J. Microbiol. Biotechnol. (2001), **11**(3), 443–451

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Physiological Responses of Oxygen-Tolerant Anaerobic *Bifidobacterium longum* under Oxygen

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Received: January 11, 2001
Accepted: April 3, 2001

Abstract In order to investigate what kind of response anaerobic bifidobacteria has on oxygen stress, five oxygen-tolerant bifidobacteria were isolated from human fecal samples. All were temporarily identified as *Bifidobacterium longum* through an analysis of carbohydrate utilization patterns and cellular fatty acid profiles. In the presence of oxygen, the lag phase became extended and the cell growth was suppressed. Bifibobacterial cell was able to remove dissolved oxygen in an early stage of growth and to overcome oxygen stress to a certain extent. The cell became long in size and showed a rough surface containing many nodes which were derived from abnormal or incomplete cell division. Cellular fatty acid profiles changed remarkably under a partially aerobic condition, so that the carbon chain of cellular fatty acid became short. All the dimethyl acetals originated from plasmalogen were reduced, and cyclopropane fatty acid, 9,10-methyleneoctadecanoic acid ($C_{19:0}$cyc9,10), was increased remarkably. Oxygen stress induced a 35.5 kD protein in *B. longum* JI 1 of the oxygen-tolerant bifidobacteria, that was named Osp protein, and its N-terminal amino acid sequence was as follows: unknown amino acid-Thr-Gly-Val-Arg-Phe-Ser-Asp-Asp-Glu. Therefore, the oxygen-tolerant bifidobacteria seemed to defend against oxygen stress by increasing the content of short fatty acid and cyclopropane fatty acid, and induction of an oxygen stress protein, but not the plasmalogen.

Key words: Bifidobacteria, oxygen stress, cellular fatty acid, plasmalogen, 9,10-methyleneoctadecanoic acid, induced protein

Bifidobacteria are the predominant species of human and animal intestinal microorganisms, which are known to be beneficial to the health of their host [12, 13, 19]. Therefore, many researchers have attempted to apply bifidobacteria to food, feed, drugs, and even to kimchi [2, 35]. Unfortunately, it is not easy to use bifidobacteria in dairy fermented food and therapeutic preparations, mostly due to their vital sensitivity to oxygen stress. For the effective use of bifidobacteria, it is necessary to select oxygen-tolerant bifidobacteria and to define their physiological responses and tolerance mechanism to oxygen stress. Although *Bifidobacterium* have been known to be strict anaerobes, some strains, such as *Clostridium* sp., *Bacteroides* sp., and *Eubacterium* sp., have a considerable tolerance level to oxygen stress compared with other strict anaerobes [7, 32]. In general, reactive oxygen species (ROS) have been shown to cause some damage of DNA, RNA, protein, and lipid in living cells [3, 4, 8, 10, 15, 22, 23]. ROS are produced as an inevitable by-product of the normal aerobic metabolism. Oxygen toxicity results when the degree of oxidative stress caused by ROS exceeds the capacity of the cell defense system [5, 34]. Most aerobic and facultative anaerobic bacteria have evolved complex responses and defense mechanisms to mitigate the damaging effects of ROS [5]. In particular, the responses to oxidative stress of *E. coli*, *S. typhimurium*, and lactic acid bacteria (LAB) such as *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* have been well investigated [25]. In recent years, there has been some growing evidence of the strict anaerobe possessing a defense mechanism involving enzymatic systems for scavenging oxygen radicals [17]. However, the responses and defense mechanism of bifidobacteria to the oxygen stress have not been fully explored. In this study, oxygen-tolerant bifidobacteria were isolated from human fecal samples and their physiological responses under oxygen stress were extensively investigated.

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MATERIALS AND METHODS

Bacterial Strains and Culture Condition

B. longum ATCC 15707 and *B. adolescentis* ATCC 15703 were purchased from the Korean Collection for Type Culture (KCTC). One hundred and fifty three bifidobacterial strains were isolated from human fecal samples, and the TP plate medium [18] was used for the selective isolation process. An anaerobic growth of bifidobacteria on agar plates was carried out in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, U.S.A.). An anaerobic broth culture was prepared in a 100 ml serum bottle containing oxygen-free SMRS broth, which was lactobacilli MRS broth (Difco, U.S.A.) supplemented with 0.5 g/l of L-cysteine HCl, 0.2 g/l of Na_2CO_3, and 0.1 g/l of $CaCl_2 \cdot 2H_2O$.

