Supplemental Material

Acquired Tolerance to Oxidative Stress in *Bifidobacterium longum* 105-A via the Expression of a Catalase Gene

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table S1. Plasmids were constructed using *Escherichia coli* One Shot® Mach1™ T1® and *E. coli* One Shot *ccdB* Survival™ T1® as transformation hosts and were subsequently transferred to *B. longum* 105-A to assay catalase activity.

Media and growth conditions. *E. coli* and *B. subtilis* were grown in LB medium at 37 ℃. For anaerobic culture, *B. longum* 105-A was grown in MRS medium (Difco, Franklin, New Jersey) containing 0.34% L-ascorbic acid sodium salt (Nacalai Tesque, Kyoto, Japan), 0.02% L-cysteine (Nacalai Tesque), and 50 mM sucrose at 37 ℃. For aerobic culture, *B. longum* 105-A was grown in MRS medium containing 50 mM sucrose and 10 μM hemin (Sigma-Aldrich, St. Louis, MO) at 37 ℃. When necessary, antibiotics were used at the following concentrations: chloramphenicol (Cm; 25 μg/mL), kanamycin (Km; 50 μg/mL) and spectinomycin (Sp; 75 μg/mL). Aerated cultures (10 mL) of *Bifidobacterium* were grown in 25-mL tubes using silicone plugs with shaking at 120 rpm. Anaerobic cultures (10 mL) were grown in 12-mL test tubes with screw caps. CFUs of *B. longum* 105-A were counted as follows: appropriate sample dilutions were prepared in sucrose solution (50 mM sucrose and 1 mM triammonium citrate, pH 6.0), plated on MRS agar, and incubated for 48 h at 37 ℃ under anaerobic conditions using the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan).

DNA manipulations. General molecular biology techniques were used as described previously (4). Plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen, GmbH, Germany). The

restriction enzyme *Stu*I was used as recommended by the supplier (TaKaRa, Ohtsu, Japan). PCR amplifications were performed using *KOD*-Plus DNA polymerase (Toyobo, Osaka, Japan) and Go*Taq* DNA polymerase (Promega, Madison, Wisconsin). When required, DNA fragments were isolated from agarose gels using a NucleoSpin® Extract II kit (MACHEREY-NAGEL, Düren, Germany). DNA was extracted using an UltraClean™ Microbial DNA Isolation kit (MO BIO, Carlsbad, California). To construct expression plasmids, DNA elements were combined using the MultiSite Gateway® Pro kit (Invitrogen, Carlsbad, California). Sequence analysis was performed using an ABI3100 (Applied Biosystems, Foster City, California).

Cloning of *B. subtilis katE*. The plasmid pKKT427 is an *E. coli-Bifidobacterium* shuttle vector modified from a pBRASTA101 replicon (7). To construct the destination vector pGU100, pKKT427 was digested with *Stu*I and ligated with Reading Frame Cassette A supplied with the Gateway® Pro kit. The *B. longum* 105-A *hup* terminator (*hup*T) was amplified with the primers attB5-hupTf and attB2-hupTr. pDONR52::hupT was constructed by BP recombination using the *hup*T fragment and the pDONR-221-P5-P2 vector. The catalase gene was cloned under the control of the *hup* promoter (Hp) (6). Hp was PCR amplified from the *B. longum* 105-A genome using primers hup-f and hup-r. For KatE production, *katE* was PCR-amplified from the *B. subtilis* genome using the primers kat-f and kat-r. The HpkatE fragment was amplified by overlap-PCR with Hp and KatE using primers hup-f and kat-r. pDONR15r::HpkatE was constructed by BP recombination using the HpkatE fragment and the pDONR221-P1-P5r vector. The plasmid pBCAT001 (Fig. S1) was constructed using pDONR15r::HpkatE, pDONR52::hupT, and pGU100 with the MultiSite Gateway® Pro kit. *B. longum* 105-A was transformed with the pKKT427 and pBCAT001 plasmids.

Electroporation of *Bifidobacterium*. Electrocompetent cells were prepared as described previously (8). Electroporation was performed using a Gene-Pulser (Bio-Rad, Berkeley, California)

set at 12.5 kV/cm and 200 Ω and employing a 2-mm cuvette (time constants were between 3.9 and 4.2 ms).

Detection of catalase activity. Cultures of *B. longum* 105-A harboring plasmids were grown overnight and then inoculated at a 1:50 dilution in fresh medium supplemented with spectinomycin. At an OD_{660} of 1.0, 1.5 mL of each culture were centrifuged, and cells were washed twice with PBS and resuspended in 100 μL of PBS. Aliquots (20 μL) were mixed with 10 μL of 30% H_2O_2. The presence of catalase activity results in bubble formation. Quantitative analysis of catalase activity was performed as described previous (1, 5). With some modification, the supernatant was 32-fold concentrated by Vivaspin 20 (molecular weight cutoff 3,000; GE Healthcare). At an OD_{660} of 1.0, cultures were centrifuged, cells were resuspended in PBS, and total cellular proteins were extracted by French press (model 5615, Ohtake Works, Tokyo, Japan). The protein concentration was assayed by the Lowry method with bovine serum albumin as the standard.

Determination of H_2O_2 production. Culture supernatants were diluted 3:2 with a solution of 0.2% Triton X-100, 0.01% horseradish peroxidase, and 0.63 mM *o*-dianisidine dihydrochloride in 50 mM acetate buffer, pH 5.0. The accumulation of H_2O_2 in the culture medium was assayed spectrophotometrically at 460 nm by detecting the oxidation of *o*-dianisidine dihydrochloride.

