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O₂-inducible H₂O₂-forming NADPH oxidase is responsible for the hyper O₂ sensitivity of *Bifidobacterium longum* subsp. *infantis*

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Bifidobacteria are beneficial anaerobes, and their O₂ sensitivity levels differ among species as a function of unknown molecular mechanisms. *Bifidobacterium longum* subspecies *infantis* (*B. infantis*), a predominant colonizer of the gastrointestinal tract of infants, showed a hyper O₂-sensitive growth profile with accompanying a production of H₂O₂. In this study, we characterized an NADPH oxidase as a key enzyme responsible for this microbe's hyper O₂ sensitivity. A dominant active elution peak of H₂O₂-forming NADPH oxidase activity was detected in the first step of column chromatography, and the purified NADPH oxidase (NPOX) was identified as a homolog of nitroreductase family proteins. The introduction of the gene encoding *B. infantis* NPOX (*npoxA*) into O₂-tolerant *Bifidobacterium minimum* made the strain O₂ sensitive and allowed it to produce H₂O₂. Knockout of the *npoxA* gene in *B. infantis* decreased the production of H₂O₂ and mitigated its *B. infantis* hyper O₂ sensitivity. A transcript of *B. infantis npoxA* is induced by O₂, suggesting that the aerobic production of toxic H₂O₂ is functionally conserved in *B. infantis*.

Oxygen (O₂) has a negative effect on the growth of anaerobes^{1,2}; however, the habitats of anaerobes, such as the gastrointestinal tract, are frequently contaminated with O₂ that has been dissolved in food and beverages. Therefore, anaerobes must possess systems to adapt to aerated environments for survival in nature^{3,4}. Recent studies have identified an O₂-inducible O₂- and ROS-reducing enzyme complex in obligate anaerobes such as sulfate-reducing bacteria^{5,6}, *Clostridium*^{7,8}, and *Bacteroides*⁹. These findings indicate that these obligate anaerobes possess excellent systems to avoid oxidative damage for maintaining their obligate anaerobiosis in aerated environments.

Several gut anaerobes such as O₂-sensitive bifidobacteria and lactic acid bacteria are known to produce H₂O₂, a toxic reactive oxygen species (ROS), after exposure to O₂, which inhibits the cell growth^{10–12}. These anaerobes do not harbor the above-mentioned genes encoding the ROS-reducing enzyme complex in their genomes. NAD(P)H peroxidase or alkylhydroperoxide (Ahp) reductase systems (AhpF-AhpC or thioredoxin reductase-AhpC system), which detoxify H₂O₂ to H₂O using NAD(P)H in aerobes and facultatively anaerobes^{13–16}, have been identified in these anaerobes^{17–19}; however, exogenous H₂O₂ production has been detected in these anaerobes^{20,21}. These results suggest that H₂O₂ production and decomposition are controlled *in vivo* and play a role in the biological functions of these bacteria in aerated environments. H₂O₂ is highly toxic for a bacterium's own cells; thus, the reason for this H₂O₂ production remains unclear^{22,23}.

Members of the genus *Bifidobacterium* are gram-positive, catalase-negative anaerobes that are known to be beneficial to human health^{24–26}. The degree of O₂ sensitivity differs among species and strains in the genus^{11,27–35}. de Vries and Stouthamer (1969) proposed that O₂-sensitive *Bifidobacterium* species produce H₂O₂ under

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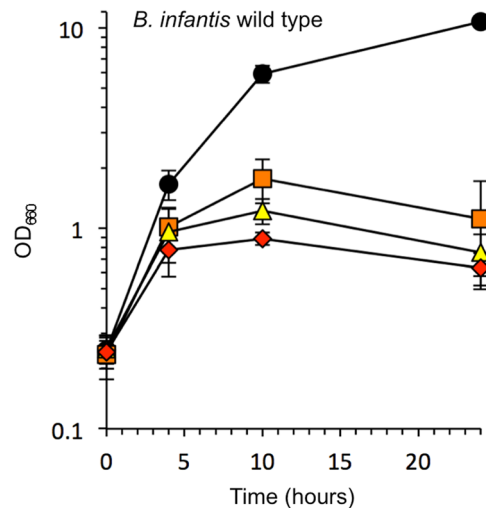


Figure 1. Growth of *Bifidobacterium infantis* JCM 1222^T in liquid shake culture at various O₂ concentrations. Circles, 100% N₂ cultures; squares, 5% O₂/95% N₂ cultures; triangles, 10% O₂/90% N₂ cultures; diamonds, 20% O₂/80% N₂ cultures. Data represent the average \pm SD of three independent cultures.

aerobic growth conditions through a reaction catalyzed by NADH oxidase activity, which can be detected in cell extracts¹¹. According to our studies using liquid shaking cultures at different O₂ concentrations, bifidobacterial strains can be classified into four groups: O₂ hypersensitive (5% O₂-sensitive), O₂-sensitive (10% O₂-sensitive), O₂-tolerant (21% O₂-tolerant), and O₂-hypertolerant (microaerophilic)^{33,36,37}. H₂O₂ production has been detected in strains belonging to the O₂-hypersensitive and O₂-sensitive groups when cell growth was inhibited by O₂³³. The majority of O₂-tolerant and hyper O₂-tolerant strains have been isolated from non-human sources such as a honeybee hindgut³⁸ and bovine rumen³⁹. *Bifidobacterium asteroides* (*B. asteroides*) was isolated from a honeybee hindgut and possesses a heme-catalase, which is a rare characteristic among bifidobacteria^{24,38,40,41}. Several reports have revealed that the addition of catalase to the culture medium^{11,33} or the introduction of ROS-detoxifying enzymes into O₂-sensitive *Bifidobacterium* strains^{42,43} improved their aerobic growth, indicating that H₂O₂ production strongly correlates with the inhibition of aerobic growth. With respect to the enzymes that produce H₂O₂ in bifidobacteria, an H₂O₂-forming NADH oxidase was identified in an O₂-sensitive strain of *Bifidobacterium bifidum* (*B. bifidum*), a type species in the genus *Bifidobacterium*, and the purified enzyme was identified as a *b*-type dihydroorotate dehydrogenase (DHOD), which is the enzyme that catalyzes oxidation of dihydroorotate to orotate in pyrimidine biosynthesis⁴⁴. This enzyme is suggested to be involved in H₂O₂ production in *B. bifidum*, but its function has not been confirmed *in vivo* because of the lack of a gene disruption technique for this bacterium.