Determination of Oxygen-Sensitivity and Identification of Oxygen-Tolerant Bifidobacteria

Oxygen-sensitivities of the isolates were determined by the method described in a previous report [1]. Analyses of carbohydrate utilization patterns and cellular fatty acid profiles were used for identifying the selected strains. Carbohydrate utilization patterns were investigated with API 50CH (bioMérieux Vitek, Inc., France) involving a 49 carbohydrate fermentation test. After 12 h of incubation in an anaerobic glove chamber, results were compared with the identification keys which were proposed by Roy *et al.* [29] and *Bergey's Manual of Systematic Bacteriology* [30]. An analysis of cellular fatty acid was completed by MIDI system (Microbial ID, Inc., U.S.A.). Cells were incubated for 48 h anaerobically in the PYG-Tween 80 media (Table 1). Extraction and analysis of the cellular fatty acid was performed by following the instruction manual for the MIDI system. Identification was carried out automatically with the Moore library of the Sherlock software.

Table 1. Composition of PYG-Tween 80 medium.

Component	Amount
Peptone	5.0 g
Pepticase	5.0 g
Yeast extract	10.0 g
Glucose	10.0 g
Tween 80	0.25 ml
Resazurin solution[a]	4.0 ml
Salt solution A[b]	40.0 ml
Hemin solution[c]	10.0 ml
Vitamin K_1[d]	0.2 ml
L-cysteine · HCl	0.5 g
Distilled water	1.0 l

[a]Resazurin solution: Dissolve 25 mg resazurin in 100 ml distilled water. [b]Salt solution: $CaCl_2 \cdot 2H_2O$ 0.25 g, $MgSO_4$ 0.20 g, K_2HPO_4 1.00 g, KH_2PO_4 1.00 g, $NaHCO_3$ 10.0 g, NaCl 50.0 g in 500 ml D.D.W. [c]Hemin solution: Dissolve 50 mg hemin in 1 ml of 1N NaOH and make up to 100 ml with D.D.W. [d]Vitamin K_1: Dissolve 0.15 mg of Vitamin K_1 in 30 ml of 95% ethanol.

Cultivation under Oxygen Stress

Two-hundred ml of SMRS broth with 6 ml of seed culture and cotton plug in a 500-ml Elenmeyer flask were incubated in the shaking water bath (Jeio Tech. Co., Ltd., Korea) at 37°C. The culture condition under oxygen was obtained by shaking at a constant rate during the incubation process. Partially aerobic and aerobic culture conditions were adjusted by shaking at a rate of 40 strokes/min and 80 strokes/min, respectively.

Monitoring of Cell Growth and Dissolved Oxygen (D.O.) under Oxygen Stress

Twelve flasks of 200 ml SMRS broth were prepared for monitoring cell growth and D.O. during the incubation process under oxygen stress. Six ml of seed culture was inoculated into each broth and sealed with cotton plugs. Six of them were incubated under a partially aerobic condition and others were incubated under an aerobic condition. Cell growth and D.O. were measured after 0, 3, 6, 8, 12, and 24 h. Cell growth was measured by its absorbance level at 600 nm. For measuring D.O., it was necessary to prevent influx of environmental oxygen. Liquid paraffin was overlaid on the culture broth with a 50-ml syringe and an O_2 electrode (Ingold Electrodes Inc.. Andover, MA, U.S.A.) was placed into the broth culture. The D.O. value was read after a stabilization process for 20 min.

Scanning Electron Microscopy

Cells which were washed twice with saline solution were attached to filter paper (Gelman, Germany) of 0.45 pore size. The pieces were then fixed by immersing them in 0.2 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2.5% paraformaldehyde for 3 h, and washed in 0.2 M of phosphate buffer three times for 15 min. They were dehydrated stepwise with 50, 70, 80, 90, and 100% ethanol, and finally with 100% ethanol three times for 15 min.

The specimens were dried in a critical point drier (Polaron, Watford, England) with carbon dioxide as a transition fluid, mounted on the metal studs, and coated with gold for 5 min. Cell shape was examined with an ambient-temperature scanning electron microscope (Atsem, JSM-5410LV, Jeol. Ltd., Japan).

Analysis of Cellular Fatty Acids

Sample preparation was performed by following the method of Shin *et al.* [16, 33]. Varian 3300 gas chromatographic system with Supelco sp2330 capillary column (length 30 m, ID 0.25 mm) and FID detector was used for analyzing cellular fatty acid. Analysis was performed isothermally at 170°C with helium as a carrier gas at a rate of 30 ml/min.

Injector and detector temperature were maintained at 210°C and 270°C, respectively.

