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Expression of Superoxide Dismutase in *Listeria monocytogenes*

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The nature and expression of superoxide dismutase (SOD; EC 1.15.1.1) in the gram-positive food-borne pathogen *Listeria monocytogenes* were examined. Metal depletion and reconstitution studies and resistance to H_2O_2 and potassium cyanide inactivation indicated that *L. monocytogenes* has a single SOD which utilizes manganese as a metal cofactor. The specific activity of SOD was unchanged in cells exposed to a heat shock at 42°C or grown in the presence of paraquat-generated superoxide anion or of metal chelators in the medium. SOD levels increased, however, as the cells progressed through the logarithmic phase of growth and into the stationary phase. Furthermore, SOD activity decreased with decreasing growth temperatures and declined concurrently with decreased growth when higher concentrations of sodium chloride were added to the medium. Cells grown anaerobically possessed relatively high levels of SOD, although these levels were about 10 to 30% lower than those of aerobically grown bacteria. Different isolates of *L. monocytogenes* were found to produce approximately equivalent levels of SOD, although greater differences in SOD expression were seen among other species of *Listeria*. When compared with *L. monocytogenes*, for example, *Listeria welshimeri* typically produced about 30% greater SOD activity, whereas *Listeria murrayi* produced about 60% less total SOD activity. Although all species of *Listeria* produced a single Mn-type SOD, differences in the relative electrophoretic mobility of the native enzymes were noted. These data suggest that the single *L. monocytogenes* SOD enzyme is constitutively produced in response to many environmental factors and may also be responsive to the cellular growth rate.

Listeria monocytogenes is a facultatively anaerobic, gram-positive bacterium which is widely distributed in soils, water, vegetation, animal silage, and other environments (10). It is pathogenic for humans, causing meningoencephalitis, perinatal infections, and septicemia, and is of particular concern to immunocompromised subpopulations (20). Because of its food-borne mode of transmission, considerable effort must be expended in detecting and preventing listerial contamination.

The virulence of *L. monocytogenes* is dependent on its ability to invade and replicate within the cytoplasm of phagocytic cells (27). While phagocytosed *Listeria* organisms eventually escape the phagosomal compartment and spread from cell to cell, for a short period they must still withstand the cytotoxic reactive oxygen intermediates, such as superoxide anion ($\dot{O}_2^-$), which are produced by macrophages and neutrophils. For this reason, it has been suggested that superoxide dismutase (SOD) and catalase, which detoxify reactive oxygen intermediates, could be likely candidates for virulence factors in *L. monocytogenes*, as is the case for a number of other bacterial pathogens (1, 2, 32, 33). Furthermore, these enzymes are critical for the survival of aerobic organisms because they eliminate the toxic oxygen by-products of metabolism. This is of relevance to the food processing industry because both superoxide dismutase and catalase are thought to be inactivated by mild heating, resulting in reduced bacterial growth in food products (6, 21).

Superoxide dismutases (EC 1.15.1.1) catalyze the conversion of superoxide anion to hydrogen peroxide through the reaction $\dot{O}_2^- + \dot{O}_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ (14). They are metalloproteins that are classified according to the nature of their metal cofactor(s): iron, manganese, or copper and zinc. Bacterial SODs tend to use Fe or Mn, and indeed many organisms possess two distinct types of SOD. *Escherichia coli*, for instance, contains both a constitutively expressed Fe-containing SOD (FeSOD) and an MnSOD which is regulated by environmental factors such as iron concentration, atmospheric oxygen, and the presence of superoxide radicals (16). Relatively little, however, is known about the expression of SOD by *L. monocytogenes* or of its role in virulence. Increased SOD activity, for example, does not correlate with increased virulence of *L. monocytogenes*, and strain-to-strain differences in total SOD activities are sometimes seen (6, 25). Early work suggested that *L. monocytogenes* had only one form of SOD containing iron as a cofactor (33). A later report, however, suggested that manganese was the metal cofactor (29), and recent DNA sequence information obtained from the cloned *L. monocytogenes* SOD gene tends to support this conclusion (4). In addition, the expression of SOD in *L. monocytogenes* has been reported to be induced by oxygenation and by the presence of superoxide anion (29, 33). Welch et al. (33) noted that the supplementation of iron-free medium with iron resulted in increased SOD specific activity, whereas Schiavone and Hassan (29) were unable to observe any significant differences in *L. monocytogenes* SOD activity between organisms grown under iron-rich conditions and those grown under iron-restricted conditions. Finally, Dallmier and Martin (7) found that the presence of sodium chloride could either increase or decrease SOD expression, depending on the concentration of NaCl used.

In an attempt to explain some of these conflicting data, we have reexamined some of the characteristics of the *L. monocytogenes* SOD in terms of the metal cofactor requirements and the effects of various environmental conditions on the level of SOD expression. In addition, SOD activity in other species of *Listeria* was studied to see whether any correlates could be drawn between SOD expression and pathogenicity. Our results support the conclusion that the SOD from *L. monocytogenes* is

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a manganese-containing enzyme which is expressed in relatively constant amounts under most environmental conditions.

