Comparative analysis of probiotic bacteria based on a new definition of core genome

Maria Satti^{*,¶}, Yasuhiro Tanizawa^{†,||}, Akihito Endo^{‡,***} and Masanori Arita^{†,§,††}

^{*}*Department of Genetics, School of Life Science
SOKENDAI (The Graduate University for Advanced Studies)
Yata 1111, Mishima, Shizuoka 411-8540, Japan*

[†]*Center for Information Biology, National Institute of Genetics
Yata 1111, Mishima, Shizuoka 411-8540, Japan*

[‡]*Department of Food and Cosmetic Science, Faculty of Bioindustry
Tokyo University of Agriculture, 196 Yasaka
Abashiri, Hokkaido 099-2493, Japan*

[§]*RIKEN Center for Sustainable Resource Science
1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama
Kanagawa 230-0045, Japan*

[¶]*msatti@nig.ac.jp*

^{||}*ytanizaw@nig.ac.jp*

^{***}*a3endou@bioindustry.nodai.ac.jp*

^{††}*arita@nig.ac.jp*

Received 30 November 2017

Revised 30 November 2017

Accepted 20 March 2018

Published 23 May 2018

The commensal genus *Bifidobacterium* has probiotic properties. We prepared a public library of the gene functions of the genus *Bifidobacterium* for its online annotation. Orthologous gene cluster analysis showed that the pan genomes of *Bifidobacterium* and *Lactobacillus* exhibit striking similarities when mapped to the Clusters of Orthologous Group (COG) database of proteins. When the core genes in each genus were selected based on our statistical definition of “core genome”, core genes were present in at least 92% of 52 *Bifidobacterium* and in 97% of 178 *Lactobacillus* genomes. Functional comparison of the core genes of the two genera revealed a significant difference in the categories “amino acid transport and metabolism” representing their difference in niche specificity. Over-represented *Bifidobacterium* protein families were primarily involved in host interactions, the complex compound metabolism, and in stress responses. These findings coincide with the published information and validate our bias-resilient definition of the core genome.

Keywords: Comparative genomics; metabolism; probiotics; pan-genome; core-genome.

[¶]Corresponding author.

This is an Open Access article published by World Scientific Publishing Company. It is distributed under the terms of the Creative Commons Attribution 4.0 (CC-BY) License. Further distribution of this work is permitted, provided the original work is properly cited.

1. Introduction

The *Bifidobacterium* was first isolated in 1899 by Henri Tissier from the feces of healthy breast-fed infants.¹ It was initially named *Bacillus bifidus* (“divided into two parts” for its Y-shape), then *Lactobacillus bifidus*, and in 1924, Orla-Jensen categorized it into an independent genus *Bifidobacterium*. The genus now represents Gram-positive, immotile, non-gas-producing, obligatory anaerobic, and Y-shaped bacteria.^{1,2}

Bifidobacterium species have been isolated from various ecological niches. In human, they are present in gastrointestinal tract (GIT) and oral cavity. They are the dominant clade of the gut microbiota of healthy breast-fed infants,³ and commensal species are considered important for microbial modulation at birth, e.g. the immune programming of its host.^{4,5} Most members of the genus are safe and nontoxic in humans. Bifidobacteria are generally host-specific and separated into human-, animal-, and insect-types. Human-type bifidobacteria are further classified as infant- (i.e. *B. bifidum* and *B. breve*) and adult-type (i.e. *B. adolescentis* and *B. catenulatum*). Certain species of bifidobacteria are found in environmental niches such as sewage, fermented products, and anaerobic digesters.⁶ Animal guts are suspected as the major source of these contaminants.