In the present study, *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) showed a hyper O₂-sensitive growth profiles. *B. infantis* has been reported as a champion colonizer in the gut microbiota of human infants⁴⁵ and is reported to confer benefits to premature infants in terms of promoting anti-inflammatory activity and decreasing the risk of necrotizing enterocolitis^{46,47}. Use of *B. infantis* as a bioactive probiotic for infants is highly anticipated; therefore, we set out to identify the mechanisms underlying its hyper-O₂ sensitivity. Here, we identified an enzyme that produces H₂O₂ in *B. infantis*. The role of this protein and the mechanisms and evolutionary significance of H₂O₂ production in this bacterium are discussed.

Results

***B. infantis* showed a hyper O₂-sensitive growth profile.** The degree of O₂ sensitivity of *B. infantis* JCM1222^T (=ATCC15697 = DSM20088) was tested at several O₂ concentrations in liquid shaking cultures. *B. infantis* grew well under anoxic conditions, and the optical density at 660 nm (OD₆₆₀) reached 8 to 10 in the final growth stage. This level of growth was one of the most productive observed in MRS medium among the tested *Bifidobacterium* species^{36,37}. Although several O₂-sensitive bifidobacterial strains, including *B. bifidum* and *Bifidobacterium longum* (*B. longum*), grew well in liquid shaking cultures at 5% O₂³³, the growth of *B. infantis* was found to be significantly inhibited at 5% O₂ (Fig. 1). Therefore, we classified *B. infantis* as O₂-hypersensitive. H₂O₂ production in the culture medium was detected at 10% and 20% O₂ (Table 1).

Elution profiles of NAD(P)H oxidase activity in the first step of column chromatography. We previously identified the enzyme fractions that are involved in H₂O₂ production in an O₂-sensitive *B. bifidum* strain using a Butyl-TOYOPEARL column⁴⁴. In this study, we performed the same experiments for *B. infantis*. Proteins in the cell free extracts from *B. infantis* were fractionated according to their hydrophobic properties using a Butyl-TOYOPEARL column (Fig. 2A). One dominant elution peak of NADPH-dependent oxidase activity was detected, whereas NADH-dependent oxidase activity was detected as a minor elution peak. These major and minor peak fractions showed an H₂O₂-forming oxidase reaction in which stoichiometric production of H₂O₂ was detected by means of the reduction of O₂. No significant NAD(P)H oxidase activity was detected in either the unbound fraction or the washed fractions in the first step of column chromatography.

O ₂ (%)	H ₂ O ₂ (μM) ^a			
	<i>B. infantis</i> wild type	<i>B. infantis</i> Δ <i>npoxA</i>	<i>B. minimum</i> pkkt427 ^b	<i>B. minimum</i> + <i>npoxA</i> in pKKT427 ^c
0	ND ^d	ND	ND	ND
5	ND	ND	ND	ND
10	29 ± 10	(3 ± 1) ^e	ND	172 ± 13
20	31 ± 6	14 ± 10	ND	169 ± 18

Table 1. H₂O₂ accumulation of *B. infantis* and *B. minimum* under 0% to 20% O₂ conditions. ^aH₂O₂ accumulation in the medium (24 h). ^bVector pKKT427 was transformed into *B. minimum*. ^cThe *B. infantis* *npoxA* gene was cloned in pKKT427 and transformed into *B. minimum*. ^dND, not detected (less than 3 μM). ^eData in parentheses are close to the reliable detection limit. Data represent the average ± SD of three independent cultures.

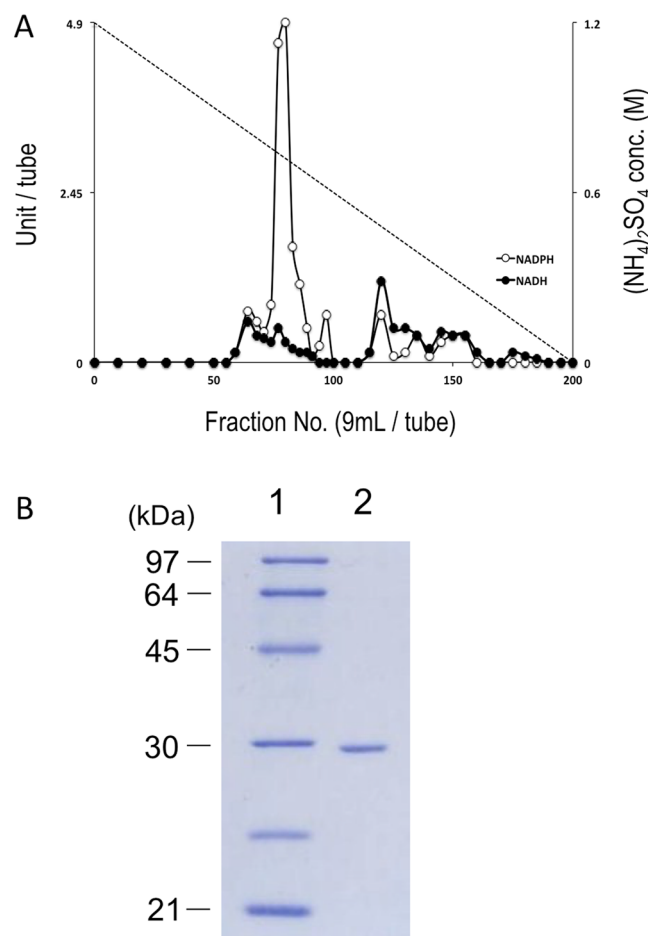


Figure 2. (A) Chromatographic elution profiles of *B. infantis* NADH and NADPH oxidases. Cell extracts, after treatment with streptomycin sulfate and ammonium sulfate, were applied to a Butyl-TOYOPEARL column equilibrated with 1.2 M ammonium sulfate in 50 mM potassium phosphate buffer (pH 7.0). After sample loading, the bound proteins were eluted with a linear gradient of ammonium sulfate, from 1.2 to 0 M, dissolved in the same buffer. Black circles, NADH oxidase activity; white circles, NADPH oxidase activity; dashed line, ammonium sulfate concentration (conc.). (B) SDS-PAGE of the purified *B. infantis* NPOX protein. After electrophoresis, the gel was stained with Coomassie brilliant blue. The protein standards (lane 1) and purified protein (lane 2) are indicated, along with the corresponding molecular masses (indicated on the left in kDa).

Purification and characterization of the NADPH oxidase in *B. infantis*. The fractions that showed NADPH oxidase activity were collected and purified by additional column chromatography steps. At each purification step, only one predominant active elution peak was detected. After the final step of affinity chromatography, the purified fractions appeared as a single band (29 kDa) by SDS-PAGE (Fig. 2B). The N-terminal sequence of the purified NADPH oxidase (NPOX) was sequenced, and the amino acid sequence (MVTNATIEALLGRRSIRKFK) showed 100% identity with the N-terminal sequence of the Blon_2447 gene product of *B. infantis* ATCC15697 in the genome database.

