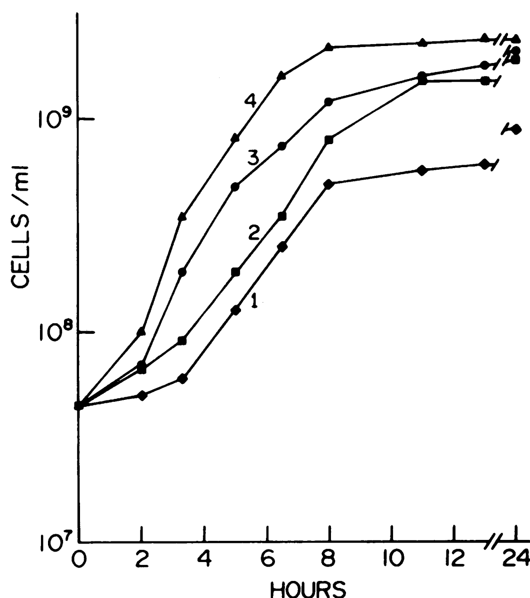

FIG. 1. *Effects of plumbagin and of pO_2 on growth of L. plantarum. L. plantarum strain 14917 was grown overnight on anaerobic glucose-APT, and this culture was used to inoculate Ryan (side-arm) flasks inside an anerobic Coy chamber. A 2% inoculum was used in all cases, and the flasks were incubated at 37°C on a shaker while growth was monitored turbidimetrically at 600 nm. Line 4, Flasks incubated under anaerobic conditions. Line 3, Flasks incubated under air. Line 2, Flasks incubated under a stream of filtered 100% O_2. Line 1, Flasks containing 10 μM plumbagin and incubated under air.*

growth under and inhibition by 1, 2, 5, 10, and 18 atm of pure O_2 on glucose-APT agar plates. After growth for 17 h at 37°C, all cells were harvested from each plate and enumerated. All grew well at 1 atm of O_2, but the cell yield at 2 atm of O_2 of *E. coli*, *B. megaterium*, and *L. plantarum* 8014 and 14917 was reduced by 98, 99, 74, and 56%, respectively. Little or no growth of any of these organisms occurred at 5 or 10 atm of O_2; however, except for *B. megaterium*, the inocula remained viable and, when subsequently incubated in air, the 5- and 10-atm plates yielded heavy growth. *S. faecalis* was highly tolerant of hyperbaric O_2, showing a 69% decrease at 10 atm and a 98% decrease at 18 atm. Growing cultures of *L. plantarum* not only were somewhat more tolerant of hyperbaric O_2 than *E. coli* and *B. megaterium* but remained viable even at 10 atm of O_2.

Mannitol- and sorbitol-APT, unlike glucose-APT, did not support anaerobic growth but, aerobically after an initial lag, supported growth equal in rate and extent to that of glucose despite the accumulation of substantial H_2O_2 (Table 1).

Tolerance for H_2O_2. Whereas the presence of the NADH-dependent peroxidase reported by other workers (13, 38) was confirmed in log-

phase glucose-APT-grown cells (data not presented), H_2O_2 accumulation late in the growth cycle, particularly on mannitol- and sorbitol-APT, indicated the lack of an efficient means of scavenging H_2O_2. The ability of stationary-phase mannitol- and sorbitol-grown cultures to remain viable in the presence of >4 mM H_2O_2 suggests a surprising level of resistance to this oxidant. In another experiment, 1.2×10^7 washed log-phase *L. plantarum* 14917 cells per ml, grown in glucose-APT, could be incubated in 4.0 mM H_2O_2 in APT-salts buffer for 40 min at 37°C with no more than 10% loss in viability.

