Note

Methionine utilization by bifidobacteria: possible existence of a reverse transsulfuration pathway

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Although bifidobacteria are already widely used as beneficial microbes with health-promoting effects, their amino acid utilization and metabolism are not yet fully understood. Knowledge about the metabolism of sulfur-containing amino acids in bifidobacteria is especially limited. In this study, we tested the methionine utilization ability of several bifidobacterial strains when it was the sole available sulfur source. Although bifidobacteria have long been predominantly considered to be cysteine auxotrophs, we showed that this is not necessarily the case.

Key words: bifidobacteria, sulfur-containing amino acid, reverse transsulfuration pathway

Bifidobacteria are well known as one of the major beneficial microbes with health-promoting effects both for humans and for animals. This has led to some bifidobacteria being used commercially in dietary probiotics, such as *Bifidobacterium*-containing yogurt. To provide health-promoting benefits, bifidobacteria should survive in the large intestine of host animals in a nutrient-poor environment. Thus, understanding how bifidobacteria acquire their own nutrition in the host intestine is important to fully utilize their health-promoting effects; however, knowledge about the nutrient utilization and metabolism of bifidobacteria is still limited to sugar-related compounds, such as oligosaccharides, and the amino acid utilization and metabolism of bifidobacteria are not yet fully understood.

There have been several reports on the amino acid requirements of bifidobacteria. In the 1950's, Hassinen and colleagues reported that *Lactobacillus bifidus* (*Bifidobacterium bifidum*) requires cysteine for its growth and that its cysteine auxotrophy is not compensated for by methionine and homocysteine [1]. More recently, Ferrario *et al.* examined the amino acid auxotrophy of 52 bifidobacterial strains and revealed that most of them did not grow in a synthetic minimum medium without cysteine [2]. In addition, most media developed for bifidobacteria so far contain cysteine as a source of sulfur or as a reducing agent [3–5]. Thus, bifidobacteria have been considered to be cysteine auxotrophs for a long time.

Recent genome sequencing analysis revealed that bifidobacteria lack the genes encoding the sulfur assimilation pathway,

including the *cysE* gene, which encodes serine acetyltransferase and is essential for cysteine biosynthesis. Therefore, bifidobacteria cannot synthesize cysteine intracellularly. Some bifidobacteria, however, possess the genes involved in the reverse transsulfuration pathway, which can synthesize cysteine from methionine using homocysteine as an intermediate, such as the *ahcY* (encoding *S*-adenosylhomocysteinase) and *luxS* (encoding *S*-ribosylhomocysteinase) genes for the *S*-adenosyl methionine (SAM) cycle, a part of the reverse transsulfuration pathway (Fig. 1; chemical structures are given in Supplementary Fig. 1). This indicates that some bifidobacterial strains can synthesize cysteine from methionine *via* the reverse transsulfuration pathway and utilize methionine as a sole sulfur source like other microorganisms, such as *Bacillus subtilis* [6], *Pseudomonas aeruginosa* [7], and *Mycobacterium tuberculosis* [8]. However, this prediction was deduced solely from genome sequence analysis and has not yet been functionally proven.

In this study, we tested the methionine utilization ability of several bifidobacterial strains when it was the sole available sulfur source. Although bifidobacteria have long been believed to be cysteine auxotrophs, we showed that this is not necessarily the case.

The bifidobacterial strains used in this study are listed in Table 1. Bifidobacterial strains, including *Bifidobacterium longum* subsp. *longum* 105-A (*B. longum* 105-A, JCM 31944) [9, 10], were routinely cultured in 1/2 MRSCS [11]. Sulfur-containing amino acid assimilation was examined in bifidobacterial minimal
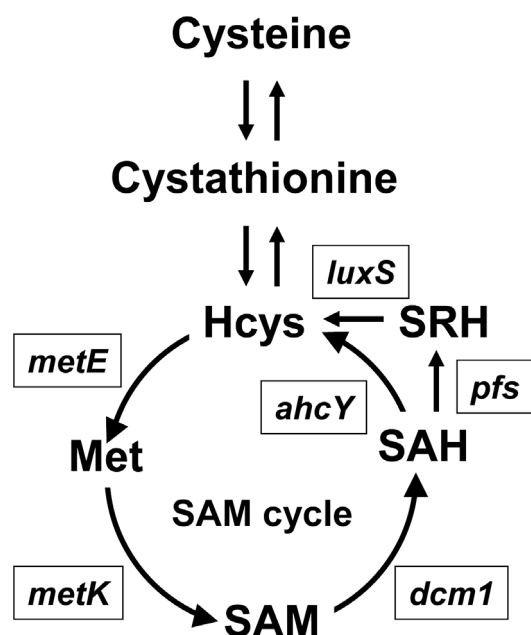

