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Acetate-producing bifidobacteria protect the host from enteropathogenic infection via carbohydrate transporters

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The human gut harbors a large and diverse community of commensal bacteria. Among them, Bifidobacterium is known to exhibit various probiotic effects including protection of hosts from infectious diseases. We recently discovered that genes encoding an ATP-binding-cassette-type carbohydrate transporter present in certain bifidobacteria contribute to protecting gnotobiotic mice from death induced by enterohemorrhagic *Escherichia coli* O157:H7. We elucidated the molecular mechanism on lethal infection in mice associated with several bifidobacterial strains by a multi-omics approach combining genomics, transcriptomics and metabolomics. The combined data clearly show that acetate produced by protective bifidobacteria acts in vivo to promote defense functions of the host epithelial cells and thereby protects the host from lethal infection. As demonstrated here, our multi-omics approach provides a powerful strategy for evaluation of host-microbial interactions in the complex gut ecosystem.

Keywords: bifidobacteria, probiotics, acetate, omics, carbohydrate transporter, genome, EHEC, gnotobiotic mouse, metabolome, gut microbiome

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The human gut is colonized by trillions of bacteria, representing hundreds of species and thousands of subspecies, and the gut microbiota has a profound influence on human physiology, immunology and nutrition.[1-4] For instance, an imbalance in the gut microbiota is linked to various disorders such as obesity, inflammatory bowl disease, metabolic syndrome, diabetes and colon cancer.[5-9] Therefore, controlling the balance of the intestinal microbiota may be one of the most effective means to maintaining good health in humans. Probiotics are defined as live microorganisms that are known to beneficially affect their hosts by either improving the imbalanced microbiota or by maintaining the healthy microbiota. Most probiotics taxonomically belong to two genera, bifidobacteria and lactobacilli, both of which inhabit various environments including the human intestine. The biological and bacteriological properties of these probiotic strains have been exclusively studied to characterize their beneficial functions.[10-12] However, the molecular mechanisms by which probiotics act on their host cells remain largely unknown.

Functions of intestinal bacteria, including probiotics, may be the consequences of host-bacterial interactions in which bacterial components or metabolites are involved.[13,14] To comprehensively analyze host-microbial interactions, we previously developed the technique, called "multi-omics."[15-18] The multi-omics approach includes various omics data obtained from genomics, transcriptomics and metabolomics of bacteria, host cells and intestinal contents. It provides us an efficient way to identify molecules and genes participating in the interactions by investigating positive and negative correlations between the omics data using appropriate statistics.

There are now several reports on probiotic inhibition of pathogenic *Escherichia coli* O157 by bifidobacteria in gnotobiotic mice.[19-21] However, the molecular mechanism for the protective effect of bifidobacteria remains unclear. Thus, we applied our multi-omics approach to elucidate the