Effects of intracellular of O_2^- production. Several compounds have been shown to increase cyanide-resistant respiration and to exacerbate oxygen toxicity in *E. coli* (19, 21). Their net effect is diversion of electron flow from the normal respiratory pathway, by a mechanism involving cyclical reduction followed by autoxidation, with a concomitant increase in the generation of O_2^- and H_2O_2. Some of the compounds found to act in this way in *E. coli* (19) were tested with *L. plantarum*. Paraquat (methyl viologen) was not active in *L. plantarum* because of the lack of a diaphorase capable of catalyzing its reduction by NAD(P)H. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), however, was effective and at 10 μM increased the respiration of *L. plantarum* on glucose-APT from 0.38 to 3.6 nmol of O_2 per 10^8 cells per min and slowed aerobic growth (Fig. 1). As expected, plumbagin had no effect on anaerobic growth even when tested at 100 μM (data not shown). Phenazine methosulfate and juglone (5-hydroxy-1,4-naphthoquinone) were also capable of increasing respiration and of exacerbating growth inhibition by O_2.

Figure 2 illustrates the ability of plumbagin to increase the respiration of intact cells in the presence of glucose or of cell extracts in the presence of NADH and plumbagin. Since NADH presumably does not readily enter intact *L. plantarum* cells (18; Fig. 2), there was very little effect of plumbagin on the oxidation of NADH by cells. Increased respiration in extracts in the presence of plumbagin was associated with the production of O_2^- (Fig. 3). Extracts of *L. plantarum* catalyzed a rapid reduction of cytochrome *c* in the presence of NADH plus plumbagin, and this was suppressed by SOD. In the absence of plumbagin there was no perceptible reduction of cytochrome *c* by a variety of combinations of cell extract, substrate, and NAD(P)H (data not shown). Plumbagin mediated only a small amount of reduction of cytochrome *c* by intact *L. plantarum* cells in the presence of glucose. This low observed O_2^- evo-

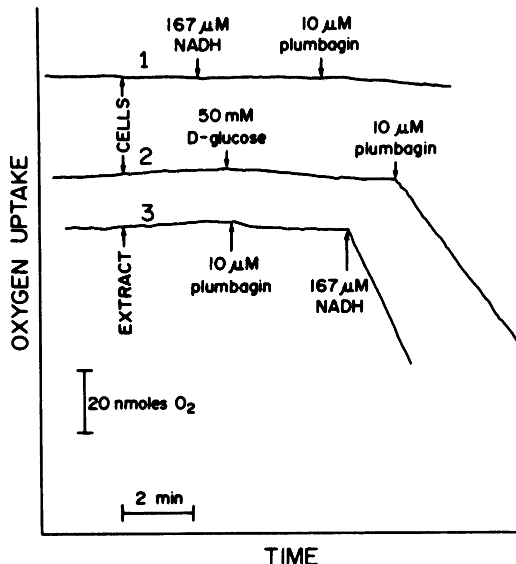

FIG. 2. *Oxygen consumption by intact cells and by cell extracts. L. plantarum strain 14917 was grown to mid-log phase on glucose-APT and was then washed in APT-salts and equilibrated with these salts for 1 h. Cell extracts were prepared from such resting cells by use of the French press and centrifugal clarification, as described in Materials and Methods. Line 1, Clark polarographic trace of 4.2×10^8 cells/ml presented first with NADH and then with plumbagin. Line 2, Obtained when the cells were presented with glucose followed by plumbagin. Line 3, Oxygen uptake of extracts of 4.2×10^8 cells/ml prepared as described under Materials and Methods. Addition of plumbagin to the extract caused no O_2 uptake, whereas subsequent addition of NADH caused a rapid uptake (0.21 µmol of O_2/min). When this order of addition to the cell extract was reversed, NADH caused an O_2 consumption of 0.09 µmol of O_2/min; subsequent addition of plumbagin again caused an uptake of 0.21 µmol O_2/min. In all cases the reaction volume was 2.0 ml and the temperature was 37°C.*

lution by whole cells strongly suggested that O_2^- production was occurring largely within the cell and that the cell wall was impermeable to O_2^-, as in the case of *E. coli* (20). The low cytochrome *c*-reducing activity that was seen could have been due to the effect of plumbagin diaphorase released by a small degree of cell lysis or by some escape of reduced plumbagin from intact cells.