Electron acceptor ^a	Relative activity (%) ^b
O ₂	100
DCIP	358
FAD	229
FMN	74
Nitrobenzene	2090
Nitrofurazone	725

Table 2. Identification of electron acceptors of *B. infantis* NPOX. ^aRelative activity (%) was calculated in comparison with the activity of NADPH oxidase. Data are the average of two independent measurements that varied by less than 5%.

The gene encoding NPOX (designated as *npoxA*) was cloned from the *B. infantis* genome, and the amino acid sequence identity (% amino acid identity calculated from ClustalW alignment) showed 100% to that derived from BLON_RS12650 (Blon_2447). The translated product of *B. infantis npoxA* showed homology to proteins of the bacterial nitroreductase family and encodes a protein of 254 amino acids that shows ~35% identity to *Escherichia coli* oxygen-insensitive NADPH nitroreductase NfsA (accession number P17117)⁴⁸, *Bacillus subtilis* NADPH-dependent nitroreductase NfrA1 (32% identity, accession number P39605)⁴⁹, and *Vibrio harveyi* NADPH-flavin oxidoreductase FRP1 (35% identity, accession number Q56691)⁵⁰. *B. subtilis* NfrA1 is reported to catalyze H₂O₂-forming NADH oxidase activity⁵¹, and *V. harveyi* FRP1 is reported to catalyze an FMN reductase reaction that is involved in a luciferase reaction that generates light in luminescent bacteria⁵². These NPOX homologs utilize NADH or NADPH or both as an electron donor to reduce substrates. The protein homologs are widely distributed in the genus *Bifidobacterium*. Homologous proteins were found in several *B. longum* strains (99% to 100%), *Bifidobacterium breve* (85% identity), *B. bifidum* (70% identity), *Bifidobacterium minimum* (*B. minimum*) (58% identity), and *B. asteroides* (52% identity). None of these proteins have been characterized in terms of function.

Enzymatic properties. The purified enzyme showed high affinity for NADPH, rather than NADH, as the electron donor. The K_m values for NADPH and NADH when O₂ was used as an electron acceptor were 1.38 ± 0.12 and 113.3 ± 7.3 μ M, respectively. The purified *B. infantis* NPOX showed a typical flavoprotein spectrum with absorbance maxima at 280, 373, and 444 nm (Fig. S2). The bound flavin was identified as FMN by HPLC analysis. In the NADPH oxidase reaction, stoichiometric production of H₂O₂ was detected following the reaction with O₂; e.g., 1 μ g enzyme consumed 28.3 nmol O₂/5 min/mL, and 13.8 nmol O₂/mL was produced after the addition of catalase. This result indicated that the purified enzyme reduces O₂ by two reducing equivalents to H₂O₂. The purified enzyme used flavins and nitro compounds as electron acceptors under anoxic conditions (Table 2). The pH optimum for the NADPH oxidase reaction was found to be 5.5–6.0. The temperature optimum was approximately 30–40 °C, and activity significantly decreased at temperatures above 40 °C. The specific activity of this protein under air-saturated conditions at 37 °C was 13.8 ± 4.3 U/mg protein.

Transformation of the *B. infantis npoxA* into an O₂-tolerant *B. minimum* strain. *B. minimum* DSM 20102^T, which is an isolate from sewage⁵³, showed an O₂-tolerant profile^{32,37}, and liquid shaking growth was not significantly inhibited in the presence of 20% O₂ (Fig. 3A). Therefore, we classified *B. minimum* as a member of the O₂-tolerant group and used this microbe for further study. The *E. coli*-*Bifidobacterium* shuttle vector pKKT427, originally developed for the transformation of *B. longum* strain 105-A^{54,55}, was successfully transformed into *B. minimum* in this study; therefore, we used this vector to express *B. infantis* NPOX in *B. minimum*. *B. infantis npoxA* was cloned into pKKT427, and then the growth of transformants was tested at different O₂ concentrations. The production of H₂O₂ was not detected in a *B. minimum* wild-type strain carrying pKKT427 in the presence of O₂ (Table 1). However, *B. minimum* carrying *npoxA* in pKKT427 showed an O₂-sensitive growth profile in the presence of 10% and 20% O₂ with accompanying the production of H₂O₂ (Fig. 3B, Table 1). These results indicated that *B. infantis* NPOX is responsible for the O₂ sensitivity of and H₂O₂ production by *B. minimum*.

The growth of a *B. infantis npoxA* gene knockout strain. Single-gene transformation of the O₂-tolerant *B. minimum* strain significantly increased H₂O₂ production and O₂ sensitivity. To determine the function of the *npoxA* product in *B. infantis*, we constructed a *npoxA* deletion mutant ($\Delta npoxA$) (Fig. S1), and then the growth of this mutant was compared with that of a wild-type strain of *B. infantis*. *B. infantis* $\Delta npoxA$ grew well at 5% O₂, and H₂O₂ production was significantly decreased at 10% and 20% O₂ (Fig. 4, Table 1). These results indicated that the *npoxA* product contributes to both aerobic growth inhibition and H₂O₂ production in *B. infantis*. Candidates for the production of residual H₂O₂ were the minor active fractions of the NADH-dependent H₂O₂-forming oxidase (Fig. 2A).

Expression profiles of genes encoding *B. infantis* NADPH oxidase and alkylhydroperoxide reductase. To determine the effect of O₂ on the expression of the gene encoding *B. infantis* NADPH oxidase (*npoxA*), we performed a Northern blot analysis. The result showed that *B. infantis npoxA* was significantly upregulated within 30 min after the start of 5% O₂ aeration (Fig. 5B). We also determined the expression of genes encoding alkylhydroperoxide reductase (Ahp), which is an enzyme complex composed of AhpF and AhpC that detoxifies ROS. TrxR is an AhpF homolog in *B. infantis*, and *ahpC-trxR* genes are tandemly located in the genome

