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NOTE

Activities of Oxidative Enzymes Related with Oxygen Tolerance in *Bifidobacterium* sp.

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To study the relationship between oxygen tolerance and enzyme activity in the oxygen metabolism of bifidobacteria, the activities of catalase, superoxide dismutase (SOD), NADH oxidase and NADH peroxidase from six typical bifidobacteria and other bacteria were assayed by spectrophotometry. Catalase activity was hardly detected in any of the bifidobacteria tested. SOD activity was detected in every species including the *Clostridium* species. In particular SOD activity was notably high in the aerosensitive *Bifidobacterium adolescentis*. This fact indicates that SOD activity is not a critical factor to ensure aerotolerance. Aerosensitive *B. adolescentis* showed very low NADH oxidative enzyme activity whereas other aerotolerant bifidobacteria exhibited considerable activity for the enzymes. It seems that detoxification of H_2O_2 by NADH oxidative enzymes might be an important factor in improving for aerotolerant bifidobacteria survival rates in an oxygen environment.

Bifidobacteria are a predominant species of human and animal intestinal microorganisms, and are known to be beneficial to the health of their hosts. Recently, many researchers have studied the introduction of these strains to food, feed, drugs, and even to Kimchi (1) in the form of viable probiotic cells (19). However, it is difficult to maintain high levels of cell viability in large scale of fermentations and in applications in the food industry because these strains are strict anaerobes (9). In general, bifidobacteria do not grow on agar plates in air. However, some strains show a considerable resistance to air (3). Many researchers (4, 15, 20) have tried to elucidate the relationship between oxygen senisitivity and enzyme activities in this microorganisms, a definite correlation has not yet been reported. McCord *et al.* (14) have reported on the distribution of catalase, superoxide dismutase (SOD) in various microorganisms. They suggested that strict anaerobes exhibited no SOD and generally no catalase activity, and all aerobic organisms containing the cytochrome system had both SOD and catalase, and aerotolerant anaerobes were devoid of catalase but exhibited SOD activity. However, Hewitt and Morris (5) have refuted their observation in that the possession of SOD was not restricted to organisms capable of growth in air.

In short, susceptability to oxygen and the correspondent enzyme activities of bifidobacteria are still unclear. From this background, the objecive of this experiment was to examine the relationship between oxygen tolerance and enzyme activities involved in oxygen metabolism in bifidobacteria. The assayed enzymes were catalase, SOD, NADH oxidase and NADH peroxidase in 6 typical bifidobacteria and other reference bacterial species showing typical responses to oxygen in the environment.

Six bifidobacteria, *B. adolescentis* ATCC 15703, *B. animalis* ATCC 25527, *B. breve* ATCC 15700, *B. bifidum* ATCC 29521, *B. infantis* ATCC 15697 and *B. longum* ATCC 15707 were obtained from the American Type Culture Collection. As reference bacteria species, *Bacillus subtilis* ATCC 14593, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 4698, *Lactobacillus casei* ATCC 9018, *Clostridium acetobutylicum* ATCC 4259 and *Clostridium perfrigens* ATCC 13124 were also purchased from ATCC.

For bifidobacteria species, *L. casei*, and *C. acetobutylicum*, supplemented MRS (SMRS) and MRS (Difco) media were prepared anaerobically in 150 ml serum vials. SMRS media consisted of 0.05% (W/V) L-cysteine HCl, 0.075% bacto agar, 0.02% sodium carbonate, and 0.01% calcium chloride dihydrate in the MRS media. Reinforced clostridial medium (RCM) (Difco) was used for *C. perfrigens*. Nutrient media were used for *B. subtilis* and *E. coli*, whereas enriched nutrient media containing 1.25% heart infusion, 0.25% yeast extract in nutrient broth were used for for *M. luteus*. All tested bacteria except *M. luteus* at 30°C were cultured at 37°C.