Analysis of Cellular Protein Pattern

Bacterial cells were cultured under partially aerobic and anaerobic conditions in 100 ml of SMRS broth, and were harvested by centrifugation at 5,000 rpm at 4°C for 10 min. Cells were washed twice with cold saline solution and resuspended in 5 ml of the solution. Cell suspensions were disrupted with sonication (Sonic Materials Inc, Connecticut, U.S.A.) for 4 min. Cell debris was removed by centrifugation at 18,000 rpm at 4°C for 10 min, and the supernatant was lyophilized and used as a cell free extract. Protein concentration was assayed by the Bradford method, using a Bio-Rad protein assay kit (Bio-Rad Laboratories., U.S.A.). In order to analyze the protein pattern of the extract, electrophoresis was carried out with a SE600 vertical slab gel unit (Hoefer Scientific Instruments, U.S.A.). SDS polyacrylamide gel [7.5% (w/v)] was used for separating large molecular weight proteins, and 12.5% (w/v) SDS polyacrylamide gel was used for small molecular weight proteins. Samples were mixed with a buffer containing 0.2 M dithiothreitol used as a reducing agent to break disulfide linkages, and then boiled for 90 sec. Fifty μg of protein was loaded per lane, and electrophoresis was performed until the dye reached the bottom of the gel. The gel was stained with Coomassie Blue R-250 for visualization.

Purification and Amino Acid Sequence Analysis

Lyophilized cell free extracts from bacterial cells under a partially aerobic condition were resuspended in D.D.W. Proteins were fractionated and divided into four parts with the FPLC system (Phamacia, Sweden) using Bio-Scale Q2, an anion exchange column (Bio Rad, U.S.A.). Tris · HCl buffer at pH 7.0 was used at a rate of 1 ml/min. Proteins were eluted with a linear gradient of 0 to 1 M of NaCl. The partially purified fraction containing the target protein was lyophilized and resuspended in 100 μl of D.D.W. Twenty five μg protein was loaded onto a 12.5% SDS polyacrylamide gel, and electrophoresis was carried out by the protocol described previously. Proteins in the gel were transferred to the PVDF (polyvinylidene difluoride) membrane with an electrotransfer system (Hoefer Scientific Instrument, U.S.A.). The target protein band was cut and a partial N-terminal amino acid was sequenced with Precise Protein Sequencing System (Applied Biosystem, U.S.A.).

RESULTS AND DISCUSSION

Selection and Identification of Oxygen-Tolerant Bifidobacteria

Among 153 bifidobacteria isolated from human fecal samples, five strains showed viability over 90% under oxygen stress (Table 2). They showed tolerance to oxygen stress greater than *B. longum* ATCC15707, which is known as the oxygen-tolerant strain [31, 32]. While 82% cells of *B. longum* ATCC 15707 survived when exposed to oxygen stress for 24 h, more than 90% of the cells five tolerant strains, (JI 1, E4, L2, JR76, and JR89) survived.

Table 2. Oxygen-sensitivities of bifidobacteria from human fecal samples.

Strains	[a]Viability (%)	Strain	Viability (%)
B. longum ATCC15707	82	IH1	89
B. adolescentis ATCC15703	67	JO3	66
JI 1[t]	97	IH6	65
E4[t]	98	MH3	83
JR76[t]	93	MS1	82
L2[t]	92	E2-18	79
JR89[t]	95	JS8	81
Y-1	62	EI 7	79
JR13	60	EI 5	65
JR4-2	62	JS9	65
J2	61	KI1	70
K-1	60	KI3	67
JR20	83	HJ	65
EH17	65	SJ	68
MS5	67	BMH3	69
JO4	71	KF94	67
K-2	84	Int-57	65
KF79	64	KF91	72
KF82	88	KF92	82

Oxygen-sensitivities were determined by Ahn *et al.*'s method [1]. [a]Viability was defined as follows. Viability (%)=[Log(CFU/ml) under oxygen stress/ Log (CFU/ml) under anaerobic condition]×100. [t]Oxygen-tolerant strains.

All the oxygen-tolerant strains could utilize L-arabinose and D-xylose. In particular, they could utilize melezitose, which is an important carbohydrate for identification of *B. longum* (Table 3). Analyses of the cellular fatty acid profiles and carbohydrate utilization patterns were conducted. They showed 49.7 to 69.8% of similarity to *B. longum*, when compared with the Moor library in the Sherlock software (Table 4). Palmitic acid ($C_{16:0}$) was the major cellular fatty acid of these strains. In particular, 2.13 to 4.25% of 9,10-methyleneoctadecanoic acid ($C_{19:0}$cyc9,10) was found. Therefore, all the oxygen-tolerant bifidobacteria, JI 1, E4, L2, JR76 and JR89, were temporarily identified as *B. longum*.