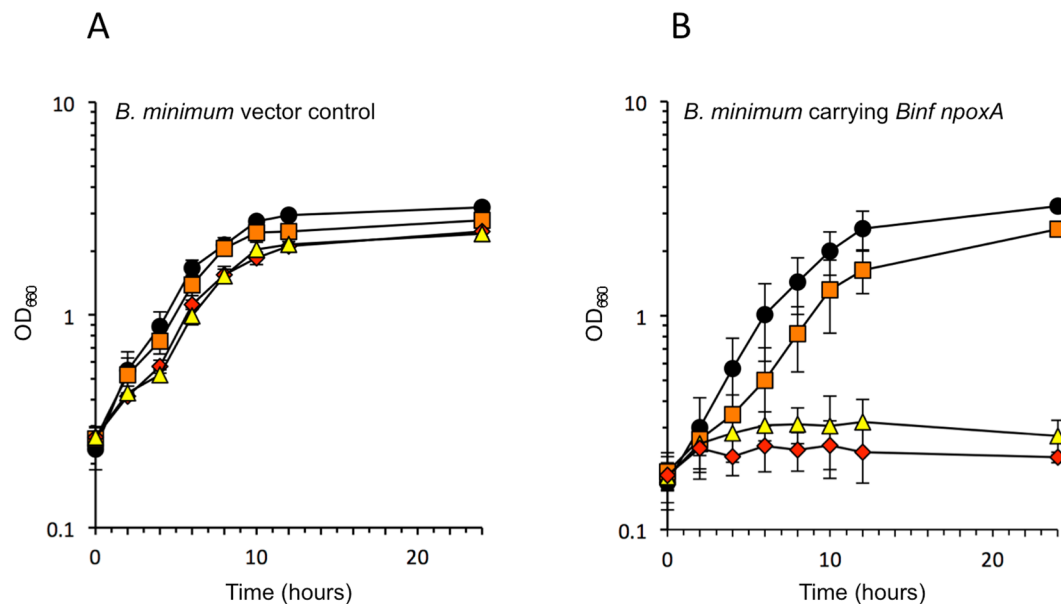


Figure 3. Growth of *Bifidobacterium minimum* DSM 20102^T in liquid shake culture at various O₂ concentrations with (B) or without (A) the *B. infantis npoxA* gene in the vector pKKT427. Circles, 100% N₂ cultures; squares, 5% O₂/95% N₂ cultures; triangles, 10% O₂/90% N₂ cultures; diamonds, 20% O₂/80% N₂ cultures. Data represent the average ± SD of three independent cultures.

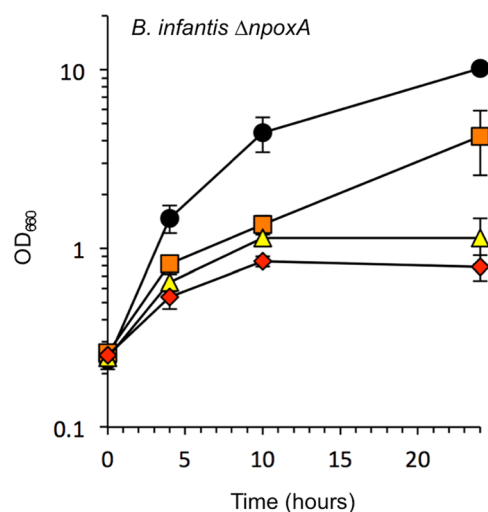


Figure 4. Growth of the Δ*npoxA* mutant of *B. infantis* in liquid shake culture at various O₂ concentrations. Circles, 100% N₂ cultures; squares, 5% O₂/95% N₂ cultures; triangles, 10% O₂/90% N₂ cultures; diamonds, 20% O₂/80% N₂ cultures. Data represent the average ± SD of four independent experiments.

(Fig. 5A). Northern analyses indicated that *B. infantis ahpC* was strongly upregulated within 10 minutes after the start of aeration (Fig. 5B). These results indicate that *B. infantis* express the genes for ROS production and for ROS detoxification in response to O₂ stress.

Discussion

The growth of bifidobacterial strains is inhibited by O₂, and this inhibition has been shown to occur in conjunction with H₂O₂ production. To date, *b*-type DHOD from an O₂-sensitive strain of *B. bifidum*, has been identified as the only purified H₂O₂-forming NADH oxidase in the genus *Bifidobacterium*. In this study, we detected a dominant H₂O₂-forming NADPH oxidase activity peak in an O₂-hypersensitive strain of *B. infantis*. This NADPH-specific peak was not detected in *B. bifidum* using the same hydrophobic column chromatography⁴⁴, thus suggesting that the mechanisms of O₂ sensitivity in these O₂-sensitive strains differ from each other.

Purified *B. infantis* H₂O₂-forming NPOX showed high similarity (~30% identity) with proteins of the bacterial nitroreductase family. *B. infantis* NPOX exhibited nitroreductase- and flavin reductase-activity under

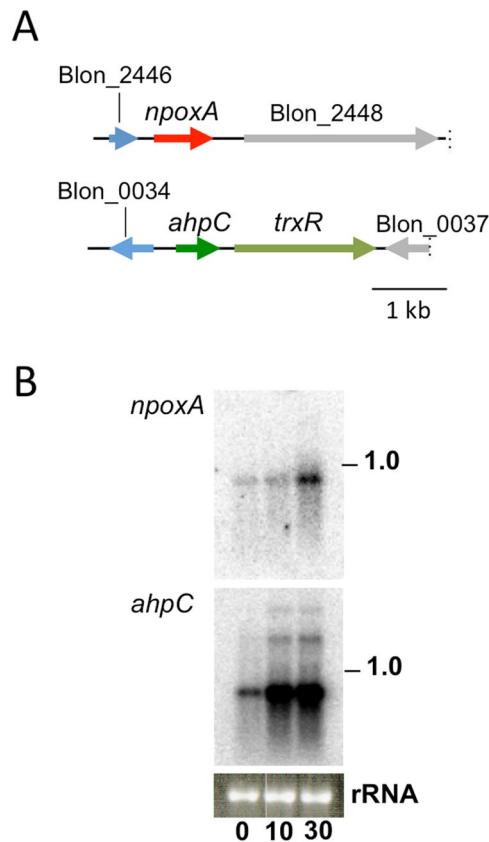


Figure 5. Genome structures and gene expression profiles of *B. infantis* *npoxA* and *ahpC*. (A) Genome structure of the *npoxA* (upper) and the *ahpC* (lower). (B) Northern blot analyses of 5 μ g of *B. infantis* total RNA probed with *npoxA* or *ahpC*. 0, immediately before the start of aeration with 5% O_2 in the mid-exponential phase; 10, after 10 min; 30, after 30 min. The estimated sizes of the observed transcripts are indicated on the right. Ethidium bromide staining of the ribosomal RNA (rRNA) to confirm equal RNA loading is shown below the autoradiogram. The membranes were reused for reprobing different probes.

anoxic conditions. The role of *B. infantis* H_2O_2 -forming NPOX under anaerobic conditions is unclear; however, based on the O_2 -inducible gene expression profile, NPOX is expected to be functionally involved in the aerobic life of *B. infantis*. Similar expression of a transcript, from a gene that encodes an NPOX ortholog (Gene ID: BL0139), was observed by Oberg *et al.* in microarray data from the H_2O_2 -sensitive strain *B. longum* D2975 and the H_2O_2 -tolerant strain *B. longum* NCC2705⁵⁶. The NPOX homolog transcript was induced only in the H_2O_2 -sensitive D2975 strain under oxidative stress. To clarify the expression of NPOX and its correlation with bifidobacterial O_2 sensitivity, it would be desirable to compare the expression profiles and kinetics of NPOX homologs in other O_2 -tolerant and O_2 -sensitive strains³⁷.