Fig. 1. Predicted sulfur-containing amino acid metabolism, including the reverse transsulfuration pathway, in bifidobacteria.

Hcys: homocysteine; Met: methionine; SAM: *S*-adenosylmethionine; SAH: *S*-adenosylhomocysteine; SRH: *S*-ribosylhomocysteine. Names set in italics in boxes represent the names of genes encoding enzymes in the *S*-adenosylmethionine (SAM) cycle. *metE*: encoding methionine synthase; *metK*: encoding methionine adenosyltransferase; *dcm1*: encoding cytosine 5-methyltransferase; *ahcY*: encoding adenosylhomocysteinase; *pfs*: encoding *S*-adenosylhomocysteine nucleosidase; *luxS*: encoding *S*-ribosylhomocysteinase.
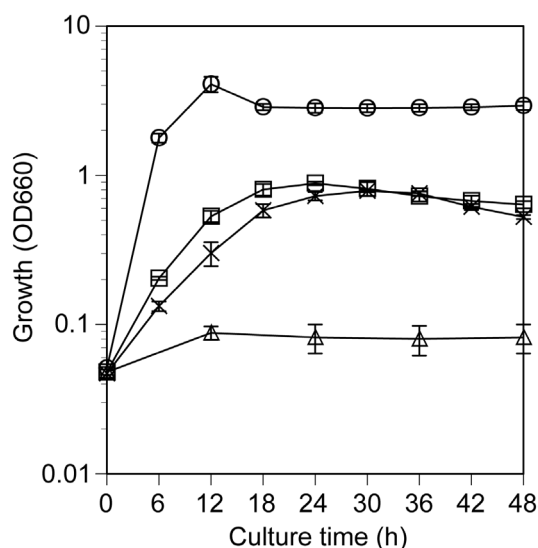
Fig. 2. Growth profiles of *B. longum* 105-A in 1/2MRSCS, BMM-S, BMM+Cys, and BMM+Met

open circle: 1/2MRSCS; open triangle: BMM-S; open square: BMM+Cys; cross: BMM+Met. The means of three independent experiments are plotted. The bars represent standard deviations.

medium (BMM; ingredients provided in Supplementary Table 1) [5] supplemented with 2 mM of cysteine (BMM+Cys) or methionine (BMM+Met), (242 mg/L for cysteine and 298 mg/L for methionine). When DL-homocysteine assimilation was tested, 4 mM of DL-homocysteine (540.7 mg/L) was added to the BMM, as only the L-enantiomer was considered to be utilized. BMM without a sulfur-containing amino acid (BMM-S) was also used as a negative control.

Bifidobacteria strains were inoculated on a 1/2 MRSCS agar plate and cultured overnight. Colonies were inoculated into 5 mL 1/2 MRSCS in a test tube until the optical density at 660 nm (OD_{660}) reached 1.5 to 2.0. After the OD_{660} value of the 1/2MRSCS culture reached around 2.0, one milliliter of culture broth was transferred into a 1.5 mL microtube and centrifuged. The cells were resuspended in 1 mL of 0.85% NaCl and then collected by centrifugation. This step was repeated twice. About 750 μL of the cell suspension was transferred into 30 mL BMM medium to give an initial OD_{660} of 0.05 (40-fold dilution). Bacterial growth was monitored by measuring the OD_{660} using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). All cultures were kept at 37°C and under anaerobic conditions (N_2:CO_2:H_2 at 8:1:1).

All amino acids used in this study were L-enantiomers, except for homocysteine. DL-homocysteine was obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. L-cystathionine was purchased from MilliporeSigma, St. Louis, MO, USA. Other chemicals used in this study were of analytical grade and commercially available.