Growth Responses of the Bifidobacteria to Oxygen Stress

Growth responses of the oxygen-tolerant bifidobacteria were investigated by comparing their growths under a partially aerobic condition at a rate of 40 strokes/min and an anaerobic condition. The patterns of the oxygen-tolerant strains, *B. longum* and *B. adolescentis*, under partially aerobic and anaerobic environments are shown in Fig. 1 (A to F). As

Table 3. Carbohydrate utilization patterns and identification of oxygen-tolerant strains with the API kit.

Carbohydrates \ Strains	JI1	L2	JR76	JR89	E4
D-Arabinose	−	−	−	−	−
L-Arabinose	+	+	+	+	+
Ribose	+	+	+	+	+
D-Xylose	+	+	+	+	+
L-Xylose	−	−	−	−	−
Adonitol	−	−	−	−	−
β-Methyl-xyloside	−	−	−	−	−
Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	−	−	−	−	−
Rhamnose	−	−	−	−	−
Dulcitol	−	−	−	−	−
Inositol	−	−	−	−	−
Mannitol	−	−	−	−	−
Sorbitol	−	−	−	−	−
α-Methyl-D-mannoside	−	−	−	−	−
α-Methyl-D-glucoside	+	+	+	+	+
N-Acetyl glucosamine	−	−	−	−	−
Amygdaline	−	−	−	−	−
Arbutine	−	−	−	−	−
Esculine	−	−	−	−	−
Salicine	−	−	−	−	−
Cellobiose	−	−	−	−	−
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Saccharose	+	+	+	+	+
Trehalose	−	−	−	−	−
Inuline	−	−	−	−	−
Melezitose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
Amidon	−	−	−	−	−
Glycogen	−	−	−	−	−
Xylitol	−	−	−	−	−
β-Gentibiose	−	−	−	−	−
D-Turanose	+	−	+	+	+
D-Lyxose	−	−	−	−	−
D-Tagatose	−	−	−	−	−
D-Fucose	−	−	−	−	−
L-Fucose	−	−	−	−	−
D-Arabitol	−	−	−	−	−
L-Arabitol	−	−	−	−	−
Gluconate	−	−	−	−	−
Identification	*B. longum*	*B. longum*	*B. longum*	*B. longum*	*B. longum*

Symbols: −; Nongrowth, +; Growth.

expected, an anaerobic environment allowed the bifidobacteria normal growth. However, maximal cell densities under a partially aerobic condition were lower than those under an anaerobic condition. Furthermore, *B. adolescentis* ATCC15703, known as the oxygen-sensitive strain [31, 32], showed no growth under a partially aerobic condition.

Lag phase under a partially aerobic condition became longer than that under an anaerobic condition. It took 12 h to reach maximal growth in the anaerobic cultivation, while it took 24 h in the partially aerobic cultivation. Although the growth rate was reduced, considerable growth rate of the selected strains was observed under the culture. However, more vigorous shaking at a rate of 80 strokes/min completely inhibited the growth of all bifidobacteria tested. In this condition, the defense capacity of the bifidobacterial cell against oxygen stress might have been limited. Changes of

Table 4. Cellular fatty acid profiles and identification of oxygen-tolerant strains with the MIDI system.

Fatty acid and its derivatives	Oxygen-tolerant strains				
	JI 1	L2	JR76	JR89	E4
Saturated fatty acid					
$C_{12:0}$ FAME[1]	2.28	0.85	2.99	1.05	1.21
$C_{14:0}$ FAME	4.63	4.69	7.65	7.37	5.64
$C_{16:0}$ FAME	35.4	32.9	26.4	29.4	28.4
$C_{18:0}$ FAME	7.24	5.37	5.81	6.19	6.52
$C_{11:0}$ DMA[2]	0.47	0.51	1.62	0.57	0.34
$C_{14:0}$ DMA	5.20	4.32	3.61	3.32	4.62
$C_{16:0}$ DMA	2.36	1.77	2.01	0.87	2.21
$C_{18:0}$ DMA	1.97	1.03	1.24	1.11	1.87
Unsaturated fatty acid					
$C_{14:1}$*cis*9 FAME	0.81	0.26	0.12	--[4]	0.64
$C_{16:1}$*cis*7 FAME	0.86	0.87	2.45	1.36	0.57
$C_{16:1}$*cis*9 FAME	1.10	1.09	1.89	1.76	1.12
$C_{18:1}$*cis*9 FAME	8.83	8.32	11.3	15.1	8.38
$C_{18:1}$*cis*9 DMA	10.9	13.6	12.9	12.6	12.5
Cyclopropane fatty acid					
C_{19}cyc9,10 FAME	2.13	4.25	2.82	3.81	2.57
C_{19}cyc9,10 DMA	4.24	8.33	6.88	4.63	5.01
Summed feature[3]					
Summed feature 1	2.59	1.43	1.82	1.31	1.30
Summed feature 2	0.10	--	--	--	--
Summed feature 7	3.15	3.19	4.77	3.02	3.76
Summed feature 10	3.44	1.18	2.45	2.76	3.47
Similarity (%)	*B. longum* (49.7)	*B. longum* (69.8)	*B. longum* (55.4)	*B. longum* (51.2)	*B. longum* (52.1)