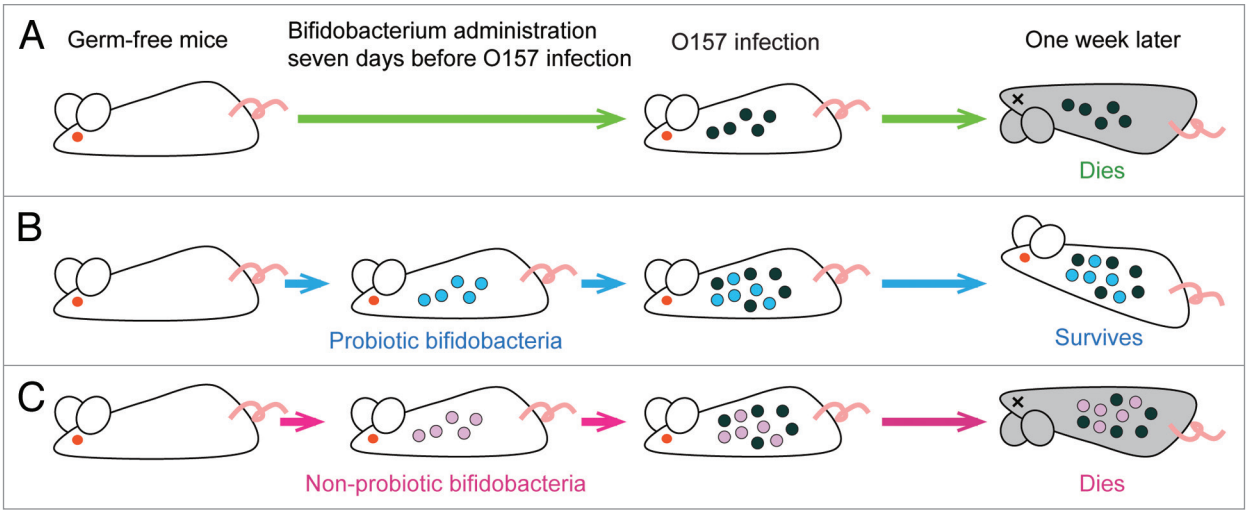

Figure 1. Simplified mouse model of lethal infection with O157. (A) GF mice fed with O157 died within seven days. (B) GF mice administered with probiotic bifidobacteria (BL, BF and BN) prior to O157 inoculation survived. (C) GF mice administered with non-probiotic bifidobacteria (BT and BA) prior to O157 inoculation died around 10 days after O157 infection.

molecular basis of the probiotic effect of bifidobacteria from O157 lethal infection in gnotobiotic mice.[22] The use of gnotobiotic mice is also valid for analyzing complex host-bacterial interactions because simplified omics data can be obtained from gnotobiotic mice that are made by colonizing germ-free (GF) mice with just one or a few known bacterial species.

Probiotic Bifidobacteria Protect Mice from Death Induced by O157 Infection

When GF mice were orally inoculated with O157, they died within seven days after O157 infection. However, the mice survived when they were colonized seven days before O157 infection with *Bifidobacterium longum* subspecies: longum JCM 1217^T (BL), infantis 157F (BF), or longum NCC 2705 (BN).[23] On the other hand, other bifidobacterial strains, *B. longum* subsp infantis JCM 1222^T (BT) and *B. adolescentis* JCM 1275^T (BA), showed no probiotic effect and failed to prevent the death of mice induced by O157 infection under the same conditions (**Fig. 1**). No significant difference was found between BL+O157 and BA+O157 mice in the number of O157 and bifidobacterial cells, the concentration of Shigatoxin (Stx), virulence gene expression by O157, the amounts of mucin and immunoglobulin A, or the pH level in the intestine. In addition, no bacterial translocation of O157 was detected in the intestine, spleen, kidney, or liver in either group of mice. However, we found that the Stx concentration in the serum was remarkably lower in BL+O157 mice than in BA+O157 mice. Histological analysis revealed a slight but reproducible inflammation, characterized by a decrease in the number of goblet cells and an increase in the infiltration of inflammatory cells, only in the distal colon of BA+O157 mice but not in BL+O157 mice seven days after O157 infection. Correspondingly, epithelial apoptosis was found to be markedly enhanced in BA+O157 mice as early as one day after O157 infection.

For the dying BA+O157 and O157 mice, gene expression profiling of the colonic epithelium corroborated the occurrence of inflammation in the mice even one day after O157 inoculation in which no microscopic inflammation was evident. Self-organized mapping followed by hierarchical clustering analysis revealed a clear difference in gene expression profiles of the colonic epithelium between the dying mice and the surviving mice. By searching the Gene Ontology database, we found that many of the genes upregulated more than 2-fold in the dying mice than in the surviving mice were assigned to the immune response and defense response categories. We then performed partial least squares-discriminant analysis (PLS-DA) and loading plot analysis, the statistical techniques referred to as multivariate analysis. PLS-DA enables us to extract genes of which the mRNA amount significantly differed from between samples by comparing the quantitative data obtained from microarray and quantitative polymerase chain reaction (qPCR) experiments. PLS-DA followed by qPCR indicated remarkable upregulation of inflammation-related genes such as the *Reg* family and *Cxcl* chemokines in the dying mice. The upregulation of these genes is correlated with an increase in epithelial cell apoptosis in the dying mice, suggesting that O157 induces colonic epithelial apoptosis before onset of mucosal inflammation, and that such inflammation is prevented by the colonization of BL in the gut.