If the effect of plumbagin on *L. plantarum* was due to increased internal production of O_2^- and of H_2O_2, as it appears to be in *E. coli* (20, 21), then it should be entirely dependent both upon a source of electrons and upon O_2. As expected, 100 μM plumbagin had very little effect upon the viability of *L. plantarum* when

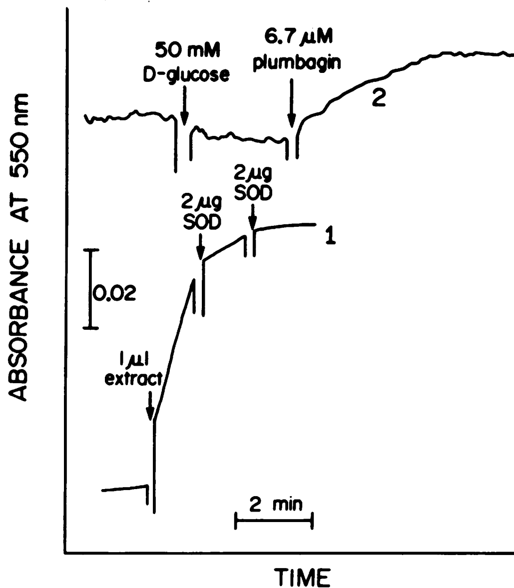

FIG. 3. *O_2^- generation by intact cells and by cell extracts. O_2^- production was followed in terms of the reduction of cytochrome c at 550 nm and at 37°C. Line 1, 3.0-ml reaction mixtures contained 6.7 µM plumbagin, 10 µM ferricytochrome c, 100 µM EDTA, 167 µM NADH, and 50 mM potassium phosphate, pH 7.8. Where indicated, 1 µl of extract containing 8 µg of protein was added, followed by two successive 2-µg portions of SOD. Line 2, Reaction mixture contained log-phase cells grown in glucose-APT and diluted to 60 µg of cell protein per 3-ml reaction volume (10 µM ferricytochrome c, 100 µM EDTA, and 50 mM potassium phosphate at pH 7.8). Where indicated, glucose and then plumbagin were added.*

incubated aerobically with these cells for 100 min in APT-salts, pH 6.7, at 37°C. In contrast, when 50 mM glucose was also present, there was a 98% loss of viability. The oxygen requirement of plumbagin toxicity was demonstrated by placing 0.5 µmol of plumbagin in 4.0-mm wells in glucose-APT agar plates that had been seeded with a lawn of *L. plantarum*. The plates were incubated anaerobically, aerobically, or under 100% O_2 overnight at 37°C. In the absence of O_2 there was no zone of growth inhibition around the wells, whereas aerobically the area of inhibition was 1,200 to 1,400 mm^2 and under 100% O_2 it was 1,600 to 1,800 mm^2. Several SOD-containing organisms were similarly compared with *L. plantarum* for their susceptibilities to growth inhibition by plumbagin under aerobic conditions. The zones of growth inhibition were 560, 385, 367, 301, and 82 mm^2 for *S. lactis*, *L. plantarum* ATCC 14917, *B. subtilis*, *B. megaterium*, and *S. faecalis*, respectively. The lethality of plumbagin could have been due to H_2O_2, to O_2^-, or to both of these acting in concert.

SOD assays. The xanthine oxidase-cytochrome *c* assay for SOD was performed on cell extracts of both strains of *L. plantarum*, grown aerobically in glucose-APT. Assays were also performed on cell extracts derived from cells stressed by growth in 10 µM plumbagin plus 100% O_2 and on extracts of vigorously respiring cells grown on sorbitol-APT and on mannitol-APT. Cyanide-resistant respiration, a high environmental pO_2, and plumbagin have all been shown to correlate with large increases in the total SOD of *E. coli* (15, 18, 19), but no true SOD (or catalase) activity was seen in *L. plantarum* extracts under these conditions. There was some apparent inhibition of cytochrome *c* reduction by the most concentrated cell extracts (5 to 26 mg of protein/ml), but this could be entirely accounted for by a small amount of noncatalytic cytochrome *c* oxidation and a slight inhibition of xanthine oxidase activity by cell extracts, as measured by a decreased rate of urate production assessed at 295 nm.