Earlier studies demonstrated that administration of certain species/strains of bifidobacteria elicits beneficial effects on host health; they include alleviation of atopic dermatitis and allergies, immune modulation, and shortening of diarrhea.^{7,8} Consequently, certain species/strains of the genus *Bifidobacterium* are used commercially as probiotics in the food industry, especially in dairy products. The genus is well adapted to the gut due to its ability to utilize host-derived oligosaccharides with sialic acid and human milk oligosaccharides (HMOs).⁹ Its characteristic metabolism is a pathway called the “bifidus shunt”, which can produce 5 adenosine triphosphates (ATPs) per 2 glucose molecules (standard glycolysis produces 2 ATPs per 1 glucose) via fructose-6-phosphate phosphoketolase (EC 4.1.2.2).¹⁰ More than 8% of its total genes are involved in the carbohydrate metabolism.¹¹ However, the specificity or particularity of these pathways remains to be investigated.

Large-scale sequencing can reveal the genomic characteristics of probiotic bacteria. The first complete genome in *Bifidobacterium* was reported for *B. longum* subsp. *longum* NCC2705.¹² Subsequent comparative studies involved as many as 52 taxa comprised of 43 species and 9 subspecies.^{13–16} We now provide an accurate and consistent genomic annotation for this beneficial genus and compare its probiotic characteristics with the commensal probiotic genus, *Lactobacillus*. Our reference annotation library is freely available on our web annotation server. We also propose a new definition of the “core genome” and reveal genus-specific metabolic functions for *Bifidobacterium* and *Lactobacillus*.

From a bioinformatics perspective, our proposal for the “core genome” is an important step forward. Traditionally, its definition was “the set of genes included in all genomes” under investigation. Based on this definition, the size of the core genome decreases as the number of sequenced genomes increases.¹⁶ This data-dependent

definition raises two problems: how to estimate the number of genomes required for determining the core genome, and how to identify the core genome among the obtained sequences. The first problem is solved easily. When the number of completely shared genes is plotted against the number of species, it drops sharply until the number of species is around 10; it then decreases very slowly as the number of species increases. This empirical knowledge implies that the core genome should be estimated from 10–20 genomes. It is more difficult to solve the second problem: how to identify the core genes in these genomes. If we posit that the core should be shared among all genomes, then genes for ribosomes and translation are over-represented. On the other hand, if we posit that conservation in 80% of the genomes delineates the core, spurious genes may slip in. Our simple criterion is to use the Clusters of Orthologous Group (COG) database to identify a stable core. This bias-resilient method is useful for many activities such as creating genome models, assessing type strains, and characterizing bacterial genus, especially when its member species contain very different genomes.

2. Methods

2.1. Construction of the *Bifidobacterium* gene library

Consistent genome annotation requires a reference library (pairs of sequences and their functional annotation) that is compatible with the published complete genome annotations. To prepare the library, we collected the complete genomes of 67 *Bifidobacterium*, 8 lactic acid bacteria strains and *Bacillus subtilis* contained in the NCBI Assembly Database (Table 1). The full genome sequences in the current study are available from GenBank. The protein sequences for all the genomes were extracted and subjected to orthologous clustering. Orthologous clusters were generated using GET_HOMOLOGUES software.¹⁷ The parameters for clustering were: *E*-value threshold of 10^{-5} , minimum percentage coverage of 75%, and Markov clustering algorithm using normalized BLAST scores (OrthoMCL).¹⁸ A total of 144,028 identified protein sequences were grouped into 21,255 clusters. The 12,545 singletons were discarded and the remaining 8710 clusters were analyzed. To obtain their protein names, gene symbols, and EC numbers, we referred to our existing library for *Lactobacillus*.¹⁹ Among the 8710 clusters, we identified the functions of 6697 clusters. This indicates that close to 80% of the shared genes in *Bifidobacterium* have orthologs in *Lactobacillus*. The functions of the remaining 2013 gene clusters were sought manually by referencing the NCBI Conserved Domain Database (NCBI-CDD) using Reverse Position-Specific BLAST. This yielded that 15 *Bifidobacterium* genomes were close duplicates, and they were excluded from further analysis. The remaining 52 genomes were analyzed. Independently, the 45,038 gene clusters in 178 *Lactobacillus* genomes were obtained from our DAGA library.¹⁹ A newly sequenced *Bifidobacterium* can be easily annotated through this system at <https://dfast.nig.ac.jp/>.

