J. Microbiol. Biotechnol. (1998), **8**(4), 305 - 309

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

False Positive SOD Activity of *Bifidobacterium* spp. Grown in MRS Medium

CHANG, WOO-SUK AND JAE-SEONG SO*

Department of Biological Engineering, Inha University, Inchon 402-751, Korea

Received: December 19, 1997

Abstract The superoxide dismutase (SOD) activity of seven *Bifidobacterium* spp. strains was examined by an indirect SOD assay method. Some *Bifidobacterium* spp. showed significant levels of SOD activity. However, we could not observe any significant differences between anaerobic and aerobic cultures. Furthermore, although several *Bifidobacterium* spp. exhibited some degree of tolerance to paraquat which produces superoxide radicals, the apparent SOD activity of these strains was not correlated with their resistance to paraquat. In addition, when we added increasing amounts of manganese or iron to MRS medium which had been prepared without either of the metal ions, the apparent SOD activity of cell free extracts (CFEs) was increased with increasing concentration of both metal ions. To our surprise, the heat-denatured CFEs also showed nearly identical correlative patterns. Based on these results, the apparent SOD activity was likely due to a nonenzymatic dismutation. These results strongly suggest that high concentration of divalent metal ions (Mn^{2+}, Fe^{2+}) in MRS medium result in nonenzymatic dismutation which can lead to false positive SOD activities in *Bifidobacerium* spp.

Key words: Superoxide dismutase, nonenzymatic dismutation, *Bifidobacterium* spp., MRS medium

Oxygen is beneficially used by many organisms in respiration and other biochemical reactions. However, oxygen causes certain toxicities and some mutations due to its reactive derivatives [5]. Consequently, most aerobic organisms require defense systems to protect themselves against reactive oxygen species [12]. Superoxide dismutase (SOD) is one of the most important factors in oxidative defense systems. From studies on aerobic organisms, four types of SODs (Mn-SOD, Fe-SOD, Cu/Zn-SOD, Ni-SOD) have been identified [7, 14]. Each of the SODs requires the indicated metal cofactor. In several aerotolerant bacteia including *Lactobacillus plantarum*, however, a nonenzymatic dismutation system which can accumulate high intracellular levels of Mn^{2+} [1, 2], and stoichiometrically remove superoxide radicals, has evolved [6].

Recently, the isolation and characterization of SOD structural genes from strictly anaerobic prokaryotes (*Porphyromonas gingivalis, Bacteroides fragilis, Methanobacterium thermoautotrophicum* strain ΔH) have been reported [8, 12]. *Bifidobacterium* spp., gram-positive strict anaerobes, are known to be beneficial to the health of humans and other animals [15]. *Bifidobacterium* spp. play an important role in restriction of the growth of putrefactive bacteria by maintaining the lower pH in the large intestine. In addition, it has been documented that *Bifidobacterium* spp. are effective against aging processes, cancer, and immune response [10]. Although *Bifidobacterium* spp. have been known to be strictly anaerobic, a number of researchers including our group have noticed a significant level of oxygen tolerance exhibited by some strains of *Bifidobacterium* spp. [16]. Therefore, we were interested in finding whether *Bifidobacterium* spp. possess SOD.

In this study, we were unable to confirm the presence of SOD in examined *Bifidobacterium* spp. upon extensive search. Instead, we found a significant level of dismutation activity. We conclude that this nonenzymatic activity may be due to the presence of divalent metal ions in the MRS medium which contains high concentrations of manganese and iron.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *B. adolescentis* ATCC 15706, *B. animalis* ATCC 25527, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, and

*Corresponding author
Phone: 82-32-860-7516; Fax: 82-32-875-0827;
E-mail: sjaeseon@dragon.inha.ac.kr