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Catalase, SOD, cytochrome c, xanthine, xanthine oxidase and NADH were obtained from Sigma. Other reagent grade chemicals were used through the experiment.

Oxygen sensitivity of bifidobacteria was determined by plate counting of late exponential phase cells gradiently diluted onto the modified bifidobacteria selective (MBS) media plates which were exposed to air at 37°C for 24 h. MBS agar medium consisted of 10 g trypticase (BBL), 5 g proteose peptone No. 3 (Difco), 3 g ammonium sulfate, 2 g potassium phosphate (monobasic), 1 g potassium phosphate (dibasic), 0.2 g magnesium sulfate, 0.5 g L-cysteine HCl, 10 g sodium propionate, 25 ml oligosaccharide mixture, 15 g agar, 1 ml resazurin (0.1%) per litre of distilled water, pH 7. The used oligosaccharide mixture consisted of 20% fructooligosaccharide, 10% galactooligosaccharide, and 10% isomaltooligosaccharide in distilled water. This mixture solution was separately autoclaved and added when needed to prepare MBS media. Dilution solution was prepared anaerobically in anaerobic pressure tubes. The solution contained 4.5 g potassium phosphate, 6.0 g sodium phosphate (monobasic), 0.5 g L-cysteine HCl, 0.5 g Tween 80, 0.5 g bacto agar and 1 ml resazurin (0.1% solution) per litre of distilled water. The inoculated plates were incubated in an anaerobic glove box (H_2 5%, CO_2 15%, N_2 80%) at 37°C and colonies were counted after 72 h.

Bacterial cells cultured anaerobically in 150 ml serum vials or aerobically in 250 ml flasks containing 100 ml of culture media, were harvested by centrifugation at 8,000 rpm and 4°C for 20 min. The cells were washed twice with cold saline, and resuspended in 2 ml of saline. The cell suspensions of bifidobacteria were disrupted in ice with sonication (Sonic Materials Inc. Connecticut, U.S.A.) for 10 min. The disrupted cell suspensions were centrifuged at 14,500 rpm and 4°C for 15 min to obtain the cell free extract. The protein content of the cell free extract was assayed by Bradford method using Bio-Rad protein kit reagents (Bio Rad Laboratories, U.S.A.).

Catalase activity in the cell free extract was measured according to the method of Beers and Sizer (2). The activity was measured by a decrease in absorbance at 240 nm of the molar extinction coefficient of 43.6 M^{-1} cm^{-1}. SOD activity was determined according to the method of McCord and Fredovich (13). One unit of SOD activity was defined as the protein amount of cell free extract required to inhibit the reduction rate of cytochrome c by 50% per minute at 25°C. NADH oxidase activity was assayed by determining the rate of NADH oxidation at 37°C spectrophotometrically. The reaction mixture contained 10 µl of 20 mM NADH, 2,390 µl of 50 mM phosphate buffer at pH 5.5 and 100 µl of cell free extract. The NADH peroxidase activity was assayed by measuring the rate of NADH oxidation under anaerobic condition. Fifty mM of phosphate buffer at pH 5.5 and 25 mM of hydrogen peroxide dissolved in the buffer were prepared anaerobically and kept in an anaerobic glove box at 37°C before the assay. Activity of NADH oxidase and NADH peroxidase was determined by calculation from absorbance differences at 340 nm per min from the initial linear portion of the curve with a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} (7).