MATERIALS AND METHODS

Organisms. Most strains of *L. monocytogenes* have been previously described (9) and were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) or as clinical isolates from the Royal University Hospital (Saskatoon, Saskatchewan). In addition, *L. monocytogenes* 162999 was an untyped clinical isolate from blood, while strains 151090 and SB were both blood isolates of serotypes 4b and 1/2b, respectively. Representative strains of other *Listeria* species were as previously described (8, 9). Most experiments were done with *L. monocytogenes* 35152. As obtained from ATCC, this isolate contains a mixture of virulent hemolytic and avirulent nonhemolytic variants (26). The hemolytic variant was purified from the mixture and was used throughout this work, although the same strain designation (abbreviated *Lm* 35152) was retained. Therefore, *L. monocytogenes* 35152, as used here, is identical to the previously described strain ATCC 43249 (26).

Media and growth conditions. The growth medium used for most experiments was Trypticase-soy (Tsoy) broth (Difco, Detroit, Mich.). For some experiments, Tsoy broth was metal depleted by treatment with Chellex-100 resin (Bio-Rad Laboratories, Ltd., Hercules, Calif.) as previously described (17). In most cases, bacteria were grown in 50 ml of medium in 250-ml Erlenmeyer flasks placed in water baths set to the required temperature. Aeration was achieved by shaking at 200 rpm. The extent of bacterial growth was determined by measuring the optical density of the cultures at 600 nm with an LKB spectrophotometer (Pharmacia, Uppsala, Sweden).

For anaerobic growth, an anaerobic growth chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) was used. A stringently anaerobic protocol, which involved doing all manipulations within the chamber itself, was followed. A 150-ml foam-plugged flask containing 50 ml of broth with 1 mg of sodium thioglycolate per ml and 1 μg of resazurin per ml was placed in the 37°C anaerobic chamber 24 h prior to inoculation. This medium was inoculated with a single colony of *L. monocytogenes* taken from a blood agar plate which had been incubated in the anaerobic chamber for 18 h. After another 18-h incubation, chloramphenicol (500 μg/ml) was added to the culture 30 min before it was placed in a tube containing 10 ml of crushed ice. The chilled culture was then removed from the anaerobic chamber and was centrifuged as described below.

For the heat shock experiments, a culture was grown in Tsoy broth at 37°C in a shaking water bath until cells reached the early log phase of growth. The culture was then divided in half; one half was again placed at 37°C for 30 min, while the other half was placed at 42°C for the same length of time. At this point the optical density of each culture was measured and the cells were harvested and processed as described below.

Preparation of crude bacterial cell extracts and biochemical assays. Bacterial cells were pelleted by centrifugation in a Hermle ZK364M centrifuge (Mandel Scientific Co., Ltd., Edmonton, Alberta, Canada) at 2,500 × *g*, resuspended in 200 μl of Tris-EDTA sucrose buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 25% [wt/vol] sucrose) to which was added 50 U of mutanolysin (Sigma Chemical Company, St. Louis, Mo.). The cells were incubated for 30 min at 37°C and were then transferred to a 2-ml microcentrifuge tube and sonicated on ice for 2 min at 100 W, by using the microtip of a Braunsonic 1510 sonicator (Braun Instruments, San Mateo, Calif.). Cellular debris was removed by centrifugation at 18,000 × *g* for 15 min. The supernatant was collected and added to 0.1 volume of 10 mM phenylmethylsulfonyl fluoride prior to storage at −20°C.

Total SOD activity was measured by the xanthine-xanthine oxidase method of McCord and Fridovich (24) by using 10 to 15 μl of bacterial cell extract per assay. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of ferricytochrome C by 50%. To determine the SOD specific activity, the protein concentrations of the cell extracts were determined with the Pierce BCA Protein assay (Pierce, Rockford, Ill.), by using the protocol recommended by the manufacturer.

Polyacrylamide gel electrophoresis and staining for SOD activity. Cell extracts were separated by electrophoresis on a 12.5% nondenaturing polyacrylamide gel by using the Laemmli buffer system (22) but without sodium dodecyl sulfate (SDS) in the gel or in the electrophoresis buffer. Staining for SOD activity in gels was done as described by Beauchamp and Fridovich (3). To distinguish between Fe-, Mn-, and CuZn SODs, a differential staining procedure using hydrogen peroxide and potassium cyanide (KCN) was done as described by Clare et al. (5).

Metal depletion and reconstitution of crude cell extracts. Metal depletion was done by dialyzing bacterial cell extracts against a metal-depletion buffer (20 mM 8-hydroxyquinoline, 2.5 M guanidium chloride, 5 mM Tris-HCl [pH 3.8], 0.1 mM EDTA) as described by Kirby et al. (19). Reconstitution of metal-depleted cell extracts was achieved by adding 2 mM manganese chloride or 4 mM ferric and ferrous ammonium sulfate (2 mM each ion) to the cell extracts and incubating at 4°C for 18 h. Metal-depleted and reconstituted cell extracts were tested for SOD activity by direct assay or by activity stain as described above.

RESULTS

Metal cofactor requirements of *L. monocytogenes* SOD. Early reports suggested that the *L. monocytogenes* SOD was an iron-cofactored enzyme (33), although more recent work points to manganese as the metal cofactor (29). To determine the metal requirements of the *L. monocytogenes* SOD, a crude cell extract of *Lm* 35152 was separated by nondenaturing polyacrylamide gel electrophoresis and was stained for SOD activity (Fig. 1). A single band of activity was consistently observed, and this activity was not affected by prior washing of the gel with KCN (lane 7), which is known to inactivate CuZn-containing SODs, or with H_2O_2 (lane 4), which inactivates Fe-containing SODs. As a control, a purified *E. coli* MnSOD was similarly unaffected by KCN or H_2O_2 (lanes 6 and 8), whereas an FeSOD from *E. coli* was completely inactivated after treatment with H_2O_2 (lane 5). These data suggested that the *Lm* 35152 SOD was a manganese-dependent enzyme, and, to verify this, crude cell extracts from *Lm* 35152 were treated so as to remove the native metal cofactor from all metal-containing proteins. As seen in Fig. 2 (lane 1), this treatment abolishes SOD activity as visualized by staining after gel electrophoresis. After reconstitution of metal-depleted extracts with various metal ions, it was found that manganese, but not ferric or ferrous iron, was able to restore SOD activity to the extent seen in undepleted extracts (lanes 2 and 3). Directly assaying the depleted and reconstituted cell extracts for SOD activity using the ferricytochrome C method confirmed this result in that only the manganese-reconstituted extract showed SOD activity (results not shown).