[1]Fatty acid methyl ester, [2]Dimethyl acetal, [3]Unknown fatty acid clusters, [4]Not detected.

dissolved oxygen (D.O.) and pH were also studied during the partially aerobic incubation (Fig. 2A). Any change of the culture pH seemed to be in accordance with cell growth, however, the change of D.O. was apparently different. Although the cell density did not increase, the environmental oxygen in the medium was removed within 6 h. In addition, the pH of the culture broth decreased slowly during this period. The biological significance of this is that the oxygen-tolerant bifidobacterial cell can remove the environmental oxygen to some extent and have a mechanism to mitigate oxygen toxicity. This phenomenon was shown more clearly by vigorous shaking of the culture at a rate of 80 strokes/min (Fig. 2B): Although no growth was observed, slight decreases of culture pH and D.O. were observed.

Changes of Cell Morphology under Oxygen Stress

Oxygen stress had some influence on the cell shape of bifidobacteria (Fig. 3). Cells under an anaerobic condition showed Y, V, or club-shapes, a typical shape of *Bifidobacterium* species, and their length ranged from 1.2 to 3.5 μm. However, the stressed cells did not show the typical bifidobacterial shape. Most of Y- or V-shaped cells disappeared and the cell size became longer, ranging from 5 to 7 μm. In addition, the cells showed a rough surface containing several nodes, evidence of incomplete and abnormal cell division. Although there were some experimental evidence to indicate that ROS inhibited the formation of macromolecules, such as lipid and protein, and cell proliferation [27], it was unclear as to what toxic species were responsible for those effects. Moreover, there was no report on the relationship between oxygen stress and cell proliferation of the strict anaerobic bacteria. These results suggest that oxygen indeed affects cell growth and it inhibits normal cell devision of bifidobacteria.

Changes of Cellular Fatty Acid Profiles under Oxygen Stress

Oxygen-tolerant bifidobacteria contained two interesting cellular fatty acids. First, a considerable amount of plasmalogen was found. Plasmalogen is an alk-1-enyl, acylphosphoglyceride that is found in the cell membrane of only strict anaerobic bacteria [20, 21]. It produces dimethyl acetal (DMA) in the methylation process for gas chromatographic analysis. Second, cyclopropane fatty acid was detected. Veerkamp [36] reported that bifidobacteria contain cyclopropane fatty acid, namely, 11,12-methyleneoctadecanoic acid that is called lactobacillic acid. However, the cyclopropane fatty acid of bifidobacteria is not identified as the lactobacillic acid, but