B. longum ATCC 15707 which were provided from the Korea Food Research Institute. *B. indicum* ATCC 25912 and *B. infantis* ATCC 25962 were obtained from the American Type Culture Collection. As positive and negative control strains for SOD activity, *Escherichia coli* HB101 containing plasmid pDTI-5 and *E. coli* QC774, a *sodA sodB* double mutant, were obtained from D. Touati (University of Paris, France) [9]. Commercially available SOD isolated from *Bacillus subtilis* was purchased from Sigma Chemical Co. (U.S.A.) and used as a control SOD. *Bifidobacterium* spp. were grown anaerobically or aerobically at 37°C in modified MRS medium (per liter: lactose, 10.0 g; tryptose, 3.0 g; tryptic soy broth, 10.0 g; yeast extract, 5.0 g; L-cystein HCl, 0.3 g; K_2HPO_4, 3.0 g; KH_2PO_4, 3.0 g; ammonium citrate, 2.0 g; tween 80, 1.0 g; $FeSO_4$, 35 mg; $MgSO_4$, 575 mg; $MnSO_4$, 120 mg). *E. coli* was grown in LB broth (per liter: bacto-tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 5.0 g; 1N NaOH, 1 ml) at 37°C with vigorous agitation. Ampicillin and paraquat were used at concentrations of 50 μg/ml and 0.1~100 mM, respectively. MRS medium containing $FeSO_4$ and $MnSO_4$ at final concentrations of 0 to 10 mM was used for the metal supplementation experiments [17].

Preparation of Cell-free Extracts and SOD Activity Assays

Bifidobacterium cells were harvested by centrifugation at 10,000×g for 5 min at 4°C, washed twice with 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.8; KPi-EDTA buffer), and resuspended in the same buffer. The cell suspensions were disrupted by 30-s sonication bursts for a total of 10 min on ice. Cell debris was removed by centifugation at 20,000×g for 20 min at 4°C to obtain the cell free extracts (CFEs). Protein concentration was determined by the Bradford method [3]. Quantitative SOD activity was measured by the cytochrome c method, and the activity was expressed in McCord/Fridovich units [11]. To visually identify the SOD activity, proteins in CFEs were separated on 12% native polyacrylamide gels and stained for SOD activity with nitroblue tetrazolium (NBT) [4].

RESULTS AND DISCUSSION

Effects of Oxygen and Paraquat

To examine the possible SOD activity of some *Bifidobacterium* strains, CFEs were prepared from cultures grown in anaerobic or aerobic conditions. As shown in Table 1, apparent SOD activity was observed in six out of seven *Bifidobacterium* strains examined. The activities of *B. indicum* and *B. longum* were higher in aerobic than those in anaerobic conditions, while *B. adolescentis, B. bifidum, B. breve*, and *B. infantis* exhibited lower or no activities in aerobic conditions. These results suggest that the apparent SOD activity is not related with oxygen stress in *Bifidobacterium* spp. unlike in many other SOD-containing microorganisms [9, 12, 13]. Aerobic microorganisms are known to be tolerant to paraquat which produces superoxide radicals, as SOD activity is induced by it [9]. When we examined paraquat resistance in *Bifidobacterium* spp., variable degrees of tolerance to paraquat were observed among the strains examined (Fig. 1). However, there was no correlation between the apparent SOD activity and tolerance to paraquat in *Bifidobacterium* spp. (Table 1, Fig. 1). Furthermore, we have attempted to visualize the apparent SOD activity of positive CFEs by native polyacrylamide gel electrophoresis.

Table 1. Comparison of the apparent SOD activity of seven *Bifidobacterium* spp. and *E. coli*.[a]

Strains used	Anaerobic culture (units/mg proteins)	Aerobic culture[b] (units/mg proteins)
E. coli HB101/pDTI-5	NT[c]	454
E. coli QC774	NT	ND[d]
B. adolescentis	69.4	47.8
B. animalis	ND	ND
B. bifidum	29.6	ND
B. breve	53.0	45.4
B. indicum	21.3	55.5
B. infantis	80.0	70.5
B. longum	ND	30.3

[a]CFEs were prepared from cells at early stationary phase [OD_{600}].
[b]The culture/flask ratio was 1/5 for aerobically (200 rpm) grown culture.
[c]NT, Not tested.
[d]ND, Not detected.