H_2O_2 is highly toxic to cells, and therefore, *Bifidobacterium* strains need to conserve systems to reduce the amount of H_2O_2 to avoid cell death. Alkylhydroperoxide reductase, a component of the AhpF-AhpC system that is known to decompose H_2O_2 efficiently, is widely conserved in bifidobacterial genomes. Although the function of this enzyme complex has not been characterized in the genus *Bifidobacterium*, the upregulation of *ahpC* (or the protein AhpC) in response to O_2 or H_2O_2 stress has been reported in strains such as *Bifidobacterium animalis* subsp. *lactis*³⁵ and *B. longum*⁵⁶. These data suggested that *ahpC* is involved in the oxidative stress protection of bifidobacterial strains. In the present study, *B. infantis* induced *ahpC* within 10 minutes after exposure to O_2 . Interestingly, the induction of *ahpC* was more rapid than that of *B. infantis* *npoxA* (Fig. 5). This gene expression program is considered to be important for the strain to rapidly prepare the defense system against the generation of H_2O_2 .

In this study, knockout of *npoxA* in *B. infantis* enabled the strain to grow under microaerobic conditions, with a decrease in H_2O_2 production under aerobic conditions. Improvement of bacterial O_2 sensitivity by single-gene knockout has been demonstrated in the obligate anaerobe *Bacteroides fragilis*, with deletion of *oxe* enabling the strain to grow under micro-oxic conditions (as high as 2% O_2)⁵⁷. Although the function of the *oxe* gene product has not yet been identified, a mutation in *oxe* enhanced the H_2O_2 -scavenging activity of the mutant relative to that of the wild type. The reason for the production of a toxic H_2O_2 in anaerobes has been unknown; however, it is obvious that both *B. fragilis* and *B. infantis* control the production and degradation of H_2O_2 according to their own genetic programs.

We conclude here that *B. infantis* induces the expression of NPOX in response to an increase in O_2 , leading to the production of H_2O_2 to maintain anaerobiosis. *B. infantis* $\Delta npoxA$ still produced H_2O_2 at elevated O_2

concentrations, indicating the presence of another H₂O₂-forming oxidase *in vivo*. A *b*-type DHOD, that is conserved in the genome of *B. infantis*, is a likely candidate. Further characterization of the residual H₂O₂ production in *B. infantis*, as well as analysis of the enzyme kinetics of the O₂-tolerant *B. minimum* NPOX homolog, will be needed to describe the mechanisms of anaerobiosis in these strains.

Methods

Bacterial strain and growth conditions. *B. infantis* (*Bifidobacterium longum* subsp. *infantis*) JCM 1222^T (=ATCC 15697^T, DSM 20088^T) was used in this study. *B. infantis* was grown anaerobically at 37 °C in modified MRS medium (pH 6.7). The culturing conditions under several O₂ concentrations were as described previously³³.

Chemicals. All chemicals were of analytical grade. β-NADPH, β-NADH, FAD, FMN, riboflavin, nitrobenzene, nitrofurazone, and 2,6-dichlorobenzenoneindophenol (DCIP) were from Sigma. Water was prepared with an Ellix-10 Milli-Q ultra-pure water system (Millipore, Tokyo, Japan).

Enzyme purification. Microaerobically grown *B. infantis* cells (cultured statically but stirred with a magnetic stirrer in 3 liters of medium in a 5-liter flask with a cotton plug) were harvested for enzyme purification. NAD(P)H oxidase activity was assayed spectrophotometrically in 1 ml of air saturated 50 mM potassium phosphate buffer (pH 6.5) at 37 °C. The reaction was started by the addition of enzyme solution, and the decrease in absorbance at 340 nm ($\epsilon_{340} = 6,220 \text{ M}^{-1}\cdot\text{cm}^{-1}$) was monitored with a spectrophotometer (HITACHI U-3300, Hitachi, Japan). One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NAD(P)H per minute.

Microaerobically grown *B. infantis* cells (80 g) were suspended in 240 ml of 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM DTT, 0.2 mM PMSE, and 5 mM EDTA, and then the cells were disrupted by treatment with a French pressure cell at 140 MPa. All purification procedures were carried out at 4 °C or on ice. Cell free extracts (CFE) were obtained by removing cell debris by centrifugation at 39,000 g for 15 min. The cytoplasmic solution obtained by ultracentrifugation at 100,000 g for 2 h was treated with 10% streptomycin sulfate (dissolved in 10 mM potassium phosphate buffer, pH 7.0) to remove nucleic acids. After centrifugation at 39,000 g for 15 min, the supernatant was fractionated by the stepwise addition of solid ammonium sulfate (20%). The supernatant obtained after centrifugation at 30,000 g was dissolved in 50 mM potassium phosphate buffer, pH 7.0, containing 1 M ammonium sulfate, 5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1 mM dithiothreitol (DTT). After centrifugation at 39,000 g for 15 min, the supernatant was applied on a hydrophobic interaction chromatography using Butyl-TOYOPEARL 650 s (TOSOH, Tokyo, Japan) column chromatography. The enzyme was eluted with a linear gradient from 1.2 M to 0 M ammonium sulfate dissolved in the same buffer. After the Butyl-TOYOPEARL chromatography, active fractions of NADPH oxidase activity were obtained. The solution after Butyl-TOYOPEARL chromatography was dialyzed against 50 mM potassium phosphate buffer, pH 6.5, containing 0.5 mM EDTA, 0.2 mM PMSF, and 0.1 mM DTT for 6 h, and this procedure was repeated 3 times using freshly prepared buffer each time. The enzyme solution was then applied to DEAE Sephacel (GE Healthcare, USA) column chromatography. The column was sequentially washed with the same buffer containing 0 mM and 150 mM NaCl, and eluted with buffer containing 250 mM NaCl. The active fractions were pooled and dialyzed for 12 h against 10 mM potassium phosphate buffer, pH 6.5, containing 0.5 mM EDTA and 0.1 mM DTT and subjected to hydroxyapatite (WAKO, Japan) column chromatography. After applying the enzyme solution to the hydroxyapatite column, the protein was sequentially washed with 10 mM and 50 mM potassium phosphate buffer, pH 6.5, and eluted with 100 mM potassium phosphate buffer, pH 6.5. The active fractions were collected and concentrated using Amicon Ultra centrifugal filter units (30,000 Da cutoff; MILLIPORE, Cork, Ireland). The concentrated enzyme was applied to POLOS HQ/H (PerSeptive Biosystems, Framingham, USA) column chromatography. The column was sequentially washed with 50 mM potassium phosphate buffer (pH 6.5), then eluted with a linear gradient of 0 mM–250 mM NaCl. The active fraction was collected and concentrated using Amicon Ultra. After this chromatography, the enzyme purity in the active fractions was checked by SDS-PAGE with Coomassie Brilliant Blue G-250 staining or silver staining. By using this enzyme concentrator, the basal buffer of the enzyme solution was changed to 50 mM potassium phosphate buffer, pH 6.5, by diluting the concentrated enzyme solution 100-fold with the new buffer, concentrating to the original volume, and then repeating the dilution and concentration steps; the second concentrated enzyme solution was subjected to enzyme assay. Protein concentration was determined by the dye-binding assay⁵⁸.