These cell extracts were also subjected to polyacrylamide gel electrophoresis, followed by activity staining for SOD. This procedure will detect 10 ng of SOD, yet even when 1 mg of cell protein was loaded onto each gel, no typical achromatic SOD bands were seen. A single, very faint band coincident with a heavy band of protein was seen with strain 8014. This was presumably an artifact of the activity-staining procedure or a nonspecific O_2^- scavenger of very low activity, since no SOD activity could be detected by the standard solution assay.

***L. plantarum*, Mn(II), and O_2^- scavenging.** For over 30 years the lactobacilli have been known to require large amounts of Mn for growth (28). More recently, Mn(II) has been shown to be effective in scavenging O_2^- (5, 25, 27, 35), except in the presence of EDTA (27, 35, 36). Since xanthine oxidase is susceptible to an activity-dependent inactivation by trace metals (12), 0.1 mM EDTA is routinely included in the SOD assay; in the presence of EDTA, no O_2^--scavenging activity could be detected in *L. plantarum*. Omission of EDTA from the assay mixture permitted undialyzed *L. plantarum* cell extracts to demonstrate an O_2^--scavenging activity equivalent to 24 to 80 U of SOD per mg of cell extract protein. Addition of EDTA to the assay immediately and completely eliminated the inhibition of cytochrome *c* reduction. Controls demonstrated that even in the absence of EDTA, extracts had little effect on xanthine oxidase activity and that the xanthine oxidase was only slightly inactivated during the time of the assays.

Several lines of evidence suggest that this EDTA-sensitive O_2^--scavenging activity is due

to free or loosely bound Mn within the cell. The activity was completely resistant to boiling and increased somewhat upon repeated freezing and thawing. Extracts of *L. plantarum* were rich only in Mn, and only Mn showed substantial loss after the dialysis, which also markedly decreased the O_2^--scavenging activity of the extracts (Table 2). In contrast, the SOD activities of *E. coli* and *S. faecalis* were largely unaffected by EDTA. To ensure that this apparent EDTA-sensitive inhibition of cytochrome *c* reduction was not an artifact of the xanthine oxidase-cytochrome *c* assay, several other SOD assays were also used (Table 3). Despite standardization of the assays with a known amount of purified bovine erythrocyte SOD, there was a certain amount of variation in the values obtained by the different assays; however, Mn(II) and extracts of *L. plantarum* were clearly seen to scavenge O_2^-. Furthermore, the specific activity and concentrations of Mn determined to be present in these extracts could account for all the O_2^--scavenging activity measured. Washed cells of both strains were pelleted by centrifugation (8,700 × *g*, 5 min), and the pellets were found to contain 12 to 19 mM Mn. If one allows 25% for interstitial space, the cells themselves must contain 16 to 25 mM Mn.

The data in Table 2 indicate that *L. plantarum* contained very little Fe. Indeed, it appeared possible that this organism requires no Fe at all and that the low levels found represented contamination. *L. plantarum* strain 14917 was grown in glucose-APT containing 23, 1.3, and <0.2 μM Fe. The latter condition was achieved by treating the components with Chelex-100 to remove adventitious Fe and by adding 36 μM EDDA to sequester residual Fe. It should be noted that EDDA competes effectively enough with the siderophores of enteric bacteria for iron to inhibit their growth even in iron-sufficient media (31, 34). Iron limitation had no effect on the growth of *L. plantarum* (Fig. 4).