Table 1. The list of genomic sequences used for constructing the reference library.

Accession	Bioproject	Biosample	TaxID	Organism
GCA_000007525.1	PRJNA328	SAMN02603675	206672	<i>B. longum</i> NCC2705
GCA_000008945.1	PRJNA18773	SAMN02603512	205913	<i>B. longum</i> DJO10A
GCA_000010425.1	PRJNA16321	SAMD00061080	367928	<i>B. adolescentis</i> ATCC 15703
GCA_000020425.1	PRJNA17189	SAMN02598380	391904	<i>B. longum</i> subsp. <i>infantis</i> ATCC 1569 = JCM 1222 = DSM 20088
GCA_000021425.1	PRJNA19423	SAMN02603485	442563	<i>B. animalis</i> subsp. <i>lactis</i> AD011
GCA_000022705.1	PRJNA32897	SAMN02603172	580050	<i>B. animalis</i> subsp. <i>lactis</i> Bl-04
GCA_000022965.1	PRJNA32893	SAMN02603171	555970	<i>B. animalis</i> subsp. <i>lactis</i> DSM 10140
GCA_000024445.1	PRJNA17583	SAMN02603177	401473	<i>B. dentium</i> Bd1
GCA_000025245.1	PRJNA42883	SAMN02603131	552531	<i>B. animalis</i> subsp. <i>lactis</i> BB-12
GCA_000092325.1	PRJNA47579	SAMN02603181	759350	<i>B. longum</i> subsp. <i>longum</i> JDM301
GCA_000092765.1	PRJNA32515	SAMN02603934	573236	<i>B. animalis</i> subsp. <i>lactis</i> V9
GCA_000164965.1	PRJNA51963	SAMN02603395	883062	<i>B. bifidum</i> S17
GCA_000165905.1	PRJNA42863	SAMN02603510	702459	<i>B. bifidum</i> PRL2010
GCA_000166315.1	PRJNA53143	SAMN02603469	890402	<i>B. longum</i> subsp. <i>longum</i> BBMN68
GCA_000196555.1	PRJDA32047	SAMD00060951	565042	<i>B. longum</i> subsp. <i>longum</i> JCM 1217
GCA_000196575.1	PRJDA32051	SAMD00060953	565040	<i>B. longum</i> subsp. <i>infantis</i> 157F
GCA_000210755.1	PRJNA45963	SAMEA3138379	722911	<i>B. longum</i> subsp. <i>longum</i> F8
GCA_000213865.1	PRJNA51077	SAMN00100758	866777	<i>B. breve</i> ACS-071-V-Sch8b
GCA_000219455.1	PRJNA66401	SAMN02603656	1035817	<i>B. longum</i> subsp. <i>longum</i> KACC 91563
GCA_000220135.1	PRJNA13487	SAMN02604112	326426	<i>B. breve</i> UCC2003
GCA_000220885.1	PRJNA67865	SAMN02604350	1042403	<i>B. animalis</i> subsp. <i>lactis</i> CNCM I-2494
GCA_000224965.2	PRJNA71815	SAMN02604239	1075106	<i>B. animalis</i> subsp. <i>lactis</i> BLC1
GCA_000265095.1	PRJNA21077	SAMN02603486	484020	<i>B. bifidum</i> BGN4
GCA_000269965.1	PRJDA32049	SAMD00060952	391904	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697 = JCM 1222 = DSM 20088
