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

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For improvement of tolerance to oxidative stress in *Bifidobacterium longum* 105-A, we introduced the *Bacillus subtilis* catalase gene (*katE*) into it. The transformant showed catalase activity (39 U/mg crude protein) in the intracellular fraction, which increased survival by ~100-fold after a 1-h exposure to 4.4 mM H_2O_2, decreased *de novo* H_2O_2 accumulation, and increased survival in aerated cultures by 10^5-fold at 24 h. The protection level was better than that conferred by exogenously added catalase.

As probiotics, some species of *Bifidobacterium* are widely used in various therapeutics (11, 19) and food products (9, 10, 27). To be effective, bifidobacteria must remain viable until reaching the intestinal tract. However, as bifidobacteria are obligate anaerobic bacteria, their sensitivity to O_2 limits their survival and use in industrial applications (14, 24).

Superoxide anion (O_2^-) is a biologically quite toxic component (7). It produced by a one-electron reduction of dioxygen, which occurs widely in nature under aerobic conditions. The aerobic organisms express superoxide dismutase, which decomposes O_2^- and produces H_2O_2 (3, 7). In the presence of ferrous iron(II) or some other transition metals, H_2O_2 is converted to an OH radical by the Fenton reaction, which shows the highest toxicity among active oxygen (2, 3, 6). Thus, H_2O_2 decomposition is one of the most important processes in cell survival under aerobic conditions. Bifidobacteria also have an oxidase function which enables them to use O_2 as an electron acceptor to reduce to H_2O and H_2O_2 under aerobic conditions (5, 16, 21).

Eukaryotes have catalase and glutathione peroxidase (GPX) as an H_2O_2-decomposing system to survive under aerobic conditions (1, 3, 13). Little is known about GPX activity in prokaryotes, and GPX gene and glutathione synthesis pathways have not been detected in obligate anaerobes, including bifidobacteria (13, 20, 23). Catalase is an enzyme commonly found in aerobes and facultative anaerobes but is absent in almost all obligate anaerobes, including bifidobacteria (1, 3). NADH peroxidase was found in bifidobacteria, and some types of peroxidases were predicted, including thiol peroxidase, alkyl hydroperoxide reductase, and peptide methionine sulfoxide reductase. However, these peroxidases are unable to decompose the H_2O_2 produced by bifidobacteria under aerobic conditions (23, 26).

Catalases are classified in 2 groups, depending on the type of metal cofactors: heme-dependent catalase and manganese-dependent catalase (3, 7). *B. subtilis* KatE (heme-dependent catalase) is a well-known catalase that is used to improve the viability of some species of bacteria via heterologous expression (4, 18, 22). In this study, to reduce the toxicity from active oxygen, we therefore investigated the effects of expressing *B. subtilis* heme-dependent catalase on the oxidative stress resistance of *Bifidobacterium longum* 105-A. For comparison, the effects of exogenously added catalase on *B. longum* were also tested.

To express *katE* of *B. subtilis*, we constructed an expression vector, pBCAT001, derived from plasmid pKKT427 (28), by inserting the *B. subtilis* KatE gene within the promoter and termina-

TABLE 1 Numbers of CFU/ml and survival rates after H_2O_2 exposure for 1 h

Growth phase (OD_{660})	Strain	CFU/ml after exposure to[a]: 0 mM H_2O_2	CFU/ml after exposure to[a]: 4.4 mM H_2O_2	Survival rate[b]	Fold survival rate increase[c]
Exponential (0.6)	*B. longum* 105-A(pKKT427)	$(7.00 \pm 0.34) \times 10^8$	$(1.20 \pm 0.20) \times 10^5$	0.00017	
	B. longum 105-A(pBCAT001)	$(8.00 \pm 0.16) \times 10^8$	$(1.64 \pm 0.26) \times 10^7$	0.021	120
Stationary (1.0)	*B. longum* 105-A(pKKT427)	$(1.68 \pm 0.22) \times 10^9$	$(2.10 \pm 0.40) \times 10^6$	0.0013	
	B. longum 105-A(pBCAT001)	$(1.56 \pm 0.34) \times 10^9$	$(2.00 \pm 0.14) \times 10^8$	0.13	103

[a] Data are the means ± standard deviations from three independent experiments.
[b] Survival rates were determined by comparing the colony counts for exposure to 0 versus 4.4 mM H_2O_2 for 1 h at 37°C.
[c] Survival rate increases were compared for *B. longum* 105-A(pBCAT001) and *B. longum* 105-A(pKKT427).