The purified enzyme was subjected to SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (NIPPON GENETICS, Tokyo, Japan). The N-terminal amino acid sequence was determined by the Edman degradation method using the peptide sequencer described previously⁷. The UV-Vis absorption spectrum was recorded on a Hitachi U3300 spectrophotometer (Hitachi, Tokyo, Japan) using a 1 cm path length quartz cuvette.

The bound flavins were extracted and determined by HPLC analysis with fluorescence detector according to a previously described method using a CAPCELL PACK C18 column (4.6 mm I.D. × 150 mm L; SHISEIDO CO., LTD. Tokyo, Japan) with 5 mM ammonium acetate in methanol as the mobile phase⁷. Riboflavin, FAD and FMN were used as standards.

Enzyme properties. The optimal pH of the purified enzyme was determined in 50 mM potassium phosphate buffer in the pH range of pH 5.0 – pH 8.0 at 37 °C, which is the optimum growth temperature for *B. infantis*. The temperature optimum of the purified enzyme was determined in 50 mM potassium phosphate buffer, pH 6.5, over a temperature range from 25 to 60 °C.

H₂O₂ production by the *B. infantis* NAD(P)H oxidase reaction was detected while monitoring O₂ production using an oxygen electrode by adding catalase into the reaction vessel^{8,33,44}. Catalase catalyzes the stoichiometric

conversion of 1 mol H₂O₂ to 1 mol H₂O and 1/2 mol O₂. The H₂O₂-forming type NAD(P)H oxidase produces 50% O₂ after the addition of catalase to the total amount of O₂ consumed by the NAD(P)H oxidase reaction.

The substrate specificity of the purified enzyme was assayed spectrophotometrically at 37 °C using 50 mM potassium phosphate buffer, pH 6.5. Electron acceptors for a purified enzyme were investigated by adding various substrates (final concentration, 50 μM) to the anaerobic cuvette together with the enzyme solution. For all acceptors except O₂, the reactions were carried out under anoxic conditions by purging the anaerobic glass cuvette containing the reaction buffer with O₂-free argon gas. NAD(P)H:O₂ oxidoreductase activity was assayed under air saturated condition. NAD(P)H (final 150 μM) was used as an electron donor for the enzyme. For the reactions of NAD(P)H:acceptor oxidoreductase, one unit of activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NAD(P)H ($\epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$) per minute. For the assay of DCIP, one unit of activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of DCIP ($\epsilon_{600} = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$) per minute. The values for relative activities (%) and the specific activities (U/mg protein) are the average of two independent measurements that varied by less than 5%. The apparent *K_m* values for NADH and NADPH were determined by varying the concentrations of both NAD(P)H in 50 mM potassium phosphate buffer, pH 6.5 using the Enzyme Kinetics Module 1.3 (SigmaPlot 11, SYSTAT Software, Chicago, IL). Initial rates were determined from linear plots of NAD(P)H reduction.

Northern hybridization. Northern hybridization was performed as described previously⁸. Total RNA (5 μg) extracted from *B. infantis* cells harvested before or after the 5% O₂ stress was loaded on agarose gels and blotted onto nylon membranes (Hybond N⁺, GE Healthcare, Cicgo, IL, USA). The membrane was then probed with the ³²P-labeled gene probe of *B. infantis npoxA*. The membrane was subsequently stripped of the probe and re-probed with a probe of *B. infantis ahpC*. Northern hybridization using the same membrane was repeated twice to confirm the results. The following oligonucleotide primer pairs were used to amplify the probes for *B. infantis npoxA*: 5'-ATGGTTACCAACGCAACAAT and 52-CTAATCCAGACGGAAGCCCT, *B. infantis ahpC*: 5'-ATGACTCTTCTGCAGCATGA and 52-TCACAGCTGGCCGACGAGGT. Before hybridization with a different probe, the membrane was washed at 90 °C for 2 minutes in a stripping buffer (5% SDS in 50 mM Tris-HCl, pH 7.5) and reused for the next northern analysis by re-probing with a different gene probe.

Expression of *B. infantis npoxA* in *B. minimum*. *B. infantis npoxA* including its promoter region was amplified by PCR using the forward primer (5'-TAAAAGCTTTGGATGATGGTTTCTGTGG) included a *Hind*III restriction site, and the reverse primer (5'-TAAGCGGCCCGCCTAATCCAGACGGAAGCCCTG) included a *Not*I restriction site. The purified PCR product was digested with *Hind*III and *Not*I and ligated into *Hind*III- and *Not*I-digested pKKT427 vector⁵⁴, resulting in the plasmid pBInpoxA, which was transformed into *B. minimum* by electroporation. *B. minimum* cells carrying pBInpoxA or cells carrying a control vector pKKT427 were tested the growth in MRS medium containing spectinomycin (Sp) (80 μg/ml) under several O₂ concentrations.

Gene knockout of *npoxA*. A plasmid pKO403Cm- Δ *npoxA* for the knockout of the *B. infantis npoxA* gene was constructed as follows (Fig. S1). The PCR primers were designed according to the *npoxA* gene sequence (BLON_RS12650) and the flanking genomic regions of *B. infantis* JCM1222¹ (=ATCC15697) (GenBank Accession no. NC_011593). To obtain the *npoxA* deletion DNA fragments, upstream (1.35 kb) and downstream (1.36 kb) regions of the putative *npoxA* gene of *B. infantis* were amplified by PCR using the primers shown in Fig. S1B. The produced DNA fragments were connected to the upstream and downstream regions of the Sp resistance gene (*Sp^r*) by Golden Gate Assembly⁵⁹ with *Sap*I as the Type IIS restriction enzyme. The obtained fragments were cloned into pKO403Cm, a *Bifidobacterium-Escherichia coli* shuttle vector⁶⁰, which carries a Golden Gate cloning site, chloramphenicol (Cm) resistance gene (*Cm^r*), and temperature-sensitive replicon origin⁶⁰. *B. infantis* cells were transformed with pKO403Cm- Δ *npoxA* by electroporation⁵⁵. After transformation, the cells were spread and cultured at 42 °C on MRS plates containing Sp (50 μg/ml). The transformants were transferred onto two MRS agar plates containing either Sp or Cm. The obtained Sp-resistant/Cm-sensitive transformants were selected as candidates for the double-crossover mutant. The deletion of *npoxA* was confirmed by PCR using Primer-1 and Primer-2 (Fig. S1C) and nucleotide sequencing to confirm the precise disruption.