Acetate Produced by Probiotic Bifidobacteria Prevents Colonic Epithelial Cell Death Induced by *E. coli* O157 Infection

We next addressed how BL, but not BA, can prevent O157-induced epithelial apoptosis. We hypothesized that differences in metabolic capability between BL and BA in the gut habitat may involve distinct responses to the host colonic epithelium, eventually leading to the prevention of O157-induced apoptotic cell death by BL. To examine the difference

between BL and BA, we analyzed the colonic epithelial transcriptome by microarray and the fecal metabolome by NMR in both BL-monoassociated (BL) and BA-monoassociated (BA) mice, and compared the data. Transcriptome analysis of the colonic epithelium found that only 24 genes, with no apparent mutual correlation, differed in expression level by more than 2-fold between BL and BA mice. Covariation analysis between the transcription of the 24 genes and the fecal metabolome in BL and BA mice indicated a negative correlation between the expression level of mouse genes such as Apoe, C3 and Pla2g2a and the amount of fecal metabolites, mostly carbohydrates. These mouse genes are known to be transcriptionally upregulated for cellular energy metabolism and during anti-inflammatory response. Therefore, these results implied that fecal metabolites in BL mice contain a factor(s) that acts toward the maintenance of host homeostasis by enhancing the expression of genes related to cellular energy metabolism and anti-inflammatory response in the colonic epithelium.

To identify the metabolite(s) responsible for the protective responses of the colonic epithelium, we analyzed and compared the fecal metabolome in mice monoassociated with preventive strains (BL and BF) and non-preventive strains (BA and BT) by PLS-DA. We found a remarkable difference in the fecal metabolite composition between mice monoassociated with the preventive and non-preventive strains. Loading-plot analysis and ^{1}H-^{13}C NMR measurement identified a significantly lower amount of carbohydrates in the feces of mice colonized with BL and BF than that in mice colonized with BA and BT. These data indicate that the preventive ability of bifidobacteria from O157 lethal infection clearly correlates with their efficiency for carbohydrate consumption. We also quantified the amount of short-chain fatty acids (SCFAs) such as formate and acetate in feces, since SCFAs are major end products of carbohydrate fermentation by bifidobacteria. The data showed that only the concentration of acetate was significantly higher in feces in the mice colonized with the preventive strains than in those with the non-preventive ones. These results indicate a correlation between the high amount of fecal acetate and the high preventive ability of BL and BF from O157 lethal infection. We further examined the action of acetate on human colonic epithelial Caco-2 cells in vitro and found that acetate induced gene expression of Apoe, C3 and Pla2g2a and inhibited an increase in permeability of the epithelial monolayer caused from O157-induced cell death. These in vitro results are consistent with the in vivo data obtained from the murine distal colon mentioned above. Additionally, acetate was found to prevent the translocation of Stx from the luminal side toward the basolateral side of the colonic epithelial monolayer, without changing the O157 growth rate or its virulence gene expression. These data strongly suggested that the high amount of acetate produced by the preventive bifidobacteria accompanying the high consumption of carbohydrates acts on the colonic epithelium to enhance the anti-apoptotic or anti-inflammatory responses, resulting in blocking the infiltration of lethal amounts of Stx from the gut lumen into the bloodstream.

Carbohydrate Transporters in Bifidobacteria Confer the Probiotic Effect on *E. coli* O157 Lethal Infection