Mn, in contrast, was avidly taken up from the medium, and any limitation in its availability limited growth. Thus, decreasing Mn below 30 μM in the glucose-APT medium severely decreased growth (Fig. 5). It should be noted that

TABLE 3. *Comparisons of assays for scavenging of* O_2^-

Sample	Assay used[a]			
	Xanthine oxidase-cytochrome *c*	Xanthine oxidase-NBT	Photochemical *o*-Dianisidine	Photochemical NBT
Cell extract of *L. plantarum* 14917 grown on mannitol-APT	62	79	22	76
Cell extract of *L. plantarum* 14917 grown on glucose-APT	50	75	29	73
Cell extract of *S. faecalis* grown on glucose	56	44	22[b]	77
Cell extract of *S. faecalis* as above + 10 μM EDTA	43	36	13[b]	50
1 μM $MnSO_4$[c]	2.3 2.0[d]	2.4	2.8	2.1

[a] In all assays, 1 U is defined as the amount of the test material required to inhibit by 50% or, in the case of the photochemical *o*-dianisidine assay, to double the base-line rate. NBT, Nitro Blue Tetrazolium.

[b] Extracts of *S. faecalis* exhibited disproportionately low O_2^--scavenging activity in the photochemical *o*-dianisidine assay.

[c] $MnSO_4$ activity was entirely eliminated in all assays by 10 μM EDTA.

[d] Initial rate obtained in 50 mM pyrophosphate buffer instead of in the phosphate buffer used routinely. In pyrophosphate, the O_2^- scavenging by Mn(II) ceased after it had been converted to Mn(III)- pyrophosphate.

TABLE 2. *Metal content and* O_2^-*-scavenging activity of cell extracts*

Organism	Dialysis[a]	Metal concn (μg/mg of protein)					O_2^- scavenging (U/mg of protein)	
		Fe	Mn	Cu	Co	Zn	−EDTA	+0.1 mM EDTA
L. plantarum 14917	−	0.011	8.8	0.08	0.02	0.03	39.4	<1.0
	+	0.038	2.2	0.09	0.02	0.03	2.3	<1.0
L. plantarum 8014	−	0.021	9.3	0.10	0.04	0.04	52.2	<1.0
	+	0.089	0.9			0.04	1.5	<1.0
E. coli B 23226	−	0.74	0.2	10.10	0.02	0.02	29	26
S. faecalis	−		1.0				56	43

[a] Dialysis was against several changes of 50 mM potassium phosphate, pH 7.8, at 4°C over a period of 24 h. *L. plantarum* extracts gained iron from the phosphate buffer during this dialysis.

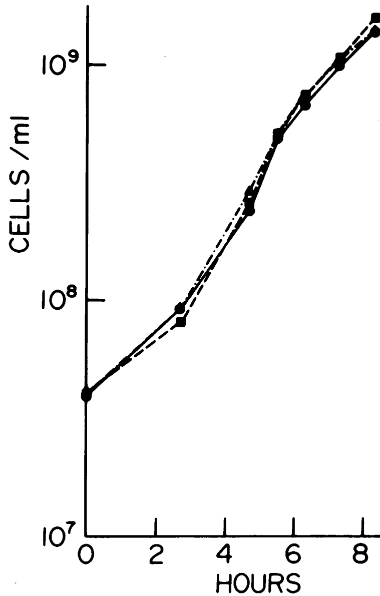

FIG. 4. *Effect of Fe concentration on the growth of L. plantarum strain 14917. The inoculum was grown overnight on aerobic glucose-APT containing 1.4 μM Fe. This inoculum was then diluted 100-fold into glucose-APT containing 23 μM Fe (●), 1.4 μM Fe (■) and <0.2 μM Fe plus 36 μM EDDA (▲). Aerobic growth at 37°C was followed turbidimetrically.*