References

- McCord, J. M., Keele, B. B. Jr. & Fridovich, I. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **68**, 1024–1027 (1971).
- Morris, J. G. Oxygen and the obligate anaerobe. *J. Appl. Bacteriol.* **40**, 229–244 (1976).
- Pan, N. & Imlay, J. A. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. *Mol. Microbiol.* **39**, 1562–1571 (2001).
- Baughn, A. D. & Malamy, M. H. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. *Nature* **427**, 441–444 (2004).
- Gomes, C. M. *et al.* Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. *J. Biol. Chem.* **272**, 22502–22508 (1997).
- Lumppio, H. L., Shen, N. V., Summers, A. O., Voordouw, G. & Kurtz, D. M. Jr. Rubrerythrin and rubredoxin oxidoreductase in *Desulfovibrio vulgaris*: a novel oxidative stress protection system. *J. Bacteriol.* **183**, 101–108 (2001).
- Kawasaki, S. *et al.* Adaptive responses to oxygen stress in obligatory anaerobes *Clostridium acetobutylicum* and *Clostridium aminovalericum*. *Appl. Environ. Microbiol.* **71**, 8442–8450 (2005).
- Kawasaki, S., Sakai, Y., Takahashi, T., Suzuki, I. & Niimura, Y. O₂ and reactive oxygen species detoxification complex composed of O₂-responsive NADH:rubredoxin oxidoreductase – flavoprotein A2 – desulfoferredoxin operon enzymes, rubperoxin, and rubredoxin in *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **75**, 1021–1029 (2009).
- Mishra, S. & Imlay, J. A. An anaerobic bacterium, *Bacteroides thetaiotaomicron*, uses a consortium of enzymes to scavenge hydrogen peroxide. *Mol. Microbiol.* **90**, 1356–1371 (2013).

10. Whittenbury, R. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. *J. Gen. Microbiol.* **35**, 13–26 (1964).
11. de Vries, W. & Stouthamer, A. H. Factors determining the degree of anaerobiosis of *Bifidobacterium* strains. *Arch. Mikrobiol.* **65**, 275–287 (1969).
12. Thomas, E. L. & Pera, K. A. Oxygen metabolism of *Streptococcus mutans*: uptake of oxygen and release of superoxide and hydrogen peroxide. *J. Bacteriol.* **154**, 1236–1244 (1983).
13. Niimura, Y., Poole, L. B. & Massey, V. *Amphibacillus xylanus* NADH oxidase and *Salmonella typhimurium* alkylhydroperoxide reductase flavoprotein components show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of *S. typhimurium* alkyl-hydroperoxide reductase 22-kDa protein component. *J. Biol. Chem.* **270**, 25645–25650 (1995).
14. Poole, L. B. & Ellis, H. R. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry* **35**, 56–64 (1996).
15. Baker, L. M., Raudonikiene, A., Hoffman, P. S. & Poole, L. B. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J. Bacteriol.* **183**, 1961–1973 (2001).
16. Seaver, L. C. & Imlay, J. A. Alkyl hydroperoxide reductase in the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* **183**, 7173–7181 (2001).
17. Higuchi, M. *et al.* Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. *J. Bacteriol.* **181**, 5940–5947 (1999).
18. Klijn, A., Mercenier, A. & Arigoni, F. Lessons from the genomes of bifidobacteria. *FEMS Microbiol. Rev.* **29**, 491–509 (2005).
19. Xiao, M. *et al.* Oxidative stress-related responses of *Bifidobacterium longum* subsp. *longum* BBMN68 at the proteomic level after exposure to oxygen. *Microbiology* **157**, 1573–1588 (2011).
20. Pericone, C. D., Overweg, K., Hermans, P. W. & Weiser, J. N. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect. Immun.* **68**, 3990–3997 (2000).
21. Pridmore, R. D., Pittet, A. C., Praplan, F. & Cavadini, C. Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. *FEMS Microbiol. Lett.* **283**, 210–215 (2008).
22. Imlay, J. A. Pathways of oxidative damage. *Annu. Rev. Microbiol.* **57**, 395–418 (2003).
23. Mishra, S. & Imlay, J. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* **525**, 145–160 (2012).
24. Jones, D. & Collins, M. D. Irregular, nonsporulating Gram-positive rods, p. 1261–1434. In Sneath, P. H. A., Mair, N. S., M. Sharpe, E. & Holt, J. G. (eds), *Bergey's manual of systematic bacteriology*. Williams and Wilkins Co, Baltimore. (1986).
25. Leahy, S. C., Higgins, D. G., Fitzgerald, G. F. & van Sinderen, D. Getting better with bifidobacteria. *J. Appl. Microbiol.* **98**, 1303–1315 (2005).
26. Riviere, A., Selak, M., Lantin, D., Leroy, F. & Vuyst, L. D. Bifidobacteria and butyrate producing colon bacteria: importance and strategies for their stimulation in the human gut. *Front. Microbiol.* **7**, 979 (2016).
27. Shimamura, S. *et al.* Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. *J. Dairy. Sci.* **75**, 3296–3306 (1992).
28. Meile, L. *et al.* *Bifidobacterium lactis* sp. nov., a moderately oxygen-tolerant species isolated from fermented milk. *Syst. Appl. Microbiol.* **20**, 57–64 (1997).
29. Ahn, J. B., Hwang, H. J. & Park, J. H. Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. *J. Microbiol. Biotechnol.* **11**, 443–451 (2001).
30. Talwalkar, A. & Kailasapathy, K. Metabolic and biochemical responses of probiotic bacteria to oxygen. *J. Dairy. Sci.* **86**, 2537–2546 (2003).
31. Gonzalez, R., Blancas, A., Santillana, R., Azaola, A. & Wachter, C. Growth and final product formation by *Bifidobacterium infantis* in aerated fermentations. *Appl. Microbiol. Biotechnol.* **65**, 606–610 (2004).
32. Simpson, P. J., Stanton, C., Fitzgerald, G. F. & Ross, R. P. Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. *J. Appl. Microbiol.* **99**, 493–501 (2005).
33. Kawasaki, S., Mimura, T., Satoh, T., Takeda, K. & Niimura, Y. Response of microaerophilic *Bifidobacterium* species, *B. boum* and *B. thermophilum*, to oxygen. *Appl. Environ. Microbiol.* **72**, 6854–6858 (2006).
34. Kawasaki, S. *et al.* Effect of CO₂ on colony development by *Bifidobacterium* species. *Appl. Environ. Microbiol.* **73**, 7796–7798 (2007).
35. Ruiz, L. *et al.* Molecular clues to understand the aerotolerance phenotype of *Bifidobacterium animalis* subsp. *lactis*. *Appl. Environ. Microbiol.* **78**, 644–650 (2012).
36. Kawasaki, S. Response of *Bifidobacterium* species to oxygen, p 103–110. In Sonomoto, K. & Yokota, A. (eds), *Lactic Acid Bacteria and Bifidobacteria*, Caister Academic Press, Norfolk, UK. (2011).
37. Kawasaki, S., Watanabe, M., Fukuya, S. & Yokota, A. Stress responses of Bifidobacteria: oxygen and bile acid as the stressors, p 131–143. In Mattarelli, P., Biavati, B., Holzappel, W. & Wood, B. J. B. (eds), *The bifidobacteria and related organisms*, Academic Press, Oxford, UK. (2017).
38. Scardovi, V. & Trovatelli, L. D. New species of bifido bacteria from *Apis mellifica* L. and *Apis indica* F. A contribution to the taxonomy and biochemistry of the genus *Bifidobacterium*. *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg.* **123**, 64–88 (1969).
39. Scardovi, V., Trovatelli, L. D., Biavati, B. & Zani, G. *Bifidobacterium cuniculi*, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and *Bifidobacterium pseudocatenulatum*: four new species and their deoxyribonucleic acid homology relationships. *Int. J. Syst. Bacteriol.* **29**, 291–311 (1979).
40. Hayashi, K. *et al.* Purification and characterization of oxygen-inducible haem catalase from oxygen-tolerant *Bifidobacterium asteroides*. *Microbiology* **159**, 89–95 (2013).
41. Bottacini, F. *et al.* *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect Gut. *PLoS One* **7**, e44229 (2012).
42. He, J., Sakaguchi, K. & Suzuki, T. Acquired tolerance to oxidative stress in *Bifidobacterium longum* 105-A via expression of a catalase gene. *Appl. Environ. Microbiol.* **78**, 2988–2990 (2012).
43. Zuo, F. *et al.* Homologous overexpression of alkyl hydroperoxide reductase subunit C (ahpC) protects *Bifidobacterium longum* strain NCC2705 from oxidative stress. *Res. Microbiol.* **165**, 581–589 (2014).
44. Kawasaki, S., Satoh, T., Todoroki, M. & Niimura, Y. *b*-type dihydroorotate dehydrogenase is purified as a H₂O₂-forming NADH oxidase from *Bifidobacterium bifidum*. *Appl. Environ. Microbiol.* **75**, 629–636 (2009).
45. Underwood, M. A., German, J. B., Lebrilla, C. B. & Mills, D. A. *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. *Pediatr. Res.* **77**, 229–235 (2015).
46. Sela, D. A. *et al.* The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proc. Natl. Acad. Sci. USA* **105**, 18964–18969 (2008).
47. Underwood, M. A. *et al.* *Bifidobacterium longum* subsp. *infantis* in experimental necrotizing enterocolitis: alterations in inflammation, innate immune response, and the microbiota. *Pediatr. Res.* **76**, 326–333 (2014).
48. Zeno, S. *et al.* Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. *J. Bacteriol.* **178**, 4508–4514 (1996).
49. Zeno, S., Kobori, T., Tanokura, M. & Saigo, K. Purification and characterization of NfrA1, a *Bacillus subtilis* nitro/flavin reductase capable of interacting with the bacterial luciferase. *Biosci. Biotechnol. Biochem.* **62**, 1978–1987 (1998).
50. Lei, B., Liu, M., Huang, S. & Tu, S. C. *Vibrio harveyi* NADPH-flavin oxidoreductase: cloning, sequencing and overexpression of the gene and purification and characterization of the cloned enzyme. *J. Bacteriol.* **176**, 3552–3558 (1994).