GCA_000277325.1	PRJNA156973	SAMN02603214	1168290	<i>B. animalis</i> subsp. <i>lactis</i> B420
GCA_000277345.1	PRJNA156975	SAMN02603215	742729	<i>B. animalis</i> subsp. <i>lactis</i> Bi-07
GCA_000304215.1	PRJNA83029	SAMN02604114	1147128	<i>B. asteroides</i> PRL2011
GCA_000347695.1	PRJNA179141	SAMN02603226	1254439	<i>B. thermophilum</i> RBL67
GCA_000414215.1	PRJNA186412	SAMN02604238	1281781	<i>B. animalis</i> subsp. <i>lactis</i> Bl12
GCA_000568955.1	PRJNA214949	SAMN03081478	1385938	<i>B. breve</i> 12L
GCA_000568975.1	PRJNA214950	SAMN03081479	1385939	<i>B. breve</i> JCM 7017
GCA_000569015.1	PRJNA214951	SAMN03081480	1385940	<i>B. breve</i> JCM 7019
GCA_000569035.1	PRJNA214954	SAMN03081481	1385941	<i>B. breve</i> NCFB 2258
GCA_000569055.1	PRJNA214955	SAMN03081482	1385942	<i>B. breve</i> 689b
GCA_000569075.1	PRJNA214956	SAMN03081483	936351	<i>B. breve</i> S27
GCA_000695895.1	PRJNA245929	SAMN02797738	28025	<i>B. animalis</i>
GCA_000706765.1	PRJNA206546	SAMN05771123	1341694	<i>B. indicum</i> LMG 11587 = DSM 20214
GCA_000730205.1	PRJNA251950	SAMN02843216	216816	<i>B. longum</i>
GCA_000737865.1	PRJNA239194	SAMN02666223	1687	<i>B. coryneforme</i>
GCA_000737885.1	PRJNA240293	SAMN02673695	1680	<i>B. adolescentis</i>
GCA_000772485.1	PRJNA191592	SAMN03093230	1300227	<i>B. longum</i> subsp. <i>longum</i> GT15
GCA_000800455.1	PRJNA234365	SAMN03253090	1447716	<i>B. kashiwanohense</i> PV20-2
GCA_000800475.2	PRJNA234364	SAMN03253091	1447715	<i>B. pseudolongum</i> PV8-2
GCA_000816205.1	PRJNA225662	SAMN02726251	1316911	<i>B. animalis</i> subsp. <i>lactis</i> KLDS2.0603
GCA_000817045.1	PRJNA271239	SAMN03273367	28025	<i>B. animalis</i>
GCA_000817995.1	PRJNA271241	SAMN03273368	1680	<i>B. adolescentis</i>
GCA_000818055.1	PRJNA252546	SAMN02941905	302911	<i>B. animalis</i> subsp. <i>lactis</i>
GCA_000829295.1	PRJDB3123	SAMD00019943	216816	<i>B. longum</i>
GCA_000966445.2	PRJNA278963	SAMN03435124	1683	<i>B. angulatum</i>
GCA_001025135.1	PRJDB56	SAMD00061040	500634	<i>B. bifidum</i> ATCC 29521 = JCM 1255 = DSM 20456
GCA_001025155.1	PRJDB55	SAMD00061039	518635	<i>B. angulatum</i> DSM 20098 = JCM 7096
GCA_001025175.1	PRJDB57	SAMD00061041	518634	<i>B. breve</i> DSM 20213 = JCM 1192