According to previous reports, the nutrient requirements of bifidobacteria vary. For example, *B. longum* 105-A reportedly requires isoleucine and tyrosine in addition to cysteine [5]. In fact, several bifidobacterial strains did not grow in the BMM+Cys used in this study (data not shown). All nine strains tested showed no growth or very slight growth in BMM-S medium, which contained no sulfur-containing amino acids (Table 1). This result confirms the previous reports that bifidobacteria cannot synthesize sulfur-containing amino acids from sulfate ion or other forms of sulfur molecule.

All nine strains grew in BMM+Cys, and Dunnett's test, with the BMM-S as the control group, was used to divide them into two groups according to maximum OD_{660} value: strains in Group 1 grew both in BMM+Cys and BMM+Met, and strains in Group 2 grew only in BMM+Cys (Table 1). Among the nine strains tested, four strains, *i.e.*, *B. longum* 105-A, *Bifidobacterium breve* JCM 1192, *Bifidobacterium longum* subsp. *suis* JCM 1269, and *B. longum* subsp. *longum* JCM 1217, showed significant growth in BMM+Met medium. On the other hand, five strains, *i.e., Bifidobacterium animalis* subsp. *lactis* JCM 10602, *Bifidobacterium animalis* subsp. *animalis* JCM 1190, *Bifidobacterium pseudocatenulatum* JCM 1200, *Bifidobacterium longum* subsp. *infantis* JCM 1222, and *B. longum* subsp. *longum* JCM 7050, did not grow significantly in BMM+Met medium. Even though one strain, *B. animalis* subsp. *lactis* JCM 10602, showed modest growth in BMM+Met medium, its growth was not statistically significant. The growth profile of *B. longum* 105-A is shown in Fig. 2. Its initial growth rate in BMM+Met medium

Table 1. Maximum growth of bifidobacteria strains cultured in 1/2MRSCS, BMM-S, BMM+Cys, and BMM+Met

Strains	Maximum Growth (OD_{660})			
	1/2MRSCS	BMM-S	BMM+Cys	BMM+Met
Group 1				
B. longum subsp. *longum* 105-A	4.08	0.088 ± 0.009^a	$0.884 \pm 0.024^{b/*}$	$0.787 \pm 0.016^{b/*}$
B. breve JCM1192	3.12	0.133 ± 0.010^a	$0.909 \pm 0.097^{b/*}$	$0.448 \pm 0.140^{b/*}$
B. longum subsp. *suis* JCM1269	4.20	0.112 ± 0.005^a	$0.443 \pm 0.073^{/*}$	$0.315 \pm 0.082^{b/*}$
B. longum subsp. *longum* JCM1217	3.81	0.066 ± 0.001^a	$0.329 \pm 0.095^{b/*}$	$0.254 \pm 0.035^{ab/*}$
Group 2				
B. animalis subsp. *lactis* JCM10602	3.35	0.088 ± 0.004^a	$1.28 \pm 0.547^{b/*}$	0.531 ± 0.158^{ab}
B. animalis subsp. *animalis* JCM1190	3.10	0.055 ± 0.010^a	$0.612 \pm 0.280^{b/*}$	0.150 ± 0.055^a
B. pseudocatenulatum JCM1200	4.34	0.071 ± 0.001^a	$1.83 \pm 0.112^{b/*}$	0.123 ± 0.021^a
B. longum subsp. *infantis* JCM1222	5.90	0.100 ± 0.009^a	$0.892 \pm 0.133^{b/*}$	0.098 ± 0.019^a
B. longum subsp. *longum* JCM7050	4.90	0.040 ± 0.002^a	$0.601 \pm 0.038^{b/*}$	0.054 ± 0.006^a

Values are presented as means ± standard deviations (n=3). An asterisk (*) indicates a significant difference compared with BMM-S by Dunnett's test ($p<0.05$). Different superscript letters (a or b) indicate a significant difference by Tukey's test ($p<0.05$).

was slower compared with that in BMM+Cys medium; however, the OD_{660} value reached the same level after 24 hr in both media.

These results clearly indicate that some bifidobacterial strains can utilize methionine as the sole sulfur-containing amino acid source and that these strains are not cysteine auxotrophs. The four strains that grew in BMM+Met may possess the reverse transsulfuration pathway, which would enable them to utilize methionine as the sole sulfur-containing amino acid source, similar to other bacteria [6–8].