The effect of environmental factors on SOD expression. (i) Growth phase and temperature. A batch culture of *Lm* 35152 was grown aerobically at 37°C, and aliquots were removed at

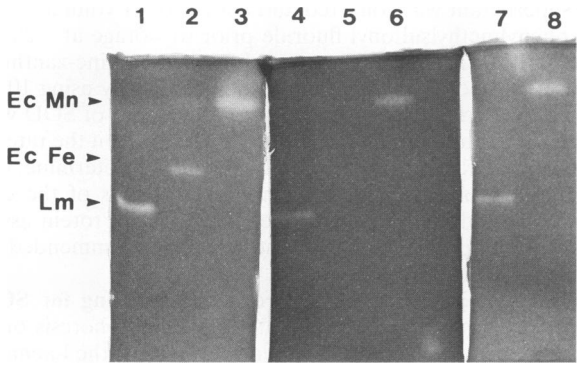

FIG. 1. Differential staining for SOD activity. Crude cell extracts from *L. monocytogenes* ATCC 35152 (lanes 1, 4, and 7) or purified preparations of *E. coli* MnSOD (lanes 3, 6, and 8) or FeSOD (lanes 2 and 5) were separated on a 12.5% nondenaturing polyacrylamide gel and treated with KCN or H_2O_2 prior to staining for SOD activity. Lanes 1 to 3, no treatment prior to staining; lanes 4 to 6, gel washed with 5 mM H_2O_2 prior to staining; lanes 7 and 8, gel washed with 2 mM KCN prior to staining. Arrowheads denote (top to bottom) the positions of the *E. coli* MnSOD, the *E. coli* FeSOD, and the SOD from an *Lm* 35152 crude cell extract.

various points throughout the growth curve. The SOD specific activity in crude cell extracts was found to closely parallel the population growth curve of the culture (Fig. 3), increasing substantially as cells progressed from early to late logarithmic growth, with highest activities seen in early-stationary-phase cells. A decline in SOD activity was seen only in late-stationary-phase cultures and corresponded to a decrease in the number of viable bacterial cells (data not shown). To assess the effect of growth temperature on SOD activity, cultures of *Lm* 35152 were incubated at between 10 and 37°C and were harvested in the late logarithmic phase of growth. Care was taken to ensure that cultures from the different temperatures had all reached approximately the same optical density prior to harvesting, so that differences in SOD activities would not be attributable to differences in bacterial growth phase. SOD specific activity was lowest in cells grown at 10°C and highest in bacteria cultured at 37°C (Table 1). To determine whether a brief heat shock could further stimulate SOD activity, an early-log-phase culture of *Lm* 35152 was placed at 42°C for 30 min. No change in the SOD specific activity could be seen relative to a control culture at 37°C (Table 2).

FIG. 2. SOD activity of metal-depleted and reconstituted crude cell extracts of *L. monocytogenes* ATCC 35152. Metal-depleted cell extracts were incubated with metal ions as described in Materials and Methods and then separated on a 12.5% nondenaturing polyacrylamide gel and stained for SOD activity. Lane 1, metal-depleted cell extract; lane 2, metal-depleted cell extract + 2.0 mM Mn^{2+}; lane 3, metal-depleted cell extract + 4.0 mM Fe^{3+}/Fe^{2+}; and lane 4, non-metal-depleted cell extract control.

(ii) Anaerobic growth. Cultures of *Lm* 35152 grown under stringently anaerobic conditions contained SOD at an average specific activity of 12.5 ± 1.7 U (triplicate experiments). Comparable cultures grown aerobically contained 16.4 ± 1.6 U of SOD, an increase of about 25%. Because other reports suggested that SOD activity in *L. monocytogenes* increased by as much as 60% when bacteria were grown aerobically (29), we tested a number of other strains under anaerobic conditions to ensure that the data from *Lm* 35152 were not anomalous. The results from triplicate experiments were as follows: *L. monocytogenes* RUH 162999 anaerobic SOD specific activity, 15.2 ± 0.2 U, aerobic SOD specific activity, 16.6 ± 0.3 U; strain RUH SB anaerobic activity, 11.2 ± 0.8 U, aerobic activity, 16.4 ± 0.6 U; and strain ATCC 15313 anaerobic activity, 15.8 ± 1.3 U, aerobic activity, 18.2 ± 0.5 U. In no case was the difference in SOD activity between anaerobic and aerobically grown cells greater than about 30%.

(iii) Superoxide anion. Superoxide anion may be formed intracellularly by the presence of paraquat, an aromatic compound that undergoes redox cycling within cells (15). Increasing concentrations of paraquat added to Tsoy broth were seen to reduce the growth of *Lm* 35152 slightly but did not significantly alter the specific activity of SOD in cell extracts (Table 2).