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tor of *hup* of *B. longum* and then introduced it into *B. longum* 105-A and *Escherichia coli* UM255 (by a method described in the supplemental material; also see Table S1 and Fig. S1 in the supplemental material). To determine the activity of KatE, hemin (10 μM) (Sigma-Aldrich, St. Louis, MO) was added to the medium because bifidobacteria do not synthesize heme (23) (see Table S2 in the supplemental material). We analyzed the crude extracts from *E. coli* UM255(pKKT427, pBCAT001) and *B. longum* 105-A(pKKT427, pBCAT001) by SDS-PAGE. 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 (see Fig. S2 in the supplemental material). We considered this weak expression was caused by the low plasmid copy number ($\approx$10 copies per cell), transcription, and translation strengths (12, 25). The catalase activity was examined by detecting bubble (O_2) formation upon the addition of 30% H_2O_2 to the cell pellet. Catalase activity was only detected in the cell fraction—39 U/mg crude protein in *B. longum* 105-A(pBCAT001) versus less than 0.1 U/mg in *B. longum* 105-A(pKKT427)—under anaerobic conditions (optical density at 660 nm [OD_{660}] of 1.0), whereas the extracellular catalase activity was less than 0.4%.

We investigated the effect of KatE on the short-term H_2O_2 tolerance of *B. longum* 105-A. The survival rates of *B. longum* 105-A(pKKT427 pBCAT001) were determined by incubating cultures for 1 h in MRS medium with 4.4 mM H_2O_2 at 37°C. The survival rates of *B. longum* 105-A(pBCAT001) during the exponential and stationary phases were significantly increased by 120- and 103-fold, respectively, compared to that of *B. longum* 105-A(pKKT427) (Table 1). The data in Table 1 demonstrate that exponential-phase cells were more sensitive to H_2O_2 than stationary-phase cells.

We also investigated the physiology of *B. longum* 105-A under aerobic conditions. *B. longum* 105-A(pKKT427, pBCAT001) reached a maximum growth rate at 12 h after inoculation under anaerobic conditions. The growth of *B. longum* 105-A(pBCAT001) was partially inhibited, and that of *B. longum* 105-A(pKKT427) was nearly stopped under aerobic culture conditions (Fig. 1A). To measure growth rates, cells were cultured and then plate counted. Although most of the *B. longum* 105-A(pKKT427) specimens survived 12 h of aerobic culture, cell growth began to sharply decrease and became almost unculturable after 24 h in aerobic culture. However, *B. longum* 105-A(pBCAT001) exhibited a high rate of survival (1×10^7 CFU/ml) at 24 h and only became unculturable after 48 h under aerobic conditions (Fig. 1B). The results demonstrate that the presence of KatE protected *B. longum* 105-A from aerobic culture-induced death.

The concomitant generation of H_2O_2 was also measured (Fig. 1D). The accumulation of H_2O_2 in *B. longum* 105-A(pKKT427) increased for 18 h and peaked at 0.1 mM. H_2O_2 was scavenged by the genetically expressed catalase during the exponential phase, and it did not begin to accumulate in the medium of *B. longum* 105-A(pBCAT001) until the stationary phase. At this time, the cells became unculturable (Fig. 1B). Interestingly, the decrease in the growth of *B. longum* 105-A(pKKT427) was faster than that of *B. longum* 105-A(pBCAT001). This might be because the concentration of H_2O_2 in *B. longum* 105-A(pKKT427) was 2.4-fold higher than that of *B. longum* 105-A(pBCAT001) in aerated cultures. *B. longum* 105-A(pBCAT001) survived longer due to the increased period of time in which H_2O_2 had not accumulated. This difference in growth rates suggests that H_2O_2 was primarily responsible for *B. longum* 105-A becoming unculturable under aerobic conditions.