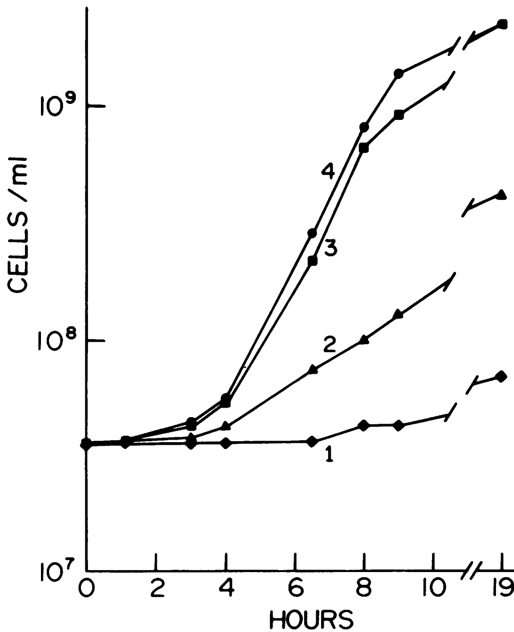

FIG. 5. *Effect of Mn concentration on the growth of L. plantarum strain 14917. The inoculum was grown overnight on aerobic glucose-APT containing 1.6 μM Mn. This inoculum was then diluted 100-fold into glucose-APT containing 0.5 (line 1), 1.6 (line 2), 30 (line 3) and 710 (line 4) μM Mn.*

Mn in the medium initially containing 710 μM Mn was depleted by 15 to 20% by the growing cells, whereas essentially all of the Mn was depleted by cultures growing on medium whose initial Mn concentration was 30 μM.

Physiological significance of O_2^- scavenging by Mn(II) in *L. plantarum*. *Lactobacillaceae* are almost unique in requiring media rich in Mn. In contrast, most common heterotrophic bacteria grow well in media containing less than 1 μM Mn. The data in Table 2 demonstrate that 75 to 90% of the Mn in extracts of *L. plantarum* was dialyzable and that this dialyzable Mn was responsible for the O_2^--scavenging activity. Was this activity important for the survival of *L. plantarum?* In one approach to this question, *L. plantarum* was grown in glucose-APT containing 710, 30, and 1.7 μM Mn(II) and the resultant cells were assayed for content of Mn and for O_2^--scavenging activity. Total Mn content of the cells and O_2^--scavenging activity fell in parallel (Table 4). The avidity with which these cells took up Mn and their ability to deplete the medium are indicated by the last column of Table 4, which gives the ratio of the Mn concentration in the cell paste to that in the original medium.

If O_2^- scavenging is an important function of Mn(II), then the effects of Mn deprivation should be more severe aerobically than anaerobically. *L. plantarum* was grown on glucose-APT containing 710 or 5 μM Mn(II), anaerobically and under 100% O_2. Growth was almost equally rapid at 710 and at 5 μM in the absence of O_2 (Fig. 6). In contrast, when oxygen was present, growth was much more dependent upon an abundance of Mn. Another way of observing this effect involved adding 0.09 μmol of Mn(II) to 4.0-mm wells in glucose-APT agar containing only 1.7 μM Mn(II) and seeded with lawns of *L. plantarum*. These plates were incubated at 37°C at 0.0, 0.2, 1.0, and 2.0 atm of O_2. A halo of denser

TABLE 4. *Effect of Mn concentration in growth medium on Mn content and O_2^--scavenging activity of L. plantarum 14917*

Mn in medium[a] (μM)	Activity[b] (U/mg of protein)	Mn in pellet[c] (mM)	Mn in extract (μg/mg of protein)	Mn in cell walls[d] (μg/mg of protein)	Ratio of Mn concn, pellet/medium[a]
710	23	20	11	27	28
30	8.4	5.4	3.2	5.3	180
1.7	0.7	1.6	0.7	2.2	940

[a] Concentration in fresh medium.

[b] O_2^--scavenging activity was measured in the xanthine oxidase-cytochrome *c* assay.

[c] Pellet denotes the cell paste collected by centrifugation.

[d] Cell walls were cells disrupted with the French press, collected by centrifugation, and measured without washing.