51. Cortial, S. *et al.* NADH oxidase activity of *Bacillus subtilis* nitroreductase NfrA1: insight into its biological role. *FEBS Lett.* **584**, 3916–3922 (2010).
52. Li, X., Chow, D. C. & Tu, S. C. Thermodynamic analysis of the binding of oxidized and reduced FMN cofactor to *Vibrio harveyi* NADPH-FMN oxidoreductase FRP apoenzyme. *Biochemistry* **45**, 14781–14787 (2006).
53. Scardovi, V. & Trovatielli, L. D. *Bifidobacterium animalis* (Mitsuoka) comb. nov. and the “*minimum*” and “*subtile*” groups of new bifidobacteria found in sewage. *Int. J. Syst. Bacteriol.* **24**, 21–28 (1974).
54. Tanaka, K., Samura, K. & Kano, Y. Structural and functional analysis of pTB6 from *Bifidobacterium longum*. *Biosci. Biotechnol. Biochem.* **69**, 422–425 (2005).
55. Yasui, K. *et al.* Improvement of bacterial transformation efficiency using plasmid artificial modification. *Nucleic. Acids. Res.* **37**, e3 (2009).
56. Oberg, T. S., Ward, R. E., Steele, J. L. & Broadbent, J. R. Transcriptome analysis of *Bifidobacterium longum* strains that show a differential response to hydrogen peroxide stress. *J. Biotechnol.* **212**, 58–64 (2015).
57. Meehan, B. M., Baughn, A. D., Gallegos, R. & Malmay, M. H. Inactivation of a single gene enables microaerobic growth of the obligate anaerobe *Bacteroides fragilis*. *Proc. Natl. Acad. Sci. USA* **109**, 12153–12158 (2012).
58. Bradford, M. M. 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 (1976).
59. Engler, C., Gruetzner, R., Kandzia, R. & Marillonnet, S. Golden Gate Shuffling: A One-Pot DNA shuffling method based on type II restriction enzymes. *PLoS ONE* **4**(5), e5553 (2009).
60. Sakaguchi, K., He, J., Tani, S., Kano, Y. & Suzuki, T. A targeted gene knockout method using a newly constructed temperature-sensitive plasmid mediated homologous recombination in *Bifidobacterium longum*. *Appl. Microbiol. Biotechnol.* **95**, 499–509 (2012).

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Author Contributions

K.T., Ta.S., J.K., S.U. performed the experiments and analysis. I.N. performed the knockout mutation. Y.K. and To.S. supervised genetic experiments and provided useful comments. Y.N. conceived and provided useful comments. K.T. and S.K. wrote the manuscript. S.K. conceived and designed this study. All authors discussed the results and commented on the manuscript.

Additional Information

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