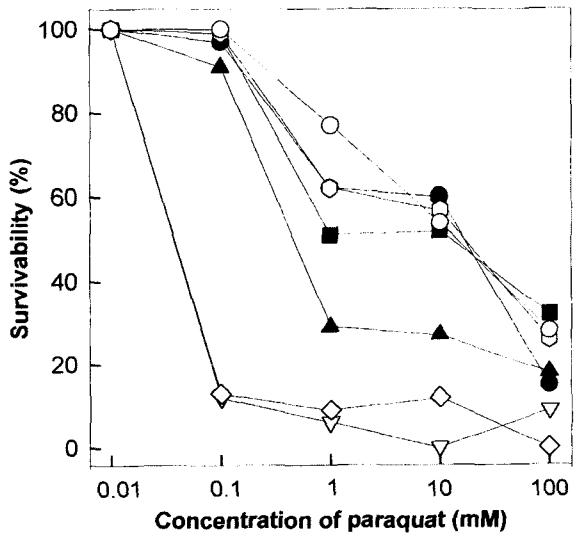

Fig. 1. Sensitivity of various *Bifidobacterium* spp. to paraquat. Survivability was calculated as % of initial [OD_{600}].
Symbols; *B. indicum* (●), *B. breve* (▽), *B. adolescentis* (■), *B. infantis* (◇), *B. animalis* (▲), *B. bifidum* (⬡), *B. longum* (○).

We were able to visualize clean SOD activity bands with controls (*E. coli* HB101/pDTI5 and *E. coli* QC774), while no activity bands were detected with positive CFEs from *Bifidobacterium* spp. (data not shown). This was in contrast to previous reports on the SOD activity of *Bifidobacterium* spp. [15, 16].

Denaturation of Cell-free Extracts by Boiling

In order to determine whether the apparent SOD activity of *B. infantis* and *B. longum* is labile to heat denaturation, we compared the activities of CFEs from the two strains before and after boiling for 10 min at 100°C. As shown in Table 2, the SOD activity of *E. coli* CFEs was completely abolished upon boiling whereas the apparent SOD activity of CFEs from *Bifidobacterium* spp. remained unaltered. Based on the results from this boiling experiment, it appears that the apparent SOD activity is likely to be due to nonenzymatic dismutation. Otherwise, the *Bifidobacterium* spp. should have possessed highly heat-resistant SOD enzyme, which is extremely difficult to visualize by the conventional NBT gel staining method.

Nonenzymatic Dismutation Activity by Divalent Metal Ions

Several lactic acid bacteria have evolved to a nonenzymatic dismutation system which functions by accumulating high intracellular levels of Mn^{2+} [1, 2]. MRS medium, which is widely used for culturing *Bifidobacterium* spp., contains a high amount of $MnSO_4$ (120 mg/l, ca. 0.5 mM). Therefore, the apparent SOD activity of *Bifidobacterium* spp. grown in MRS medium might be resulted from the nonenzymatic dismutation system based on high intracellular Mn^{2+} levels. In order to corroborate this notion, we carried out metal supplementation experiments as described in the Materials and Methods. Modified MRS medium which had been prepared without manganese and iron was supplemented with increasing concentrations of $MnSO_4$ and $FeSO_4$. As shown in Fig. 2A, the apparent SOD activity of boiled CFEs from *B. infantis* gradually increased with increasing concentrations of $MnSO_4$ added to the modified MRS medium. However, the boiled CFEs from control *E. coli* HB101/pDTI-5 showed no SOD activity regardless of manganese supplementation. Similar results were obtained from the iron supplementation experiment (Fig. 2B). *B. longum* also showed similar results to *B. infantis* (data not shown). These results support our notion that the apparent SOD activity of *Bifidobacterium* spp. is due to a nonenzymatic dismutation system

Table 2. Effect of heat denaturation on the apparent SOD activities of *B. infantis*, *B. longum*, and *E. coli*.[a]

Strains used	Nondenaturation (units/mg proteins)	Heat denaturation[b] (units/mg proteins)
E. coli HB101/pDTI-5	454	ND[c]
E. coli QC774	ND	ND
B. infantis	70.5	70.1
B. longum	30.3	30.3

[a]CFEs were prepared from cells at early stationary phase [OD_{600}].
[b]CFEs were denatured by boiling for 10 min at 100°C.
[c]ND, Not detected.