Growth of 6 strains of bifidobacteria were compared after exposed to air for 3, 10 and 24 h. As shown in Fig. 1, the growth of 5 species apart from the *B. adolescentis* species were not affected by exposure to air for 3, 10, or even 24 h. These 5 species of bifidobacteria showed a high tolerance to oxygen. *B. adolescentis* was the only strain among the bifidobacteria showing an inhibition of growth under aerobic conditions. Three species of bifidobacteria, *i.e.*, *B. longum*, *B. breve*, and *B. infantis*, displayed trace levels of catalase activity compared to other 3 speices. Organisms in the aerobic category, *E. coli*, *B. subtilis* and *M. luteus* showed 141, 1,659, and 2,522 units of catalase activity, respectively. Meanwhile anaerobes, *i.e.*, *C. acetobutylicum*, *C. perfrigens*, *L. casei*, and the rest of the bifidobacteria strains examined, *i.e.*, *B. adolescentis*, *B. animalis*, and *B. bifidum*, did not show any catalase activity as expected. The presence of catalase activity in the 3 bifidobacteria contrasted with the absence of this enzyme's actvities in the other three bifidobacteria.

In this study, SOD activity was detected in all tested

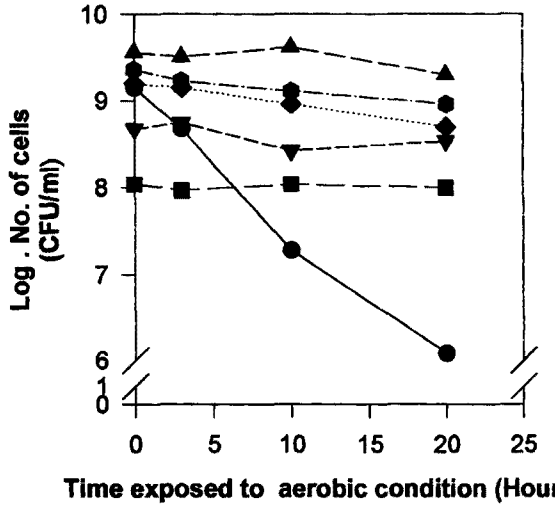

Fig. 1. Comparison of aerotolerance of 6 typical strains of bifidobacteria.

Cells taken from SMRS media culture in the late exponential phase were diluted gradiently and inoculated on the MBS medium. The plates were incubated at 37°C for 72 h anaerobically before counting colonies. Symbols: *B. adolescentis* (●), *B. animalis* (■), *B. breve* (▲), *B. bifidum* (▼), *B. infantis* (◆), and *B. longum* (⬢).

organisms and ranged from 13 units/mg to 278 units, and showed 21 fold variation. The activity of *B. adolescentis*, *B. breve*, and *E. coli* ranged from 108 to 278 units at the high level, which was more than those of the aerobes. *C. acetobutylicum*, a strict anaerobe, showed the lowest activity level, but still remained at the detectable level.

The activitiy levels of NADH oxidase in bifidobacteria ranged from 0.04 unit (*B. adolescentis*) to 1.19 unit (*B. longum*) and those of other organisms ranged from either nothing (*C. acetobutylicum* and *M. luteus*) or 0.03 units (*B. subtilis*) to 3.71 units (*L. casei*). The activity of *B. adolescentis* was the lowest among the tested bifidobacteria. The activity of *L. casei* was highest than in any other organism used in this study.

The activities of NADH peroxidase were detected in every species tested, ranging from 0.01 unit for *C. acetobutylicum* to 18 units for *L. casei*. The activity of *B. adolescentis* was the lowest among the other bifidobacteria, whereas the activity of *L. casei* was markedly higher than any other bacteria. This tendency was the same as for NADH oxidase. Aerobes, *B. subtilis*, *E. coli* and *M. luteus*, showed higher levels of catalase activity and showed an extremely low level of NADH oxidase and NADH peroxidase.

The results of O_2 related enzyme activity show that the distribution of SOD, NADH oxidase and NADH peroxidase activity does not strictly depend on any aerotolerant or the anaerobic character of the organisms and needs more complex interpretation.