To explore both the genetic and functional differences in metabolic capacity between the preventive and non-preventive bifidobacterial strains, we completely sequenced the genomes of the three strains (BL, BF and BT), and compared them with each other and with those of other bifidobacterial strains (BA and BN) publicly available.[23,24] We predicted protein-coding genes in these five genomes using Glimmer 2.0 and compared them by reciprocal BLASTP. Comparative analysis identified two syntenic loci which are conserved in all three preventive strains (BL, BF and BN) but are absent in the two non-preventive ones (BA and BT). Functional annotation indicated that the two loci contained the genes encoding ATP-binding cassette (ABC)-type carbohydrate transporters, which are assigned to COG1879, COG1172 and COG1129 in the NCBI COG (clusters of orthologous groups) database (**Fig. 2**). Substrates for these transporters unique to the preventive bifidobacteria were predicted to be ribose, mannose and fructose.[25,26] We experimentally assessed the substrates for these transporters by metabolic profiling using ^{13}C-labeled glucose and fructose in vitro followed by PLS-DA. The results showed that the preventive bifidobacterial strains have the higher consumption rate of fructose and the higher production rate of acetate than the non-preventive strains. In addition, mannose was found to be a preferable substrate for the preventive strains, but neither fructo-oligosaccharide (FOS) nor lactose were.

To confirm the contribution of the ABC-type carbohydrate transporter genes to the prevention of O157 lethal infection, we generated a mutant strain of BN (BNKO) in which one of the ABC-type transporter genes was knocked out using homologous recombination. In an in vitro culture test, BNKO showed significantly reduced abilities in both fructose consumption and acetate production rates compared with the parental BN strain. Also, an in vivo O157 infection experiment using GF mice showed significant reduction in survival rate of the BNKO-associated mice compared with that of the BN-associated mice. We also generated a BA-derived strain (BAtg) that carries the recombinant plasmid exogenously expressing the ABC-type carbohydrate transporter. The BAtg-associated mice survived significantly longer after O157 infection than the BA-associated mice. These data indicate that carbohydrate transporters in bifidobacteria are responsible for the probiotic effect on *E. coli* O157 lethal infection.

Endogenous Acetate is Sufficient for the Prevention of Mice From O157 Lethal Infection

The data shown above clearly demonstrates that the high amount of acetate produced by bifidobacteria in the intestine is efficient for the prevention from O157 lethal infection in mice. This finding led us to test whether the administration of acetylated starch alone can also prevent O157 lethal infection in mice or not, since the starch is gradually hydrolyzed by the intestinal bacteria to release a high