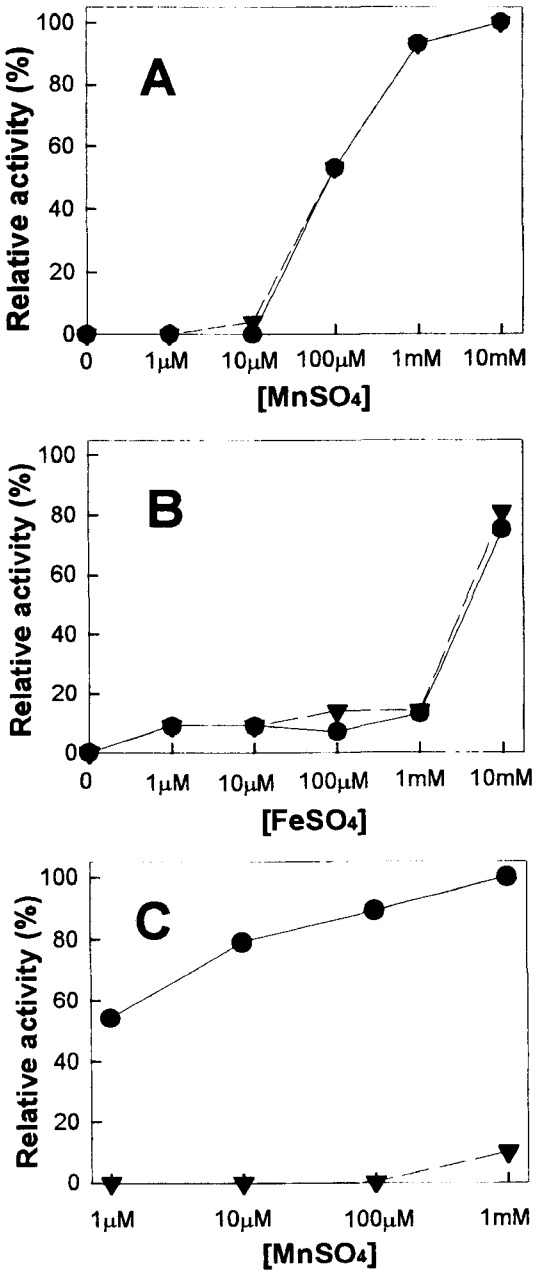

Fig. 2. Effects of metal ions on dismutation activity.

CFEs were prepared from cells after 12 hr incubation in the modified MRS medium supplemented with indicated concentrations of $MnSO_4$ or $FeSO_4$. A, *B. infantis* with [$MnSO_4$]; B, *B. infantis* with [$FeSO_4$]; C, *E. coli* HB101/pDTI-5 with [$MnSO_4$]. Symbols: nondenatured CFEs (●), heat-denatured CFEs (▼).

utilizing divalent metal ions such as Mn^{2+} and Fe^{2+} which are present at significant concentrations in MRS medium. The assay method for SOD activity was based on the inhibition of cytochrome c reduction by SOD [11]. In this indirect assay system, there seems to always be a possibility of false positive SOD activity resulting from a nonenzymatic dismutation system, in addition to the actual SOD enzyme.