(iv) Iron restriction. The effects of growth in iron-restricted media on the SOD activity of *Lm* 35152 are shown in Table 2. Iron was chelated by the addition of either 2,2′-dipyridyl or ethylenediamino-*N*,*N*′-dihydroxyphenylacetic acid (EDPA) to the Tsoy medium, or it was removed by treating the medium with Chellex resin. The specific activities of SOD, however, were not greatly different in bacteria grown in these media from those in bacteria grown in untreated Tsoy broth. Because the Chellex resin is also able to remove other metals, including manganese (17), we also determined the SOD activity in cells grown with ferric iron, manganese, or both metals. Again, no effect on SOD specific activity could be seen.

(v) Salt concentration. The effects of osmolarity on SOD specific activity and on bacterial growth were determined by growing *Lm* 35152 in Tsoy broth containing various concentrations of sodium chloride. As shown in Fig. 4, bacterial growth was optimal at salt concentrations of about 2% but was greatly reduced as the concentration of NaCl increased. The SOD specific activity tended to parallel the bacterial growth yield, with highest levels occurring at between 1 and 4% NaCl and decreased levels of activity occurring in cells grown in 6 and 8% NaCl.

SOD activity in other *L. monocytogenes* strains and *Listeria* species. Finally, strains of *L. monocytogenes* other than *Lm* 35152 were examined to determine the variability in SOD expression under a defined set of conditions. We included ATCC strains as well as recent clinical isolates. All strains were grown aerobically in Tsoy broth until the early stationary phase and were then assayed for SOD activity. The data in Table 3

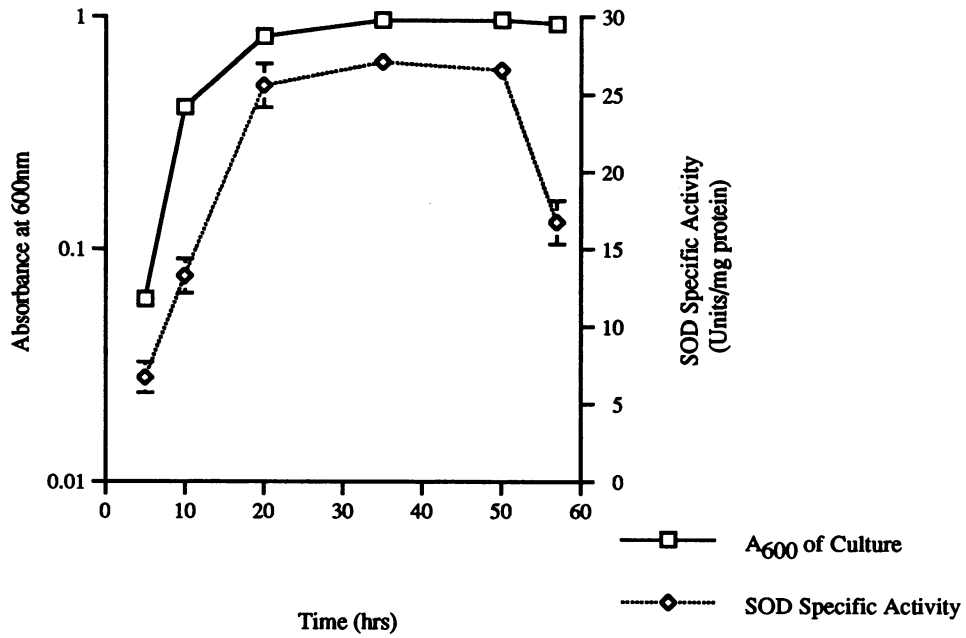

FIG. 3. SOD specific activity as a function of growth for *L. monocytogenes* ATCC 35152. Bacteria were incubated aerobically at 37°C, and growth was monitored spectrophotometrically. Crude cell extracts collected at various time points were assayed for SOD activity.

indicate that all strains of *L. monocytogenes* expressed SOD activity to approximately the same extent. No differences were seen between clinical isolates and ATCC reference strains or between different serotypes of *L. monocytogenes*.

A further comparison was done between *L. monocytogenes* and other species of *Listeria* (Table 3). In this case the SOD activities were more varied, ranging from 6.7 U of SOD in *Listeria murrayi* to more than 21 U in *Listeria welshimeri*. Crude cell extracts from different *Listeria* species were also separated by electrophoresis on native polyacrylamide gels and were stained directly for SOD activity after treatment with H_2O_2. Some differences in the relative mobilities of the SOD proteins were noted, especially for the SODs from *Listeria innocua* and *L. murrayi* (Fig. 5). As was the case with *L. monocytogenes*, the SODs from other *Listeria* species appeared to be of the manganese cofactored type, since all were resistant to inactivation by H_2O_2.

DISCUSSION

In this report we have examined some of the characteristics of SOD in *L. monocytogenes* and, for comparative purposes, have looked at the SOD activity in other species of *Listeria*. Because of inconsistencies in the reported literature regarding the nature of the metal cofactor in the *L. monocytogenes* SOD, we first established that it was a manganese-containing enzyme. The SOD proved to be resistant to both KCN and H_2O_2, which are known to inactivate CuZn- and Fe-containing SODs, respectively. Removal of the metal ion from the SOD abolished activity, but reconstitution with manganese was able to restore it. The inability of iron to similarly restore SOD activity suggests that these two metals are not interchangeable and that therefore the *L. monocytogenes* SOD is not of the cambialistic type (14, 23). Furthermore, by staining cell extracts separated on native polyacrylamide gels, we consistently observed only a single band of SOD activity, regardless of the environmental conditions under which the bacteria had been grown. This is in

TABLE 1. Specific activity of SOD of *Lm* 35152 grown at different temperatures

Temperature (°C)	A_{600}[a]	SOD specific activity (U/mg of protein)[b]
10	0.674	10.9 ± 0.2
20	0.643	17.2 ± 0.2
30	0.642	18.5 ± 0.3
37	0.650	19.3 ± 0.3

[a] Optical density of culture at time of cell harvesting.
[b] Values are means ± standard deviations of triplicate assays.