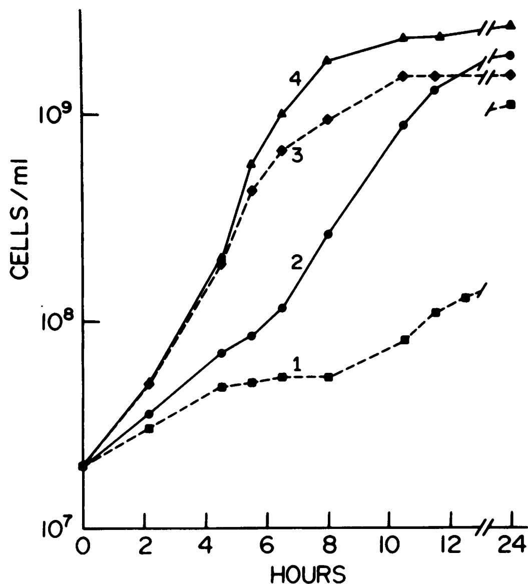

FIG. 6. *Effect of Mn concentration on oxygen sensitivity of L. plantarum strain 14917. Ryan flasks were inoculated in the Coy chamber, as in Fig. 1, with an anaerobic 12-h culture grown in glucose-APT containing 1.6 μM Mn. Half of the flasks containing media with either 710 (line 4) or 1.6 (line 3) μM Mn were sealed in the Coy chamber and were subsequently incubated anaerobically. The remaining flasks, also containing medium with either 710 (line 2) or 1.6 (line 1) μM Mn were removed from the chamber, flushed with sterile 100% O_2, and incubated.*

cell growth was seen around the Mn-containing wells, and this effect became more pronounced with increasing pO_2. It is thus clear that Mn(II) affords oxygen tolerance to *L. plantarum*.

DISCUSSION

As previously reported (16, 30), *L. plantarum* lacks true SOD. However, our earlier view that there is no need for defenses against O_2^- in *L. plantarum* because this organism does not reduce O_2 is no longer tenable. Thus, when growing on mannitol or sorbitol, it is obligately aerobic, respires at a substantial rate, and can then accumulate up to 7 mM H_2O_2 in the culture medium. Furthermore, *L. plantarum* showed resistance to the toxic effects of plumbagin and hyperbaric oxygen comparable with several SOD-containing microorganisms, strongly suggesting the presence of effective oxygen-detoxifying mechanisms.

L. plantarum does then require a defense against O_2^-, and that defense appears to be provided by intracellular Mn(II). Lactobacilli and closely related organisms exhibit an unusually high requirement for manganese (28), a need not shared by most other heterotrophic bacteria. Thus, batches of commerical nutrient broth, Trypticase soy broth, tryptone, and brain heart infusion media were found to contain 0.017, 0.140, 0.110, and 0.028 μM Mn, respectively. These media will suffice to grow most bacteria. In contrast, media such as APT, MRS, and that of Henderson and Snell (22), which were expressely devised for lactobacilli, contain 710, 331, and 578 μM Mn, respectively.

Extracts of *L. plantarum* were as able to intercept O_2^- as were extracts of aerobically grown *E. coli*. However, the activity in *L. plantarum* differed from that in *E. coli* by being dialyzable and eliminated by EDTA. The cell extracts of *L. plantarum* were rich in Mn(II), and 75 to 90% was dialyzable. All of the O_2^--scavenging activity of *L. plantarum* extracts could be accounted for by this dialyzable Mn(II). Indeed, $MnSO_4$ at a concentration equal to that of the dialyzable Mn(II) of the cell extracts mimicked the activity of these extracts.

The Mn (II) in *L. plantarum* appeared to be an important component of its defense against oxygen toxicity. Growth in Mn-deficient medium yielded Mn-poor cells which, in turn, yielded cell extracts with decreased ability to scavenge O_2^-. Mn deficiency caused an enhanced sensitivity to oxygen. Abundant Mn was thus much more important for growth of *L. plantarum* in the presence of O_2 than in its absence.

The lethality of plumbagin for washed cells of *L. plantarum* only when oxygen and a carbon and energy source was present argues for the effect being due to a toxic product of oxygen reduction. Although Mn(II) has been clearly shown here and elsewhere to effectively scavenge O_2^-, reports exist in the literature of Mn apparently scavenging both singlet oxygen (6) and the hydroxyl radical (10, 11), as well as increasing the resistance of spores to heat and ionizing radiation (1, 2).