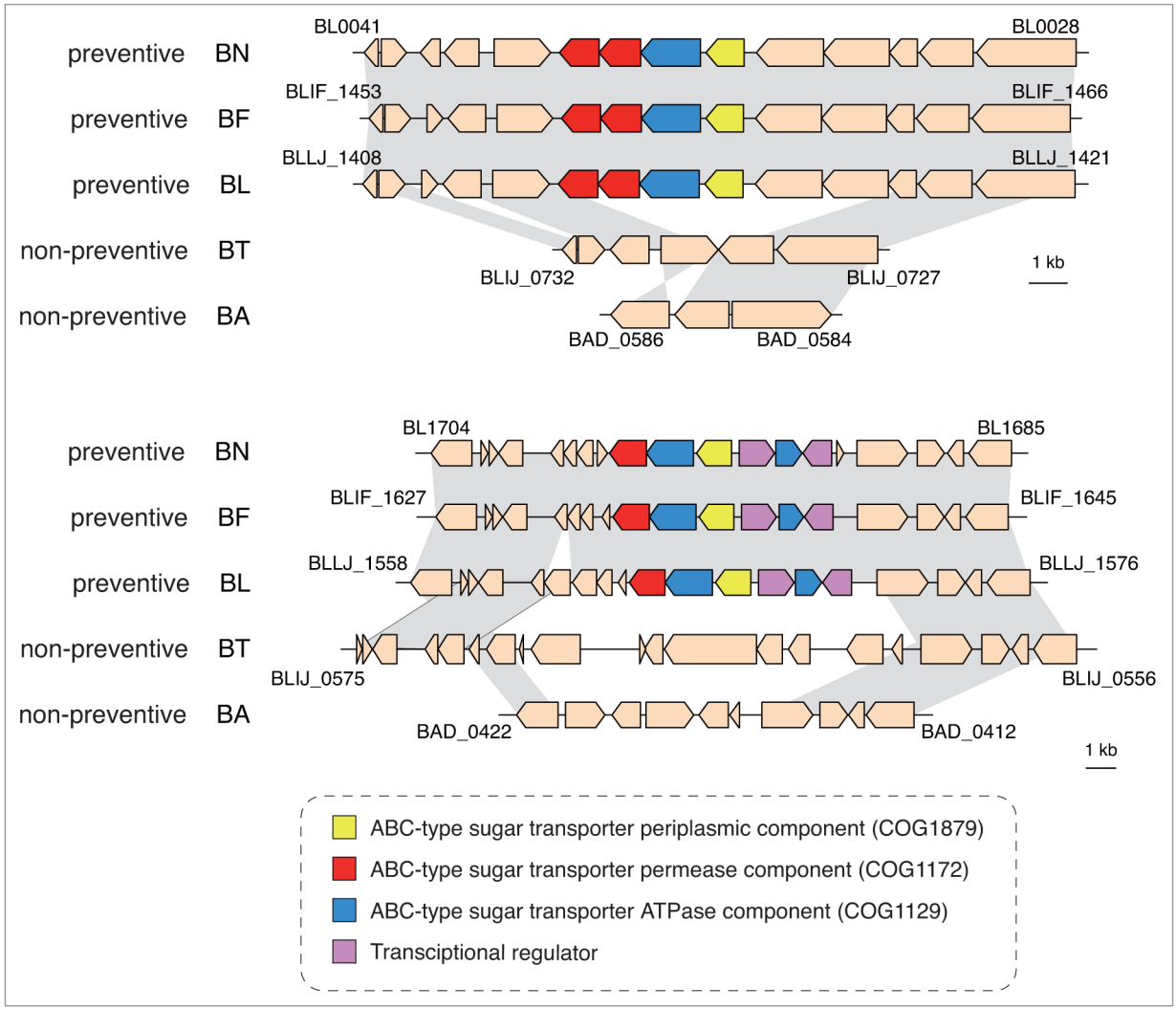

Figure 2. Comparison of regions containing the genes encoding "probiotic transporters" between the preventive and non-preventive bifidobacterial genomes. Genes and their orientations are depicted with arrows, and the genes for "probiotic transporters" are shown in red, blue and yellow. Gray bars indicate orthologous regions between the genomes shown.

amount of acetate in the intestinal tract.[27] When BA+O157 mice were fed a diet rich in acetylated starch, the amount of acetate in the feces significantly increased and the survival rate of the mice was dramatically improved similar to that of BL + O157 mice fed a normal diet. Thus, the data indicate that the elevated amount of exogenous acetate in the intestine is crucial for the prevention of O157 lethal infection. Acetylated starch can be used as prebiotics.

Taken together, this study clearly demonstrates that fructose and its ABC-type carbohydrate transporter in the preventive bifidobacteria largely contribute to the production of acetate that mediates anti-apoptotic and anti-inflammatory responses in the host colonic epithelium, leading to the protection of mice from O157-induced death (**Fig. 3**).

In this study, of more importance is the identification of carbohydrate transporters that are directly involved in conferring a probiotic effect to bifidobacteria. These "probiotic transporter" genes are encoded only in a subset of the *B. longum* lineage, and the genes highly similar to the "probiotic transporter" genes were also detected in several human gut microbiomes.[18] These findings suggest that the "probiotic transporter" genes are distributed in the human gut microbiota with a varying proportion. It will be valuable to assess the correlation between the degree of infectious severity and the presence of these genes by conducting a survey of the "probiotic transporter" genes for individuals involved in O157 or other bacterial outbreaks.

SCFAs, including acetate, generated by commensal bacteria in the intestine have long been implicated in a variety of beneficial effects on the host.[28,29] Also, acetate is generally rich in the colon of healthy subjects and various intestinal microbes have the ability to produce acetate by metabolizing carbohydrates. This study clearly shows that both bacteria-produced and endogenous acetate has a beneficial effect on the function of host epithelial cells, so that acetate production may be a general beneficial effect of probiotic strains. There have been several reports showing