Survival after short-term H_2O_2 exposure. To test the level of H_2O_2 tolerance of *B. longum* 105-A with or without *katE*, exponential-phase (OD_{660} = 0.6) or stationary-phase (OD_{660} = 1.0) cultures were incubated with 0 or 4.4 mM H_2O_2 for 1 h at 37 ℃ in the presence of 10 μM hemin. After 1 h, H_2O_2 was eliminated by the addition of exogenous bovine catalase (10 U/mL, Sigma), and culture dilutions were plated on MRS agar and cultured for 48 h under anaerobic conditions. Survival rates (fold changes) were generated by comparing the CFU for 0 versus 4.4 mM H_2O_2 exposure.

Survival rate increases were compared for *B. longum* 105-A (pBCAT001) and *B. longum* 105-A (pKKT427).

Aerated cultures. MRS medium containing 10 μM hemin was inoculated with 200 μL of anaerobically cultured *B. longum* 105-A. Aeration was performed using 25-mL tubes containing 10 mL of medium with shaking at 120 rpm under 37 ℃. The accumulation of H_2O_2 in the culture was assayed. Viable cells were counted using two methods, plate counting as described above and flow cytometry (Beckman Coulter, Brea, CA) using the LIVE/DEAD *Bac*Light bacterial viability kit (L/D; Invitrogen).

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Figure legend

Fig. S1. The map of pBCAT001 construction using the Gateway system. P: *hup* promoter; T: *hup* terminator; *katE*: *B. subtilis* heme-dependent catalase; Sp^r: spectinomycin resistance; *repB*: *Bifidobacterium* replication initiation; *ColE1* ori: Colicine E1 origin of replication; *attB1*, *attB2*, *attB5*: Gateway system sites; bold: palindromes; italic: proposed −35 and −10; uppercase: proposed RBS; uppercase italic: the initiation codon (6).

Fig. S2. SDS-PAGE analysis of *E. coli* UM255 and *B. longum* 105-A. M: molecular mass marker (Daiichi, Tokyo, Japan); + : pBCAT001; - : pKKT427. The arrowhead indicates the KatE band of *E. coli* UM255 (pBCAT001) extract. The 77 kDa band corresponding to *B. subtilis* KatE was clearly identified in *E. coli* UM255 (pBCAT001), but was not identifiable in *B. longum* 105-A (pBCAT001) because its expression level was lower than 1/13 of that in *E. coli* UM255 (39 U/mg crude extract from *B. longum* and 511 U/mg crude extract from *E. coli*).

Table S1. Bacterial strains, plasmids, and primers used

Strain or plasmid	Characteristic(s)	Reference of Source
Bacterial strains		
Bacillus subtilis GTC01672	Wild type	GTC[c]
B. longum 105-A	Wild type	2
E. coli One Shot Mach1™ T1^R	F- *φ80(lacZ)ΔM15 ΔlacX74 hsdR(rK⁻mK⁺) ΔrecA1398 endA1 tonA*	Invitrogen
E. coli One Shot *ccd*B Survival™ T1^R	F- *mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZΔM15 ΔlacX74 recA1 araΔ139 D(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG tonA::Ptrc -ccdA*	Invitrogen
E. coli UM255	*pro leu rpsL hsdM hsdR end1 lacy katG2 katE::*Tn*10 recA*	3
Plasmids[a]		
pDONR™221-P1-P5r	pDONR™221; Cm^r Km^r; ccdB; attP1; attP5r	Invitrogen
pDONR™221-P5-P2	pDONR™221; Cm^r Km^r; ccdB; attP5; attP2	Invitrogen
pDONR15r::HpkatE	pDONR™221-P1-P5r; Km^r; carrying the *B. subtilis katE* ; *hup* promoter	This study
pDONR52::hupT	pDONR™221-P5-P2; Km^r; carrying the *hup* terminator	This study
pKKT427	Sp^r; A shuttle vector between E. coli and Bifidobacterium, 3.9 kb modification of pBRATA101	8
pGU100	pKKT427; Cm^r, Sp^r; ccdB; Reading Frame Cassette A	This study
pBCAT001	pKKT427; Sp^r; carrying the *B. subtilis katE*; *hup* promoter	This study
Primers[b]		
kat-f	atgagtgatgaccaaaaca	This study
kat-r	*ggggacaacttttgtatacaaagttgt*tcaaattcgtctatcccaat	This study
hup-f	*ggggacaagtttgtacaaaaaagcaggct*ttccgccactttgct	This study
hup-r	ttggtcatcactcataaaagcatccttcttggg	This study
attB5-hupTf	*ggggacaactttgtatacaaaagttg*ccttctgctcgtagcgatta	This study
attB2-hupTr	*ggggaccactttgtacaagaaagctgggta*tggaagcgctgaactagtcc	This study

[a] Cm^r , Km^r and Sp^r, resistance to chloramphenicol, kanamycin, and spectinomycin, respectively.
[b] Italicized oligonucleotide sequences denote the *attB* sites.
[c] Gifu Type Culture Collection (GTC), from Gifu University Culture Collection (GIFU), Department of Microbiology, Gifu University School of Medicine.

Table S2. The catalase activities of crude extract. (U/mg)

Hemin[a]	*B. longum* 105-A (wild type)	*B. longum* 105-A (pKKT427)	*B. longum* 105-A (pBCAT001)
+	nd	nd	39
-	nd	nd	nd

[a] +, addition of hemin in medium; -, no hemin in medium.
Under anaerobic condition, $OD_{660} = 1.0$.
nd, not detected. (less than 0.1 U/mg)