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 H₂O₂, decreased *de novo* H₂O₂ accumulation, and increased survival in aerated cultures by 10³-fold at 24 h. The protection level was better than that conferred by exogenously added catalase.

**TABLE 1** Numbers of CFU/ml and survival rates after H₂O₂ exposure for 1 h

<table>
<thead>
<tr>
<th>Growth phase (OD₆₆₀)</th>
<th>Strain</th>
<th>CFU/ml after exposure to a</th>
<th>Survival rate b</th>
<th>Fold survival rate increase c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mM H₂O₂</td>
<td>4.4 mM H₂O₂</td>
<td></td>
</tr>
<tr>
<td>Exponential (0.6)</td>
<td>B. longum 105-A(pKKT427)</td>
<td>(7.00 ± 0.34) × 1⁶</td>
<td>(1.20 ± 0.20) × 1⁵</td>
<td>0.00017</td>
</tr>
<tr>
<td></td>
<td>B. longum 105-A(pBCAT001)</td>
<td>(8.00 ± 0.16) × 1⁶</td>
<td>(1.64 ± 0.26) × 1⁵</td>
<td>0.021</td>
</tr>
<tr>
<td>Stationary (1.0)</td>
<td>B. longum 105-A(pKKT427)</td>
<td>(1.68 ± 0.22) × 1⁹</td>
<td>(2.10 ± 0.40) × 1⁸</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>B. longum 105-A(pBCAT001)</td>
<td>(1.56 ± 0.34) × 1⁹</td>
<td>(2.00 ± 0.14) × 1⁸</td>
<td>0.13</td>
</tr>
</tbody>
</table>

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 H₂O₂ for 1 h at 37°C.
c Survival rate increases were compared for *B. longum* 105-A(pBCAT001) and *B. longum* 105-A(pKKT427).
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 (≈10 copies per cell), transcription, and translation strengths (12, 25). The catalase activity was examined by detecting bubble (O2) formation upon the addition of 30% H2O2 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 [OD660] of 1.0), whereas the extracellular catalase activity was less than 0.4%.

We investigated the effect of KatE on the short-term H2O2 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 H2O2 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 H2O2 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 × 107 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 H2O2 was also measured (Fig. 1D). The accumulation of H2O2 in B. longum 105-A(pKKT427) increased for 18 h and peaked at 0.1 mM. H2O2 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 H2O2 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 H2O2 had not accumulated. This difference in growth rates suggests that H2O2 was primarily responsible for B. longum 105-A becoming unculturable under aerobic conditions.

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

![Figure 1](http://aem.asm.org/)
branes (17). H₂O₂ is known to damage DNA and protein (1, 3, 7, 8); however, it is unknown whether H₂O₂ 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 BacLight bacterial viability kit (L/D; Invitrogen). After 24 h in aerobic culture, B. longum 105-A containing pKKT427 remained relatively stable, and 1 × 10⁷ “viable” cells/ml were maintained (Fig. 1C); however, the survival decreased to 1 × 10⁶ to 1 × 10⁵ 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 H₂O₂ 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 un culturable after 36 h in aerated culture; however, B. longum 105-A(pBCAT001) did not become un culturable until 48 h in aerated culture. These results indicate that B. longum maintained intact cell membranes, whereas induced exogenously added catalase eliminated extracellular H₂O₂ but was unable to eliminate intracellular H₂O₂.

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 H₂O₂ 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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REFERENCES


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