TABLE 2. Specific activity of SOD of *L. monocytogenes* ATCC 35152

Treatment (concn)	A_{600}[a]	SOD specific activity (U/mg of protein)[b]
42°C heat shock	0.282 ± 0.004	7.6 ± 3.0
Heat shock control	0.257 ± 0.023	7.1 ± 1.3
No paraquat	0.658 ± 0.107	17.2 ± 1.4
Paraquat (100 μM)	0.620 ± 0.117	14.4 ± 0.4
Paraquat (300 μM)	0.559 ± 0.052	15.5 ± 1.3
Paraquat (500 μM)	0.500 ± 0.028	19.4 ± 1.0
Tsoy control	0.593 ± 0.040	16.7 ± 2.0
Tsoy + 2,2-dipyridyl (200 μM)	0.508 ± 0.068	14.1 ± 1.7
Tsoy + EDPA (500 μM)	0.650 ± 0.060	17.2 ± 0.6
Tsoy-Chellex	0.451 ± 0.085	14.1 ± 1.6
Tsoy-Chellex + Fe^{3+} (1 mM)	0.745 ± 0.032	13.9 ± 1.0
Tsoy-Chellex + Mn^{2+} (1 mM)	0.622 ± 0.044	16.4 ± 4.3
Tsoy-Chellex + Fe^{3+}/Mn^{2+} (1 mM each metal ion)	0.813 ± 0.026	14.4 ± 1.7

[a] Optical density of culture at time of cell harvesting.
[b] Values are means ± standard errors of at least three separate experiments for each treatment.

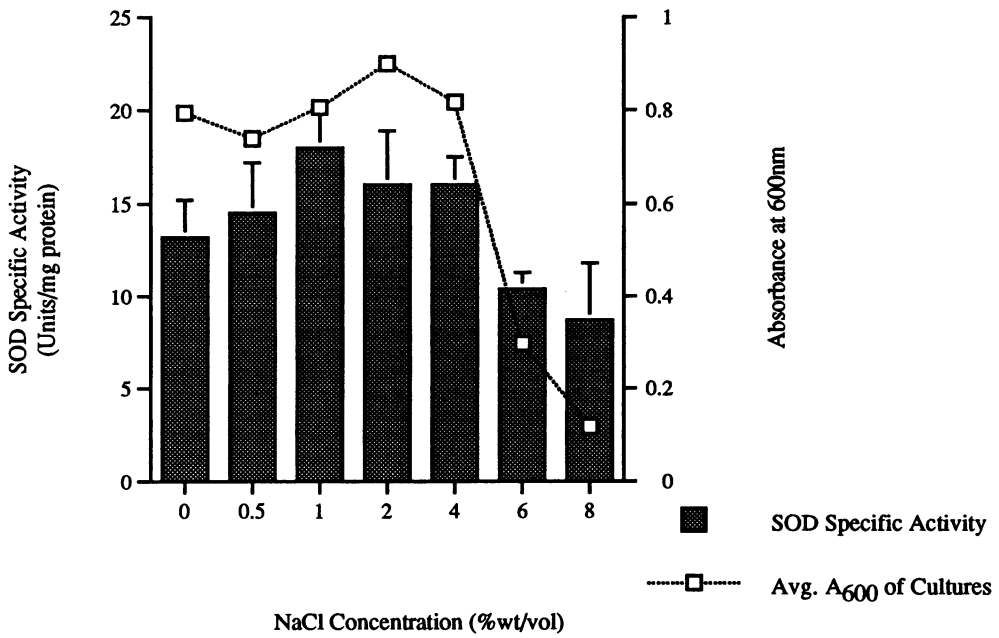

FIG. 4. Specific activities of SOD and final culture optical densities of *L. monocytogenes* ATCC 35152 after growth at 37°C in media with various levels of NaCl. SOD specific activity at each NaCl concentration is indicated by a bar, while the final optical density of each culture is indicated by the line. Values reported for SOD specific activity are means ± standard errors of four replicates.

contrast to species such as *E. coli* which contain two distinct, independently regulated, types of SOD.

Our results are consistent with *L. monocytogenes* possessing an Mn-containing SOD enzyme, as suggested by the amino acid sequence (4), and support previous results of Schiavone and Hassan (29), who reported, but gave no evidence for, a single MnSOD in *L. monocytogenes*. It is unclear how Welch et al. (33) were able to observe an FeSOD in *L. monocytogenes*, especially since we have been able to eliminate cambialism as a possible explanation. In addition, we have shown that depletion of iron from the growth medium does not significantly alter the specific activity of SOD in *L. monocytogenes*. In other organisms, the expression of FeSODs, but not MnSODs, can be repressed when the availability of exogenous iron is reduced (29). The lack of effect of iron chelators on the SOD of *L. monocytogenes* is therefore reflective of the Mn-containing nature of the enzyme.