Table 1. (Continued)

Accession	Bioproject	Biosample	TaxID	Organism
GCA_001025195.1	PRJDB58	SAMD00061042	566552	<i>B. catenulatum</i> DSM 16992 = JCM 1194 = LMG 11043
GCA_001025215.1	PRJDB61	SAMD00061045	547043	<i>B. pseudocatenulatum</i> DSM 20438 = JCM 1200 = LMG 10505
GCA_001042595.1	PRJDB59	SAMD00061043	1150423	<i>B. dentium</i> JCM 1195 = DSM 20436
GCA_001042615.1	PRJDB60	SAMD00061044	1150460	<i>B. kashiwanohense</i> JCM 15439 = DSM 21854
GCA_001042635.1	PRJDB62	SAMD00061046	1150461	<i>B. scardovii</i> JCM 12489 = DSM 13734
GCA_001263395.1	PRJNA286033	SAMN02442035	1437605	<i>B. actinocoloniiforme</i> DSM 22766
GCA_001281305.1	PRJNA270905	SAMN03271683	1682	<i>B. longum</i> subsp. <i>infantis</i>
GCA_001281345.1	PRJNA270902	SAMN03271680	1681	<i>B. bifidum</i>
GCA_001293145.1	PRJNA270904	SAMN03271682	216816	<i>B. longum</i>
GCA_001446255.1	PRJNA287223	SAMN03785818	1679	<i>B. longum</i> subsp. <i>longum</i>
GCA_001446275.1	PRJNA287222	SAMN03785819	1679	<i>B. longum</i> subsp. <i>longum</i>
GCA_001688645.1	PRJNA317592	SAMN04621611	302912	<i>B. animalis</i> subsp. <i>animalis</i> s
GCA_001719085.1	PRJNA301703	SAMN04254466	216816	<i>B. longum</i>
GCA_001725985.1	PRJNA316119	SAMN04576213	1679	<i>B. longum</i> subsp. <i>longum</i>
GCA_000006865.1	PRJNA72	SAMN02603339	272623	<i>Lactococcus lactis</i> subsp. <i>lactis</i> II 1403
GCA_000007785.1	PRJNA70	SAMN02603978	226185	<i>Enterococcus faecalis</i> V583
GCA_000008925.1	PRJNA13280	SAMN02604111	362948	<i>Lactobacillus salivarius</i> UCC118
GCA_000011985.1	PRJNA82	SAMN02603047	272621	<i>Lactobacillus acidophilus</i> NCFM
GCA_000203855.3	PRJNA356	SAMEA3138345	220668	<i>Lactobacillus plantarum</i> WCFS1
GCA_000174395.2	PRJNA30627	SAMN00002237	333849	<i>Enterococcus faecium</i> DO
GCA_000006785.2	PRJNA269	SAMN02604089	160490	<i>Streptococcus pyogenes</i> M1 GAS
GCA_000014525.1	PRJNA402	SAMN02598528	321967	<i>Lactobacillus paracasei</i> ATCC 334
GCA_000009045.1	PRJNA76	SAMEA3138188	224308	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168

2.2. Pan- and core-genome computation and COG assignment

For pan- and core-genome analysis, we used COG functional categories. Gene clusters in the 52 *Bifidobacterium* and 178 *Lactobacillus* genomes were queried against NCBI-CDD using Reverse Position-Specific BLAST; the COG category was assigned with Perl script “cdd2cog” available at <https://github.com/aleimba/bac-genomics-scripts/tree/master/cdd2cog>. The entire cluster set represents the pan genome.

A commonly used definition of the “core” genome includes only genes that are present in all genomes. By this definition, the number of core genes constantly decreases as the number of genomes grow. Our concept of “*n*-core” entails the set of genes that is conserved in *n* percent of the complete genomes. For example, in *Bifidobacterium*, the designation “100-core”, “98-core”, “96-core”, . . . , “83-core” indicates the set of genes conserved in all 52-, 51-, 50-, . . . , and 43 of the 52 genomes. We created 10 such *n*-cores (from 100- to 83-core). Each *n*-core exhibits a different distribution of 26 COG functional categories (the COG ratio) because the number of shared genes increased as *n* decreased (Table 2). For example, “translation and ribosomal structure and biogenesis” category (J) is the 5th largest group in the 96-core, but it became the second in the 98-core, and the largest in the 100-core because as many as 27 and 76 genes in J are shared among 50 and 51 genomes, respectively (Table 2). Given a set of genomes, we want to choose an appropriate *n*-core that

Table 2. Rank (rows) and number of different COG functional categories (cells) in the 10 *n*-cores (columns) of *Bifidobacterium* (Color coding: dark: 10 or more gene additions; gray: 10 > and >= 5 gene additions; light gray: 5 > and >= 2 gene additions. Some gene numbers are presented as fractions because the same gene may fall in multiple COG categories).