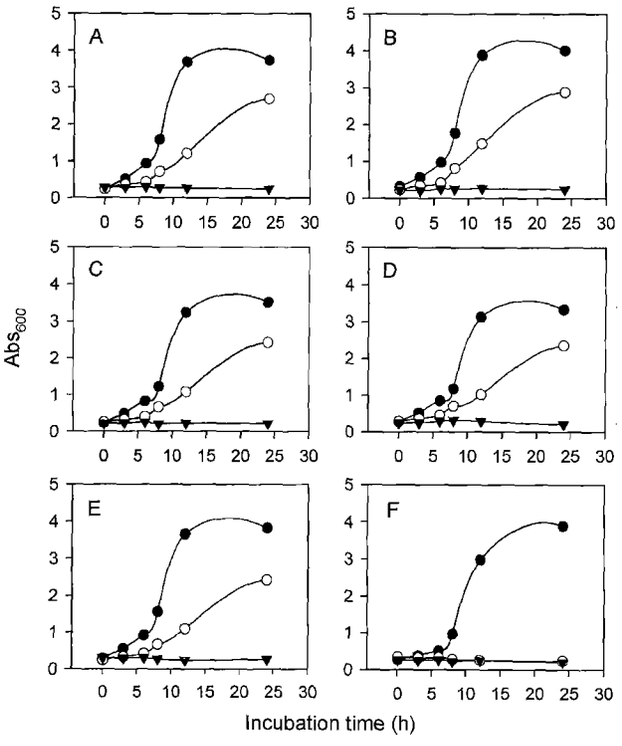

Fig. 1. Growth responses of bifidobacteria to oxygen stress.
Three % (v/v) seed culture of each strain was inoculated into SMRS broth and incubated under anaerobic, partial aerobic, and aerobic conditions at 37°C. Anaerobic culture (-●-) was performed with an oxygen-free medium. Partially aerobic culture (-○-) was performed in a shaking water bath at a rate of 40 strokes/min (37°C). Aerobic culture (-▼-) condition was obtained by shaking at a rate of 80 strokes/min. Bifidobacteria tested; A: *B. longum* JI 1, B: *B. longum* E4, C: *B. longum* L2, D: *B. longum* JR76, E: *B. longum* ATCC15707, F: *B. adolescentis* ATCC15703.

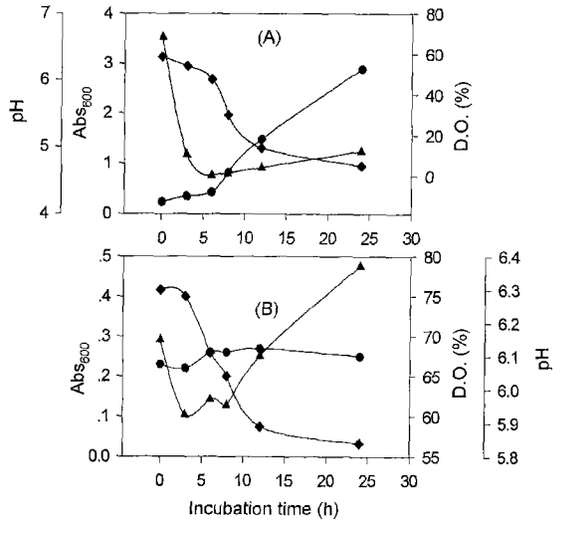

Fig. 2. Changes of D.O. and pH during incubation of oxygen-tolerant *B. longum* under oxygen stress.
B. longum JI 1, oxygen-tolerant strain, were incubated under partially aerobic (A) and aerobic conditions (B). Culture conditions were the same as described in Fig. 1. Cell growth (-●-), D.O. (-▲-), and pH (-◆-) were monitored during the cultivation.

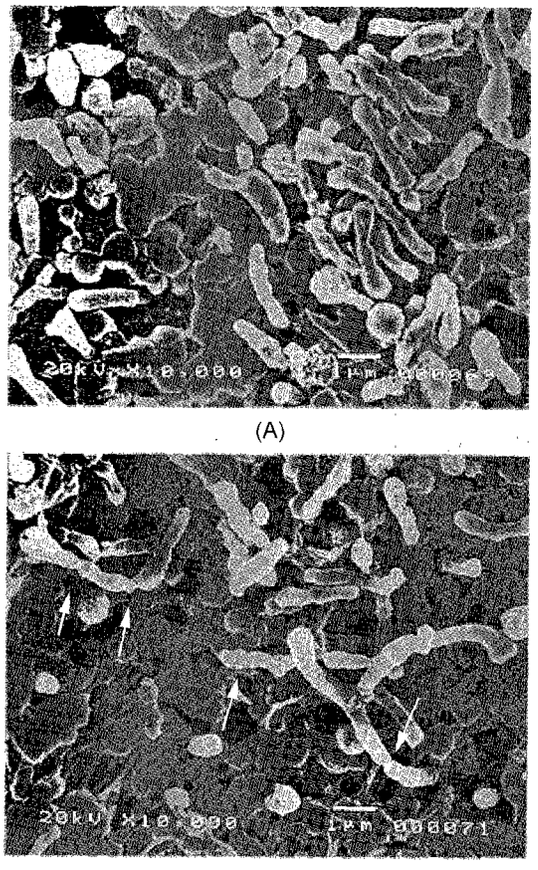

Fig. 3. Effect of oxygen stress on cell morphology of oxygen-tolerant *B. longum*.
B. longum JI 1, oxygen-tolerant strain, was incubated under anaerobic (A) and partially aerobic conditions (B) for 24 h. Cell morphology was observed with a scanning electron microscopy. Culture conditions were the same as described in Fig. 1. Arrows indicate abnormal cell surfaces resulting from oxygen stress.

as 9,10-methyleneoctadecanoic acid ($C_{19:0}$cyc9,10). These two isomers can not be separated by gas chromatographic analysis. Recently, Shin *et al.* [33] reported that the major cyclopropane fatty acid of bifidobacteria was 9,10-methyleneoctadecanoic acid as identified by GC-MASS analysis.