We have also examined the response of the *L. monocytogenes* MnSOD to changes in bacterial growth state and environmental conditions, since it is clear that in *E. coli* the MnSOD is an inducible enzyme while the FeSOD is largely constitutive. We observed that *L. monocytogenes* in late logarithmic and early stationary growth possessed about three- to fourfold higher levels of SOD than cells in early logarithmic growth, thereby suggesting some form of growth phase induction as seen for certain other metabolic enzymes (12). This

TABLE 3. Specific activities of SOD from different species of *Listeria*

Organism	SOD specific activity (U/mg of protein)[a]
Listeria monocytogenes ATCC 43256	12.0 ± 1.1
Listeria monocytogenes ATCC 35152	15.5 ± 1.9
Listeria monocytogenes ATCC 19113	17.2 ± 4.5
Listeria monocytogenes ATCC 19112	14.4 ± 2.8
Listeria monocytogenes ATCC 19111	16.6 ± 1.0
Listeria monocytogenes RUH 162999	15.6 ± 2.7
Listeria monocytogenes RUH 151090	16.0 ± 1.4
Listeria monocytogenes ATCC 15313	16.3 ± 2.0
Listeria monocytogenes RUH '58	12.9 ± 0.4
Listeria monocytogenes RUH D20	17.0 ± 1.9
Listeria monocytogenes RUH SB	17.8 ± 3.0
Listeria grayi	8.7 ± 1.0
Listeria innocua	17.3 ± 1.4
Listeria ivanovii	16.4 ± 1.6
Listeria murrayi	6.7 ± 2.0
Listeria seeligeri	17.2 ± 1.3
Listeria welshimeri	21.2 ± 3.1

[a] Values are means ± standard errors of three separate experiments.

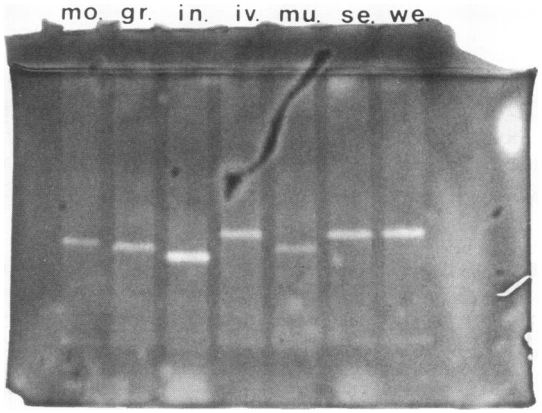

FIG. 5. SOD in different *Listeria* species. Bacterial crude cell extracts were separated on a 12.5% nondenaturing polyacrylamide gel, washed in 5 mM H_2O_2, and stained for SOD activity. Lanes: mo, *L. monocytogenes* ATCC 35152; gr, *L. grayi*; in, *L. innocua*; iv, *L. ivanovii*; mu, *L. murrayi*; se, *L. seeligeri*; we, *L. welshimeri*.

could possibly reflect the need for greater protection from accumulated toxic oxidants as the cells age. In addition, the expression of SOD in *L. monocytogenes* was found to be reduced in bacteria grown at lower temperatures, although the overall change in activity was only about 40% between 10 and 37°C cultures. Previous reports have indicated that low-temperature growth of *L. monocytogenes* may result in enhanced production of hemolysin and an overall increase in virulence (11, 18, 31). It is interesting, therefore, that SOD specific activity shows the opposite effect in response to growth temperature. It is unclear at this time whether this response of SOD is due to a temperature-dependent regulatory mechanism acting directly on SOD gene expression or to an indirect response to the differences in bacterial growth rates at different temperatures. The lower rate of growth at low temperatures would presumably reduce the demand for SOD because of reduced generation of oxygen by-products. Furthermore, we noted that SOD activity was not altered by subjecting cells to a heat shock at 42°C. In *L. monocytogenes*, many virulence-associated genes controlled by the *pfrA* regulon are induced by a heat shock (30), and in *E. coli*, the specific activity of the MnSOD is increased after heat shock (28). The fact that the *L. monocytogenes* SOD was not induced under similar conditions suggests that any regulatory mechanisms acting on SOD may operate outside of the PfrA network and could instead be tied to normal cellular metabolic processes.

In addition to the effects of growth phase and temperature, we examined the response of SOD to anaerobic growth and to the concentration of sodium chloride and the presence of paraquat in the media. Schiavone and Hassan (29) reported that SOD specific activity was two- to threefold higher when *L. monocytogenes* was grown in an aerobic environment compared with an anaerobic one. Employing a stringently anaerobic protocol similar to that used by Schiavone and Hassan, we found that anaerobically grown *Lm* 35152 had a high basal level of SOD activity and that this could be increased by about 25% when the cells were grown aerobically. A similar response was seen with three other strains of *L. monocytogenes*, including two clinical isolates and the ATCC strain 15313, which was used in the study by Schiavone and Hassan. Although we did observe a modest induction of SOD activity in aerated cultures, the magnitude of the induction in any of our strains was not as great as that reported by Schiavone and Hassan. Because of its lack of physiological relevance, we did not attempt to provide a hyperoxygenated environment, as did Schiavone and Hassan, so it is not known whether further increases in SOD expression are possible. Our results, together with those of Schiavone and Hassan, suggest that while oxygenation results in some increase in SOD expression, there is nevertheless a significant basal level of SOD activity in *L. monocytogenes* under anaerobic conditions, in which presumably the cells would not require this enzyme for detoxification purposes. Since *L. monocytogenes* contains only a single MnSOD isotype, it is probably necessary that SOD remain expressed under anaerobic conditions so as to be able to provide immediate protection against sudden oxygen exposure. This is in contrast to those organisms which have both Fe- and MnSODs, in which the MnSOD is fully repressed during anaerobic growth, although protection against oxygen stress is still possible because of continual synthesis of the FeSOD (16). In contrast to our observations of the effect of atmospheric oxygen, however, we were unable to observe an induction of SOD expression in response to superoxide anions generated by paraquat. Similar results have been reported by Schiavone and Hassan (29) and it is possible, as previously suggested (29), that the cell wall composition of *L. monocytogenes* excludes the entry of paraquat. We noted, however, that bacterial growth declined at higher concentrations of paraquat, indicating a possible toxic effect. If indeed paraquat is able to enter *L. monocytogenes*, then the lack of a SOD response is in sharp contrast to the approximately 10-fold increase in MnSOD activity induced by paraquat in *E. coli* (15).