100-core (52)	98-core (51)	96-core (50)	94-core (49)	92-core (48)	90-core (47)	88-core (46)	87-core (45)	85-core (44)	83-core (43)
J 76	E 29.5	- 16	E 16.8	- 8	K 5.5	G 9.3	G 5	G 9.3	G 3
L 25.5	J 27	E 14.5	M 6	J 5.5	G 3	I 4	S 5	R 2	V 2
R 24.6	R 19.5	R 10.5	G 6	P 5.5	S 3	G 3	E 4.3	S 2	K 2
O 21.5	- 15	L 9.5	C 5	R 5	R 2.5	P 3	F 3	- 2	M 1
G 21.3	S 13	J 8	R 4.5	L 4	V 2	R 3	R 3	D 1	O 1
- 21	L 12.8	F 7.5	I 4.3	- 4	E 2	- 3	H 2.5	M 1	E 1
M 20.5	G 11	P 6	F 4	T 3	F 2	M 2	K 2	J 1	F 1
K 19	C 9	K 5	P 3.5	K 3	O 1.5	J 1.5	I 2	C 1	P 1
E 19	M 7.5	S 5	J 3	S 3	L 1.5	E 1.5	- 2	F 1	R 1
F 17.3	F 7.5	H 4.5	S 3	F 2.5	H 1.5	F 1.5	L 1	E 0.5	S 1
C 16.8	O 7	G 3	- 3	M 2	P 1.5	D 1	P 1	H 0.5	D 0
T 12	I 6.3	T 2.5	T 2.5	O 2	D 1	T 1	M 0.5	N 0	N 0
H 10	T 6	C 2.2	D 2	C 2	M 1	V 1	N 0.5	O 0	T 0
P 9	K 5.5	M 2	O 2	G 2	T 1	C 1	U 0.5	T 0	U 0
S 9	U 5	I 2	H 1.8	D 1	J 1	A 0.5	J 0.3	U 0	W 0
I 7	P 4.5	D 1.5	K 1.5	H 1	I 1	L 0.5	D 0	V 0	Y 0
D 6	D 4	O 1	U 1	N 0	N 0.5	N 0	O 0	W 0	Z 0
U 4.5	H 3.3	V 1	A 0.5	U 0	A 0.5	O 0	T 0	Y 0	A 0
A 3.8	V 2	N 0.5	L 0.5	V 0	K 0.5	U 0	V 0	Z 0	B 0
V 3	B 0.5	U 0.5	N 0	W 0	U 0	W 0	W 0	A 0	J 0
B 2.3	N 0	A 0.5	V 0	Y 0	W 0	Y 0	Y 0	B 0	L 0
Q 1	W 0	W 0	W 0	Z 0	Y 0	Z 0	Z 0	K 0	C 0
N 0.5	Y 0	Y 0	Y 0	A 0	Z 0	B 0	A 0	L 0	H 0
Y 0.5	Z 0	Z 0	Z 0	B 0	B 0	H 0	B 0	I 0	I 0
W 0	A 0	B 0	B 0	I 0	C 0	Q 0	C 0	P 0	Q 0
Z 0	Q 0	Q 0	Q 0	Q 0	Q 0	S 0	Q 0	Q 0	- 0

avoids inappropriate deletions or inclusions. To choose the best *n* value, we first created “consensus COG ordering” based on the majority rule whereby all COG categories were ordered according to their abundance in each *n*-core and assigned their ranks. Then, the COG categories were reordered by their average rank in 10 *n*-cores. We designated this COG ordering, computed from the averages, “consensus COG ordering”. Next, each *n*-core was compared against the consensus COG ordering using the Spearman’s rank correlation coefficient, and the closest core was chosen. In *Bifidobacterium*, 773 genes present in at least 48 of the 52 genomes (92-core) were selected as the consensus core. In *Lactobacillus*, 472 genes present in at least 172 of the 178 genomes (97-core) were identified as the consensus core.