Mn(II) is known to be oxidized by O_2^- (5, 25, 26, 35). In the presence of pyrophosphate the product is a stable Mn(III)-pyrophosphate (26), and in the absence of stabilizing chelators the product is believed to be MnO_2^+(5). We noted that Mn(II) acted as a stoichiometric scavenger of O_2^- in pyrophosphate-buffered media but as a catalytic scavenger in phosphate-buffered media, all at pH 7.8. It appears likely that catalytic scavenging of O_2^- by Mn(II) depends upond reduction of the oxidized manganese product by some component of the reaction mixture. Thus, in the xanthine oxidase-cytochrome *c* assay this reduction could be caused by ferrocytochrome *c* or urate. In fact, when >2 μM Mn(II) was added to the xanthine oxidase reaction, in the presence of ferrocytochrome *c*, it caused oxidation of that

cytochrome *c*. The interior of *L. plantarum* may be assumed to abound in reductants for the oxidized manganese, since NADPH has been seen to function in this capacity (33). Mn(II) within *L. plantarum* thus eliminates O_2^- in a manner analogous to that in which a peroxidase eliminates H_2O_2. In contrast, the SOD found in most organisms eliminate O_2^- in a way analogous to the action of catalase on H_2O_2.

The ability of Mn(II) to scavenge O_2^- has previously been noted (26, 27, 35) and may at least partially explain the reports (10, 11) that Mn(II) inhibits lipid peroxidation in biological membranes. Plant material is rich in Mn, and the natural association of *L. plantarum* with fermenting plant material would allow it ready access to that metal in nature. Chloroplasts contain plentiful Mn, both free and bound, and the oxidation of Mn(II) by illuminated chloroplasts due to O_2^- has been noted by many workers (25–27, 35). Indeed, Kono et al. (26) reported the rate constant for the oxidation of Mn(II)-pyrophosphate by O_2^- to be 6×10^6 M^{-1} s^{-1} and suggested that one function of the free Mn(II), within chloroplasts, might be the scavenging of O_2^-.

The substitution in *L. plantarum* of millimolar Mn for the micromolar Mn SOD or Fe SOD present in virtually all other aerobic procaryotes examined invites comparison. On a weight-to-weight basis, $MnSO_4$ can be calculated to possess 4,900 U/mg of salt (Table 3), whereas an Mn SOD of molecular weight 40,000 has 3,000 to 3,500 U/mg of protein. Thus, in environments where adequate Mn is available, using millimolar Mn with a rate constant of 6×10^6 M^{-1} s^{-1} instead of an Mn SOD or Fe SOD with a rate constant $\geq 10^9$ M^{-1} s^{-1} would appear to result in at least comparable protection from O_2^-.

While our studies of *L. plantarum* were in progress, Götz et al. (13, 14) reported that protein-free extracts of this organism (strain 8014) could scavenge O_2^- generated by pulse radiolysis and suggested that Mn(II)-phosphate might be the active agent. When they used the xanthine oxidase-cytochrome *c* assay for scavenging of O_2^-, they noted comparatively little activity. They failed to note that the latter assay is routinely done in the presence of 100 μM EDTA, which can eliminate the activity of a like concentration of Mn(II) (14, 36).

An unusually high intracellular Mn is not the only peculiarity of *L. plantarum*. As far as is known at present, *L. plantarum*, and possibly related lactobacilli, is unique in its apparent lack of a requirement for iron. Although this might be considered an evolutionary advantage or adaptation in an organism existing solely on a substrate with low available Fe such as milk (4), *L. plantarum*, as its name suggests, is normally found associated with fermenting plant material, nearly as rich in Fe as it is in Mn. However, the absence of significant amounts of cellular Fe might be of real advantage to a catalase- and SOD-free organism living aerobically and generating H_2O_2 in minimizing Fe-catalyzed generation of oxygen free radicals in reactions of the Fenton or Haber-Weiss type (17, 39). This, in addition to the active O_2^- scavenging by intracellular Mn reported here, may explain the remarkable oxygen tolerance of this SOD-free microorganism.

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