Finally, we have shown that *L. monocytogenes* grown in the presence of increasing concentrations of sodium chloride contains greatly variable levels of SOD. The specific activity of SOD appeared to peak in cells grown in 1 to 2% NaCl and then declined substantially at 6 and 8%. A similar fluctuation in SOD activity was reported by Dallmier and Martin (7), who noted that 2.5% NaCl resulted in the highest SOD activity. These authors speculated that increased oxygen consumption by *L. monocytogenes* grown at optimal concentrations of NaCl would create a need for more SOD to detoxify oxygen intermediates. In support of this, we found that bacterial growth levels closely paralleled the changes in SOD specific activity: the least amount of growth, for example, occurred in 8% NaCl, and this culture also had the lowest enzyme levels. This again suggests that the reduced growth rate of *L. monocytogenes* under these conditions results in less demand for SOD and consequently in reduced expression of the enzyme, like the response seen previously with low-temperature growth. Nevertheless, as indicated by the continued production of SOD under anaerobic conditions, a certain minimal level of SOD expression appears to be necessary at all times, and therefore regulation of SOD activity may occur only over a relatively narrow range.

While our studies of the factors influencing SOD expression were performed with a virulent ATCC strain of *L. monocytogenes* (35152), we also examined other strains and other species of *Listeria* for SOD activity. In all cases, growth and medium conditions were kept constant, so that any differences in SOD activity could be attributed to the strains themselves. Among the *L. monocytogenes* isolates, SOD specific activity varied by no more than 30% between different serotypes and between ATCC strains and recent clinical isolates. In contrast, greater variation was noted when different species of *Listeria* were studied, although no conclusions could be drawn regarding SOD activity and relative pathogenicity. Some nonvirulent *Listeria* species such as *L. welshimeri*, for example, expressed considerably more SOD than did the virulent *L. monocytogenes*. On the other hand, *L. murrayi* produced approximately 60% less SOD than did *L. monocytogenes*. Yet *L. murrayi* is quite capable of aerobic growth, suggesting that the higher levels of SOD produced by the other species may be in excess of the level required to meet the needs of normal aerobic metabolism. Why these species continue to express high amounts of SOD is unclear.

In addition to differences in the relative levels of SOD expression between different *Listeria* species, we were able to observe differences in the mobilities of the native SOD proteins in nondenaturing polyacrylamide gels. To date, only the SODs from *L. monocytogenes* and *Listeria ivanovii* have been compared at the amino acid level, and they share 95% homology (4, 13). We have found that the SODs from these two organisms may exhibit very slight differences in relative mobilities, although the SODs from *Listeria grayi*, *L. murrayi*, and *L. innocua* show clearly distinct mobility shifts. It is unlikely that differences in metal cofactor requirements could account for these mobility differences, since the SODs from all species of *Listeria* were resistant to H_2O_2, indicating the presence of Mn as a cofactor. Instead, it is more likely that slight differences in amino acid composition may give rise to differences in the tertiary structure of the SOD proteins, and

that this is manifested as a mobility shift. A comprehensive sequence analysis of the SOD genes from the different *Listeria* species is necessary to clarify this point.

In summary, we have shown that all *Listeria* species contain a single, Mn-cofactored SOD protein and that in *L. monocytogenes* this activity appears to be mainly constitutively expressed under the growth conditions tested in this study. Regulation of SOD, if it occurs, may result in only modest changes in specific activity and may in fact be coupled to the cellular growth rate. Furthermore, our results provide support for the contention that relative levels of SOD expression in *Listeria* species may not be reflective of the degree of pathogenicity of the bacteria (25). Instead, SOD probably functions as a metabolic enzyme required to support growth of *Listeria* in aerobic environments. As such, however, the SOD enzyme may still serve as an important target in the search for effective ways of preventing or limiting growth of *Listeria* species in food products.