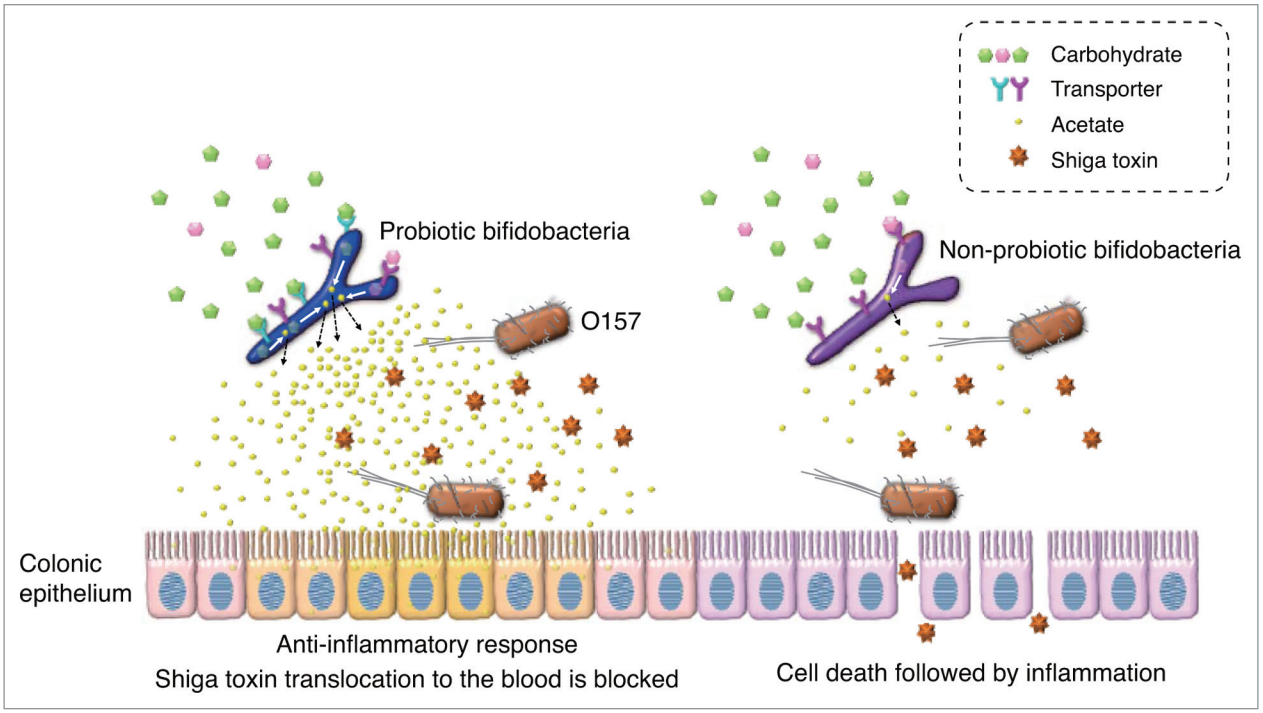

Figure 3. Schematic presentation of the overall mechanisms on protection of mice by bifidobacteria from O157 lethal infection. On the right side, non-probiotic bifidobacteria-associated mice do not produce a sufficient amount of acetate in the distal colon due to the lack of probiotic transporters. Host epithelial cell death is induced by O157 infection with slight inflammation. Stx produced by O157 leaks from the gut lumen into the blood at the inflammatory site, resulting in death of the mice due to Stx circulating throughout the body. On the left side, mice associated with probiotic bifidobacteria produce and accumulate a sufficient amount of acetate in the distal colon in the presence of probiotic transporters by which sugars are actively taken in and metabolized. Host epithelial cell death induced by O157 infection is prevented by enhancing the barrier function due to the action of acetate, resulting in protection of the mice from O157 lethal infection by preventing Stx from leaking into the blood.

that administration of probiotic bacteria similarly upregulates the expression of anti-inflammatory genes in human cells.[30] However, we do not know if the production of acetate also largely contributes to the probiotic effect of lactobacilli or other bifidobacteria not harboring "probiotic transporters." Finally, a simplified animal model system coupled with a multi-omics approach is a powerful strategy to elucidate the molecular mechanisms involved in host-microbial interactions in the complex gut ecosystem.

Disclosure of Potential Conflicts of Interest

None of the authors of this manuscript have a conflict of interest or a financial interest related to this work.

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