False Positive SOD Activity in Reaction Mixture Supplemented with Manganese

In order to substantiate the above results, we devised two experiments. The first experiment was carried out in the same way as in the metal supplementation experiment except for the use commercial SOD isolated from *B. subtilis*. As shown in Fig. 3, in the absence of manganese supplementation, the apparent SOD activity of commercial SOD was completely abolished by heat denaturation. The apparent SOD activities of nondenatured and denatured SODs increased with increasing concentration of $MnSO_4$ added to the reaction mixture. The increase in the total apparent SOD activity of nondenatured commercial SOD appears to result from the additive effects of the actual SOD activity and nonenzymatic dismutation activity by manganese. These results indicate that false positive SOD activity could be detected by the cytochrome c method due to the high concentrations of divalent metal ion such as Mn^{2+}.

In the second experiment, nonenzymatic dismutation activity of a reaction mixture with no SOD added was measured with increasing concentration of $MnSO_4$. As shown in Fig. 3B, the apparent SOD activity increased gradually with manganese added to the reaction mixture. The pattern was identical to that of nondenatured commercial SOD (Fig. 3A). These results indicate that commercial SOD was completely inactivated by boiling and manganese substituted for SOD inhibited cytochrome c reduction in the indirect SOD assay method (Fig. 3). These results are in line with our conclusion that the apparent SOD activity of *Bifidobacterium* spp. was false positive SOD activity caused by divalent metal ions such as Mn^{2+} and Fe^{2+} present at high concentrations in MRS medium.

According to a recent report by Shin and Park [16], several strictly anaerobic bacteria including *Bifidobacterium* spp. showed significant levels of SOD activity measured by the cytochrome c method. Surprisingly, *B. adolescentis* ATCC 15703 showed higher SOD activity than the *E. coli* used as a control. However, we were unable to obtain similar levels of SOD with the *Bifidobacterium* spp. examined. Moreover, the apparent SOD activity we detected was not abolished by heat denaturation nor induced by oxidative stress. Taking these results together, we conclude that the apparent SOD activity of *Bifidobacterium* spp. examined in this study is due to nonenzymatic dismutation by intracellular metal ions.

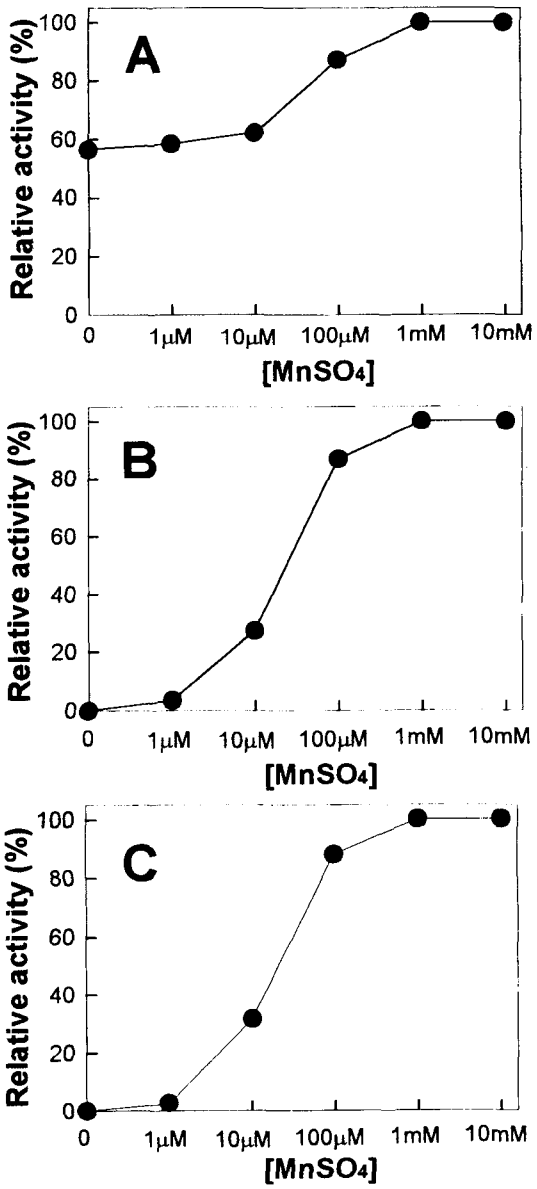

Fig. 3. Effect of manganese on the dismutation activity assay.
Relative activities were determined in the reaction mixture supplemented with indicated concentrations of $MnSO_4$. A, nondenatured *Bacillus* SOD (1 unit/mg protein); B, heat-denatured *Bacillus* SOD (1 unit/mg protein); C, buffer with $MnSO_4$ (blank reaction without SOD).