In this experiment, three bifidobacteria showed a trace level of catalase activities. The authors tried to confirm this activity qualitatively by applying 3% H_2O_2 into the cells spread onto the glass slides. However, the three bifidobacteria showed negative responses to the direct method for the viable cells. Uesugi and Yajima (21) have analyzed oxygen metabolism of strictly anaerobic 25 intestinal bacteria. They have observed that several anaerobes, bifidobacteria and bacteroides had a reasonable level of catalase activity. These results suggest that catalase may not be the only distributed for aerobes and needs more observation.

The results of SOD activity in this study revealed again that SOD levels in organisms might not be essential in differentiating strict anaerobes and aerotolerant organisms as reported in other papers (5, 8, 21). The SOD activities in this study were found in all species tested. Surprisingly, the activity of aerosensitive *B. adolescentis* was the highest in all species tested including the aerobes and anaerobes. A representive strict anaerobe, *C. acetobutylicum* had a low level of SOD activity. These results suggest that SOD may be a basic enzyme involved in the detoxification of superoxide radical O_2^- and that it is not sufficient to remove O_2-related damage because the SOD produces another toxic oxygen related metabolite, *i.e.*, H_2O_2 (18). Therefore, the bacteria devoid of catalase essentially needs another system to decompose H_2O_2 to H_2O and O_2.

The distribution of activities of NADH oxidase and NADH peroxidase showed a comparable agreement with oxygen senstivity in bifidobacteria. The activities in aerosensitive *B. adolescentis* were very low, and those in other aerotorelant bifidobacteria were relatively high.

Because accumulated H_2O_2 was not analysed in the present study, it can not be shown whether or not the reaction of NADH oxidase was by a two electron tranfer or a four electron tranfer system (20). However, the results suggest that NADH oxidative enzymes play an

Table 1. Comparison of catalase, superoxide dismutase, NADH oxidase, and NADH peroxidase activity of 6 typical strains of bifidobacteria and other species of bacteria.[a]

Species	Aerobicity	Specific activity (unit/mg protein of cell-free extract)[b]			
		Catalase	SOD	NADH oxidase	NADH peroxidase
B. adolescentis	Strict anaerobe	0	278	0.04	0.06
B. longum	Strict anaerobe	t[c]	24	1.19	1.25
B. animalis	Strict anaerobe	0	33	1.20	1.04
B. breve	Strict anaerobe	t[c]	108	0.60	0.11
B. bifidum	Strict anaerobe	0	39	0.24	0.27
B. infantis	Strict anaerobe	t[c]	57	0.68	0.27
C. acetobutylicum	Strict anaerobe	0	13	0.00	0.01
C. perfrigens	Strict anaerobe	0	32	0.50	0.02
E. coli	Facultative anaerobe	141	109	0.07	0.03
L. casei	Facultative anaerobe	0	16	3.71	18.1
M. luteus	Aerobe	2522	31	0.00	0.02
B. subtilis	Aerobe	1659	67	0.03	0.03

[a]Cell-free extracts were prepared from cells at early stationary phase. [b]The catalase and SOD actitities were evaluated by the method of Beers and Sizer (2) and McCord and Fridovich (13), respectively, NADH oxidase and NADH peroxidase activities were measured from NADH oxidation at 340 nm. [c]Trace.

important role in detoxifying oxygen damage in bifidobacteria. Many studies on the aerotolerance of lactic acid bacteria suggest that the balance and intensity of NADH oxidative enzymes probably contributes to elimination of the environmental oxygen (6, 10, 12, 15-17). The results of the present experiment show similar trends. In addition, the authors emphasize that aerotolerance in this species seems to be closely related to H_2O_2 eliminating capacity as well as the removal of a superoxide radical by SOD. Therefore, the authors suggest that it is necessary for bifidobacteria which do not use molecular oxygen as the terminal electron acceptor to be studied further and that we need to examine reaction of the NADH oxidative enzymes in detail. Mutant studies that are, aerosensitive but have high NADH oxidative enzymes and are aerotolerant but have low NADH oxidative mutants, will probably contribute to any further explanation for the oxygen tolerance mechanism of bifidobacteria.

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