In the presence of oxygen during the incubation, the cellular fatty acid profiles changed remarkably (Table 5). The content of stearic acid ($C_{18:0}$) decreased considerably, while the content of myristic acid ($C_{14:0}$) and lauric acid ($C_{12:0}$) greatly increased, indicating that the carbon chain of the cellular fatty acid became shorter. Compositional change of the fatty acid could affect the permeability and rigidity of the cell membrane where the short carbon chain in the cellular fatty acid might disturb homeostasis of the cell membrane which could be undesirable for bifidobacteria.

Changes were also found in the contents of DMA, which were originated from plasmalogen. All the DMA's were

Table 5. Changes of the cellular fatty acid composition of oxygen-tolerant bifidobacteria under oxygen stress.

Fatty acids	Composition of cellular fatty acid (%)			
	B. longum E4		*B. longum* JI 1	
	Anaerobic[a]	Partial aerobic[b]	Anaerobic	Partial aerobic
Saturated fatty acid				
$C_{12:0}$ FAME[c]	0.16	0.96	0.14	1.18
$C_{14:0}$ FAME	3.48	11.8	2.12	12.4
$C_{14:0}$ DMA[d]	1.87	[e]ND	2.86	0.20
$C_{16:0}$ FAME	33.7	36.1	32.8	27.7
$C_{18:0}$ FAME	13.6	5.36	8.97	ND
Unsaturated fatty acid				
$C_{16:1}$cis9 FAME	1.76	1.84	1.23	1.56
$C_{18:1}$cis9 FAME	18.1	16.1	21.0	22.2
$C_{18:1}$cis9 DMA	12.5	0.43	14.8	3.73
Cyclopropane fatty acid				
$C_{19:0}$cyc9,10 FAME	6.70	15.6	3.50	11.0
$C_{19:0}$cyc9,10 DMA	3.42	0.45	4.62	0.42

[a,b]Culture conditions were the same as described in Fig. 1. [c]Fatty acid methyl ester. [d]Dimethyl acetal. [e]Not detected.

reduced dramatically in the cells of partially aerobic culture. Therefore, oxygen might affect the degradation of plasmalogen, however, it was not clear why the plasmalogen was reduced under oxygen stress. Interestingly enough, the content of cyclopropane fatty acid, $C_{19:0}$cyc9,10, increased remarkably. Goldberg and Eschar [11] suggested that the amount of the cyclopropane fatty acid might be closely related to the viability of LAB after the freezing process, and in addition, it might be important in maintaining the proper fluidity of the cell membrane, thus preventing the death of certain cells which were exposed to environmental stress. Rizzo *et al.* [28] found that some LAB containing a high content of cyclopropane fatty acid showed a higher acid tolerance level than those containing a low content of cyclopropane fatty acid. According to the results by Grogan's work [14], the mutants of *E. coli* which completely lacked the synthetic activity for the cyclopropane fatty acid could grow and actually survive under normal conditions, however, the mutants were more sensitive to the freeze-thaw treatment than the mother strains. This information suggested that the cyclopropane fatty acid might play a role in defending against the environmental stress which could cause a certain disorder of the cellular fatty acid profile. Shin *et al.* [33] reported that the oxygen-tolerant *B. longum* contained $C_{19:0}$cyc9,10, while the oxygen-sensitive *B. adolescentis* did not. In this study, the oxygen-tolerant bifidobacteria defended themselves against oxygen stress by remarkably increasing the content of cyclopropane fatty acid, $C_{19:0}$cyc9,10.

Induced Protein under Oxygen Stress

Bifidobacteria under oxygen stress showed many changes in the cytoplasmic protein profiles. The amounts of some proteins were diminished and others increased. In particular, under oxygen stress, a 35.5 kD protein was induced, while the 20.8 kD protein disappeared (Fig. 4 and Fig. 5). There have been several approaches made to examine which proteins from facultative anaerobes were induced under oxidative stress [6, 9, 24, 37], however, the induced proteins in bifidobacteria have not yet been found. In this study, the induced protein of bifidobacteria under oxygen stress was purified by FPLC and SDS-PAGE, and its ten N-terminal