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REFERENCES

1. **Andersen, P., D. Askgaard, L. Ljungqvist, and J. Bennedsen.** 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. **59:**1905–1910.
2. **Beaman, L., and B. L. Beaman.** 1990. Monoclonal antibodies demonstrate that superoxide dismutase contributes to the protection of *Nocardia asteroides* within the intact host. Infect. Immun. **58:**3122–3128.
3. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
4. **Brehm, K., A. Haas, W. Goebel, and J. Kreft.** 1992. A gene encoding a superoxide dismutase of the facultative intracellular bacterium *Listeria monocytogenes*. Gene **118:**121–125.
5. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. **140:**532–537.
6. **Dallmier, A. W., and S. E. Martin.** 1988. Catalase and superoxide dismutase activities after heat injury of *Listeria monocytogenes*. Appl. Environ. Microbiol. **54:**581–582.
7. **Dallmier, A. W., and S. E. Martin.** 1990. Catalase, superoxide dismutase, and hemolysin activities and heat susceptibility of *Listeria monocytogenes* after growth in media containing sodium chloride. Appl. Environ. Microbiol. **56:**2807–2810.
8. **Deneer, H. G., and I. Boychuk.** 1991. Species-specific detection of *Listeria monocytogenes* by DNA amplification. Appl. Environ. Microbiol. **57:**606–609.
9. **Deneer, H. G., and I. Boychuk.** 1993. Reduction of ferric iron by *Listeria monocytogenes* and other species of *Listeria*. Can. J. Microbiol. **39:**480–485.
10. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
11. **Girard, K. F., A. J. Sharra, and W. A. Bardawil.** 1963. Serology of *Listeria monocytogenes*. I. Characteristics of the soluble hemolysin. J. Bacteriol. **85:**349–355.
12. **Goldie, H. G.** 1984. Regulation of transcription of the *E. coli* phosphoenolpyruvate carboxykinase locus: studies with *pck-lacZ* operon fusions. J. Bacteriol. **159:**832–836.
13. **Haas, A., and W. Goebel.** 1992. Cloning of a superoxide dismutase from *Listeria ivanovii* by functional complementation in *Escherichia coli* and characterization of the gene product. Mol. Gen. Genet. **231:**313–322.
14. **Hassan, H. M.** 1989. Microbial superoxide dismutases. Adv. Genet. **26:**65–97.
15. **Hassan, H. M., and I. Fridovich.** 1979. Paraquat and *E. coli*: mechanism of production of extracellular superoxide radical. J. Biol. Chem. **254:**10846–10852.
16. **Hopkin, K. A., M. A. Papazian, and H. M. Steinman.** 1992. Functional differences between manganese and iron superoxide dismutases in *Escherichia coli* K-12. J. Biol. Chem. **267:**24253–24258.
17. **Kadurugamuwa, J. L., H. Anwar, M. R. W. Brown, G. H. Shand, and K. H. Ward.** 1987. Media for the study of growth kinetics and envelope properties of iron-deprived bacteria. J. Clin. Microbiol. **25:**849–855.
18. **Kathariou, S., J. Rocourt, H. Hof, and W. Goebel.** 1988. Levels of *Listeria monocytogenes* hemolysin are not directly proportional to virulence in experimental infections of mice. Infect. Immun. **56:**534–536.
19. **Kirby, T., J. Blum, I. Kahane, and I. Fridovich.** 1980. Distinguishing between Mn-containing and Fe-containing superoxide dismutases in crude extracts of cells. Arch. Biochem. Biophys. **201:**551–555.
20. **Kluge, R. M.** 1990. Listeriosis—problems and therapeutic options. J. Antimicrob. Chemother. **25:**887–890.
21. **Knabel, S. J., H. W. Walker, P. A. Hartman, and A. F. Mendonca.** 1990. Effects of growth temperature and strictly anaerobic recovery on the survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. **56:**370–376.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Martin, M. E., B. R. Byers, M. O. J. Olson, M. L. Salin, J. E. L. Arceneaux, and C. Tolbert.** 1986. A *Streptococcus mutans* superoxide dismutase that is active with either manganese or iron as a co-factor. J. Biol. Chem. **261:**9361–9367.
24. **McCord, J. M., and R. Fridovich.** 1969. Superoxide dismutase: an enzyme function for bovine erythrocuprein. J. Biol. Chem. **244:**2875–2880.
25. **Myers, E. R., A. W. Dallmier, and S. E. Martin.** 1993. Sodium chloride, potassium chloride, and virulence in *Listeria monocytogenes*. Appl. Environ. Microbiol. **59:**2082–2086.
26. **Pine, L., R. E. Weaver, G. M. Carlone, P. A. Pienta, J. Rocourt, W. Goebel, S. Kathariou, W. F. Bibb, and G. B. Malcolm.** 1987. *Listeria monocytogenes* ATCC 35152 and NCTC 7973 contain a nonhemolytic nonvirulent variant. J. Clin. Microbiol. **25:**2247–2251.
27. **Portnoy, D. A., T. Chakraborti, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
28. **Privalle, C. T., and I. Fridovich.** 1987. Induction of superoxide dismutase in *Escherichia coli* by heat shock. Proc. Natl. Acad. Sci. USA **84:**2723–2726.
29. **Schiavone, J. R., and H. M. Hassan.** 1987. Biosynthesis of superoxide dismutase in eight prokaryotes: effects of oxygen, paraquat and an iron chelator. FEMS Microbiol. Lett. **42:**33–38.
30. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
31. **Stephens, J. C., I. S. Roberts, D. Jones, and P. W. Andrew.** 1991. Effect of growth temperature on virulence of strains of *Listeria monocytogenes* in the mouse: evidence for dose dependence. J. Appl. Bacteriol. **70:**239–244.
32. **Welch, D. F.** 1987. Role of catalase and superoxide dismutase in the virulence of *Listeria monocytogenes*. Ann. Inst. Pasteur Microbiol. **138:**265–268.
33. **Welch, D. F., C. P. Sword, S. Brehn, and D. Dusanic.** 1979. Relationship between superoxide dismutase and pathogenic mechanisms of *Listeria monocytogenes*. Infect. Immun. **23:**863–872.