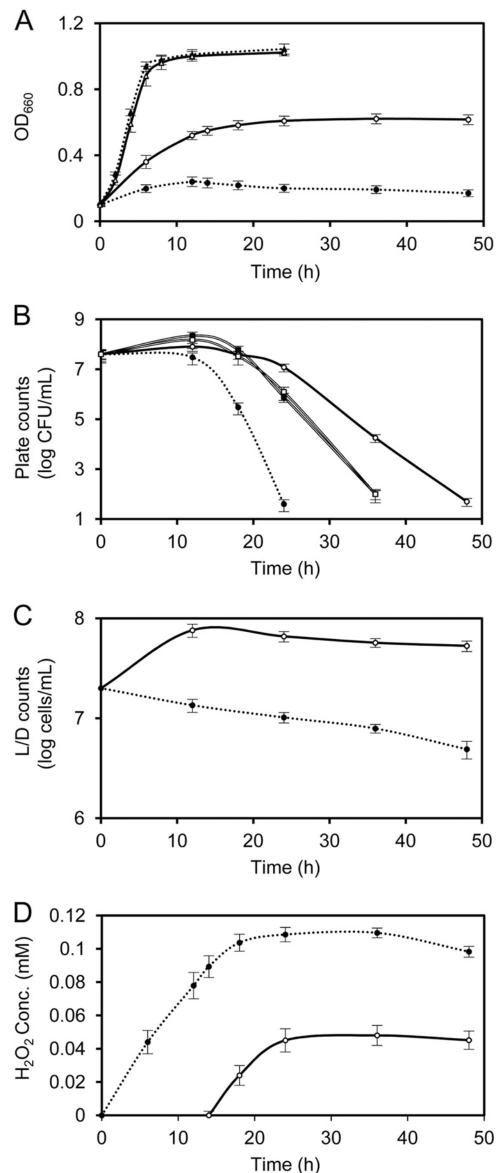

FIG 1 Cultured *B. longum* 105-A(pKKT427 or pBCAT001) under aerobic or anaerobic conditions. (A) Growth (OD_{660}) of the cultured *B. longum* 105-A strain. (B) Counts of *B. longum* 105-A cultured under aerobic conditions. (C) LIVE/DEAD (L/D) assay of *B. longum* 105-A cultured under aerobic conditions. (D) H_2O_2 accumulation by *B. longum* 105-A cultured under aerobic conditions. Dotted line, *B. longum* 105-A(pKKT427); solid line, *B. longum* 105-A(pBCAT001); solid circles, *B. longum* 105-A(pKKT427) cultured under aerobic conditions; open circles, *B. longum* 105-A(pBCAT001) cultured under aerobic conditions; solid triangles, *B. longum* 105-A(pKKT427) cultured under anaerobic conditions; open triangles, *B. longum* 105-A(pBCAT001) cultured under anaerobic conditions; solid squares, *B. longum* 105-A(pKKT427) cultured using exogenously added catalase (3,000 U/ml medium) under aerobic conditions; open squares, *B. longum* 105-A(pKKT427) cultured using exogenously added catalase (100 U/ml medium) under aerobic conditions. The results presented correspond to the averages of three different assays. Error bars correspond to the standard errors of the mean values.

Lahtinen et al. reported that *B. longum* lost culturability quickly during storage, but the cells still maintained intact mem-

branes (17). H_2O_2 is known to damage DNA and protein (1, 3, 7, 8); however, it is unknown whether H_2O_2 can easily damage the *B. longum* membrane. Therefore, we investigated whether it was possible that *B. longum* 105-A cells lost their culturability but maintained an intact membrane. These experiments were conducted using the LIVE/DEAD *Bac*Light bacterial viability kit (L/D; Invitrogen). After 24 h in aerobic culture, *B. longum* 105-A containing pKKT427 remained relatively stable, and 1×10^7 "viable" cells/ml were maintained (Fig. 1C); however, the survival decreased to 1×10^1 to 1×10^2 CFU/ml (Fig. 1B). Based on this information, we were only able to make the decision that the cells had intact membranes, but it is still unknown whether the cells were dead. To confirm whether cells maintain viability, further studies are needed, such as examining the synthesis of DNA, RNA, and protein.

Some studies reported that adding exogenous catalase to the liquid medium improved aerobic growth of bifidobacteria (5, 15). Because H_2O_2 readily diffuses across cell membranes but exogenously added catalase cannot penetrate cell membranes (1, 2), we therefore compared the culturable *B. longum* 105-A strain protected by catalase expression with the culturable *B. longum* 105-A strain protected by the addition of exogenous catalase. Although the counts of *B. longum* 105-A(pKKT427) recovered when cultured under aerobic conditions with exogenously added catalase from bovine liver (100 and 3,000 U/ml medium) (C1345-1G; Sigma), the counts of *B. longum* 105-A(pKKT427) were similar regardless of the concentration of added catalase (Fig. 1). Interestingly, the counts of *B. longum* 105-A protected by addition of exogenous catalase were nearly identical to those of *B. longum* 105-A(pBCAT001) when aerobically cultured for 18 h, although the concentrations of exogenously added catalase were much higher than the levels of expressed catalase. *B. longum* 105-A that was protected by exogenously added catalase was unculturable after 36 h in aerated culture; however, *B. longum* 105-A(pBCAT001) did not become unculturable until 48 h in aerated culture. These results indicate that *B. longum* maintained intact cell membranes, whereas induced exogenously added catalase eliminated extracellular H_2O_2 but was unable to eliminate intracellular H_2O_2.

In conclusion, we have successfully expressed *B. subtilis* KatE in *B. longum* 105-A. The KatE-expressed transformant was able to grow and survive under aerobic conditions. This finding revealed that H_2O_2 accumulation is a primary factor of growth inhibition in bifidobacteria. The addition of catalase to the medium also protects bifidobacteria from oxidative stress; however, this effect was weaker than that of heterologously expressed catalase. To our knowledge, this is the first report on protecting bifidobacteria from oxidative stress by heterologous expression of catalase.

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