The relationship between taxonomy and methionine utilization ability was inconsistent. For example, both *B. longum* 105-A and *B. longum* JCM 7050 belong to "*B. longum* subsp. *longum*" [10]; however, 105-A can utilize methionine as its sole sulfur source, whereas JCM 7050 cannot.

In order to confirm the utilization of sulfur-containing amino acids other than cysteine and methionine, the assimilation of homocysteine and cystathionine by *B. longum* 105-A was tested using BMM supplemented with DL-homocysteine and L-cystathionine. *B. longum* 105-A showed growth in both BMM+homocysteine and BMM+cystathionine that was comparable to that in BMM+Met. The maximum OD_{660} values reached 0.746 and 0.565, respectively. These are average values from two independent determinations, which gave similar results (*n*=2).

These results indicated that *B. longum* 105-A can utilize both homocysteine and cystathionine as a sole sulfur-containing amino acid source and that *B. longum* 105-A possesses both SAM cycle enzymes and enzymes for the last two steps of the reverse transsulfuration pathway (Fig. 1), *i.e.*, cystathionine β-synthase (homocysteine to cystathionine) and cystathionine γ-lyase (cystathionine to cysteine). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/pathway.html), *B. longum* 105-A has conserved all of the genes encoding its SAM cycle enzymes, *metK*, *dcm1*, *pfs*, *luxS*, and *ahcY* (Fig. 1). Taking this information into account, methionine may be converted to homocysteine *via* the SAM cycle in *B. longum* 105-A, with homocysteine subsequently converted to cysteine by cystathionine β-synthase and cystathionine γ-lyase (Fig. 1), even though the genes encoding cystathionine β-synthase and cystathionine γ-lyase have not yet been identified in bifidobacteria. Thus, *B. longum* 105-A most probably utilize

methionine through the reverse transsulfuration pathway, similar to other bacteria [6–8].

The methionine-utilizing pathways of strains other than *B. longum* 105-A in Group 1 is still unknown. For example, according to the KEGG pathway database, *B. breve* JCM1192 lacks some SAM cycle enzyme genes, such as *dcm1* and *ahcY* (Fig. 1). *B. breve* JCM1192, however, can utilize methionine as the only sulfur-containing amino acid (Table 1). Several explanations are possible for this phenomenon: i) one possible explanation is that the genes encoding SAM cycle enzymes in *B. breve* JCM1192 are not annotated correctly in the database; ii) another possibility is that an unknown pathway from methionine to cysteine other than the normal reverse transsulfuration pathway exists in *B. breve* JCM1192. Utilization tests of homocysteine and cystathionine using strains other than *B. longum* 105-A will help to further elucidate this phenomenon. If other strains in Group 1 can grow both in BMM+homocysteine and BMM+cystathionine, as in the case of *B. longum* 105-A, it would indicate that these strains can synthesize cysteine and methionine from these intermediates. Also, it would indicate that these strains probably have a reverse transsulfuration pathway as observed in other bacteria, because no metabolic pathway from methionine to homocysteine other than the SAM cycle has been reported to date. The discrepancy between the existence of predicted genes in the database and actual methionine utilization, however, remains to be clarified.

This is the first report showing that some bifidobacterial strains can utilize methionine as the sole sulfur-containing amino acid source and that cysteine is not essential for all bifidobacterial strains. In addition, homocysteine and cystathionine utilization tests strongly suggest that *B. longum* 105-A possesses a reverse transsulfuration pathway, similar to other bacteria. The whole genome sequence of *B. longum* 105-A has been analyzed [9], and a computational similarity search revealed that 105-A has putative genes of the reverse transsulfuration pathway, such as *ahcY* and *luxS* (Fig. 1). In *Helicobacter pylori, luxS* plays a main role in the transsulfuration pathway [12]. The detailed mechanism of the transsulfuration pathway of *B. longum* 105-A, however, has yet to be elucidated. A gene inactivation experiment using a targeted gene deletion system developed by our research group [11] is currently underway to clarify the detailed mechanism of methionine assimilation in *B. longum* 105-A.

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