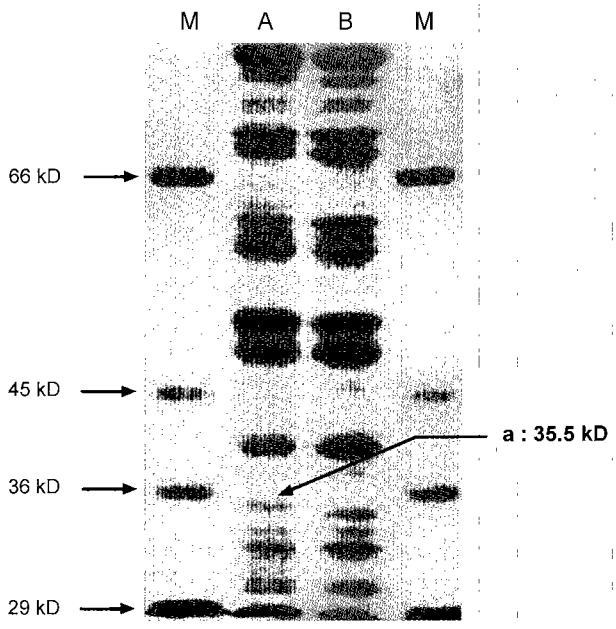

Fig. 4. Changes of large molecular weight cellular protein patterns of oxygen-tolerant *B. longum* under oxygen stress.
Culture conditions were the same as described in Fig. 1. *B. longum* JI 1, an oxygen-tolerant strain, was incubated under partially aerobic (lane A) and anaerobic (lane B) conditions for 24 h. Cellular proteins were separated by SDS-PAGE (7.5% polyacrylamide gel). The arrow indicates the protein induced under the partially aerobic condition. Lane M is the protein size maker.

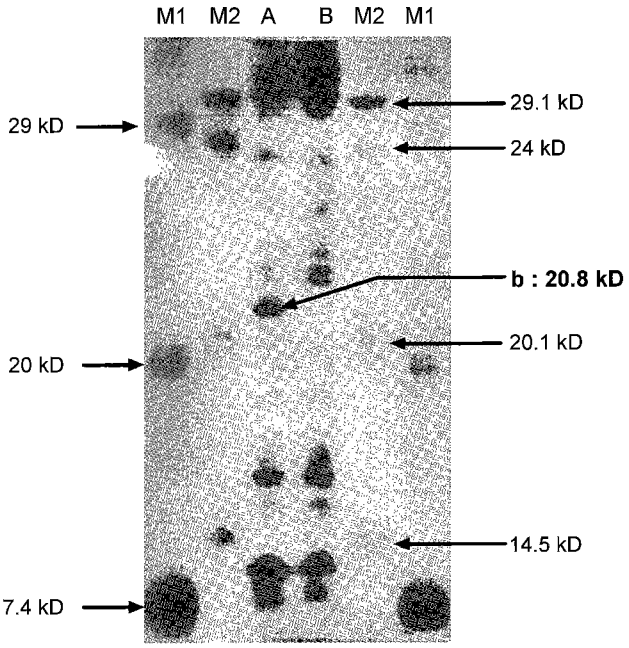

Fig. 5. Changes of small molecular weight cellular protein patterns of oxygen-tolerant *B. longum* under oxygen stress.
B. longum JI 1, an oxygen-tolerant strain, was incubated under anaerobic condition (lane A) and partially aerobic condition (lane B) for 24 h. Cellular proteins were separated by SDS-PAGE (12.5% polyacrylamide gel). Arrow b is the protein that disappeared, when it was under a partially aerobic condition. Lanes M1 and M2 represent the protein size makers.

amino acid sequence was determined (Fig. 6): recognized as unknown amino acid-Thr-Gly-Val-Arg-Phe-Ser-Asp-Asp-Glu. Homology with other proteins was investigated by FASTP, a protein sequence comparison program [26], however, it did not show any recognizable homology with any other proteins. In order to obtain more information of the induced protein, the gene that encodes this protein should be obtained and analyzed.

In summary, the physiological responses of bifidobacteria to oxygen stress were investigated. Oxygen stress had several damaging effects on bifidobacteria. Cell growth and cell proliferation were inhibited under oxygen stress. The chain length of cellular fatty acid in the cell membrane became short, which might disturb the normal fluidity and rigidity of the cell membrane. However, the oxygen-tolerant bifidobacteria that were selected in this study protected themselves by removing dissolved oxygen in the early stage of growth to a certain extent. Also, they increased the cellular content of cyclopropane fatty acid, $C_{19:0}$cyc9,10, for preventing disorder of the cell membrane. In addition, the induced protein of bifidobacteria under oxygen stress was reported for the first time in this study, however, further study is needed for the elucidation of its function and characteristics. This study presented basic information to understand the tolerance mechanism of oxygen-tolerant bifidobacteria to oxygen stress.

NH_3-Unknown[a]-Thr-Gly-Val-Arg-Phe-Ser-Asp-Asp-Glu→

Fig. 6. N-terminal amino acid sequence of the induced protein of *B. longum* JI 1 under oxygen stress.
[a]Unknown amino acid was not one of the basic set of 20 amino-acid-constituting proteins.

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