Acknowledgments

This research was supported by grants from Inha University (1997) and KOSEF (95-2-04-01-01-3). We would like to thank Dr. Soon-Young Shin at the Korea Food Research Institute for providing us with several *Bifidobacterium* spp. Thanks are also due to Professor

D. Touati at the University of Paris, France for the generous gift of *E. coli* strain QC774 and plasmid pDTI-5.

REFERENCES

1. Archiblad, F. S. and I. Fridovich. 1981. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. *J. Bacteriol.* **145:** 442–451.
2. Archiblad, F. S. and I. Fridovich. 1981. Manganese, superoxide dismutase, and oxygen tolerance in some lactic acid bacteria. *J. Bacteriol.* **146:** 928–936.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:** 248–254.
4. Charles, B. and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44:** 276–287.
5. Fridovich, I. 1983. Superoxide radical: An endogenous toxicant. *Ann. Rev. Pharmacol. Toxicol.* **23:** 239–257.
6. Gotz, F., E. F. Elstner, B. Sedewitz, and E. Lengfelder. 1980. Oxygen utilization by *Lactobacillus plantarum*. *Arch. Microbiol.* **125:** 215–220.
7. Kim, E.-J., H.-J. Chung, B. Suh, Y. C. Hah, and J.-H. Roe. 1998. Expression and regulation of the *sodF* gene encoding iron- and zinc-containing superoxide dismutase in *Streptomyces coelicolor* Muller. *J. Bacteriol.* **180:** 2014–2020.
8. Leo, M., K. Fischer, and T. Leisinger. 1995. Characterization of the superoxide dismutase gene and its upstream region from *Methanobacterium thermoautotrophicum Marburg*. *FEMS Microbiol. Lett.* **28:** 247–253.
9. Margaret, Y. G., B. R. Glick, and J. E. Thompson. 1990. Cloned manganese superoxide dismutase reduces oxidative stress in *Escherichia coli* and *Anacystis nidulans*. *Proc. Natl. Acad. Sci. USA* **87:** 2608–2612.
10. Mitsuoka, T. 1990. Bifidobacteria and their role in human health. *J. Ind. Microbiol.* **6:** 263–268.
11. McCord, J. M. and I. Fridovich. 1969. Superoxide dismutase. An enzymatic function for erythrocuperin (hemocuperin). *J. Biol. Chem.* **244:** 6049–6055.
12. Nakayama, K. 1994. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. *J. Bacteriol.* **176:** 1939–1943.
13. Park, Y.-D., K.-J. Lee, and J.-H. Roe. 1994. Effect of partial oxygen pressure on the growth and defense enzyme activities of *Streptomyces coelicolor* in continuous culture system. *Kor. J. Appl. Microbiol. Biotechnol.* **22:** 538–543.
14. Smith, M. W. and R. F. Doolittle. 1992. A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *J. Mol. Evol.* **34:** 175–184.
15. Shimamura, S., F. Abe, N. Ishibashi, H. Miyakawa, T. Yaeshima, T. Arata, and M. Tomita. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. *J. Dairy Sci.* **75:** 3296–3306.
16. Shin, S.-Y. and J.-H. Park. 1997. Activity of oxidative enzymes related with oxygen tolerance in *Bifidobacterium* sp. *J. Microbiol. Biotechnol.* **7:** 356–359.
17. Shirley, Y. R. P., J. L. Diguiseppi, and I. Fridovich. 1984. Induction of superoxide dismutase in *Escherichia coli* by manganese and iron. *J. Bacteriol.* **160:** 137–142.