2.3. Odds ratio (OR) and p-value of genus-specific functions

Statistical analysis was performed to evaluate the relative abundance of protein families in *Bifidobacterium* and *Lactobacillus* species. To identify over- and under-represented functional categories in both species, we calculated the OR using a two-by-two contingency table. It included four parameters based on the number of protein families that were (a) present or (b) absent in the respective COG category of *Bifidobacterium*, and (c) present or (d) absent in the respective COG category of *Lactobacillus*. The formula for calculating the OR was ad/bc. This value is also regarded as an approximated risk value, since the number of genes in each COG

category is much smaller than the total. From the OR, we calculated the 95% confidence interval (CI) as follows:

$$\begin{aligned} &\exp [\ln(\text{OR}) + 1.96 \sqrt{(1/a + 1/b + 1/c + 1/d)}] \quad \text{for the upper limit, and} \\ &\exp [\ln(\text{OR}) - 1.96 \sqrt{(1/a + 1/b + 1/c + 1/d)}] \quad \text{for the lower limit.} \end{aligned}$$

When D is the difference in the natural log (ln) of the upper and the lower limits, the standard error (SE) is computed as $D/(2 * 1.96)$, the z score was $\ln(\text{OR})/\text{SE}$. The corresponding p -value was obtained with R function $2 * \text{pnorm}(-\text{abs}(z \text{ score}))$.

2.4. Assignment of carbohydrate-active enzymes

Carbohydrate-active enzymes were identified based on their similarity on the Carbohydrate Active Enzymes (CAZy) database. The online tools CAZymes Analysis Toolkit (CAT) and dbCAN were applied manually.^{20,21}

3. Results and Discussion

3.1. General genomic features of the genera *Bifidobacterium* and *Lactobacillus*

The genome sequences of 52 (sub) species of *Bifidobacterium* and 178 (sub) species of *Lactobacillus* were retrieved from the NCBI Assembly database. The genome size of *Bifidobacterium* varied from 1.6 Mb for *B. commune* to 3.3 Mb for *B. biavatii* (Fig. 1(a)). The approximate G + C content ranged from 53–66% (Fig. 1(b)) and the approximate number of coding sequences (CDS) from 1200–2500 (Fig. 1(c)). The genome size of *Lactobacillus* ranged from 1.2 Mb for *L. sanfranciscensis* to 3.7 Mb for *L. pentosus* (Fig. 2(a)). The G + C content of *Lactobacillus* was lower than that of *Bifidobacterium*, it ranged from 32–57% (Fig. 2(b)).

Fig. 1. Genomic features of genus *Bifidobacterium*.

Fig. 2. Genomic features of genus *Lactobacillus*.

Phylogenetically, the two genera are distant. *Lactobacillus*, a low G + C bacillus, belongs to the phylum Firmicutes, whereas *Bifidobacterium* belongs to the phylum Actinobacteria. However, they share a common niche habitat (animal gut), energy metabolism (lactate fermentation), and both are used industrially as probiotic species. Genome-scale comparison will reveal their metabolic similarities.

3.2. COG comparison of the pan- and core-genomes of *Bifidobacterium* and *Lactobacillus*

The pan- and core-genomes of the 52 *Bifidobacterium* and the 178 *Lactobacillus* were determined as described in Sec. 2. The pan genome of *Bifidobacterium* and *Lactobacillus* included 16,232 and 45,038 gene clusters, respectively. Functional categories for all gene clusters of both genera were assigned according to the COG classification. Almost 30% of the pan genomes (9283 and 26,944 gene clusters in *Bifidobacterium* and *Lactobacillus*, respectively) remained functionally unknown and assigned to no COG category (Fig. 3). This is common to many other bacteria; close to 30% of the genes of *Escherichia coli* are functionally unknown.

The overall COG classification of the pan genomes of the two distant genera was strikingly similar. In both, the highest fractions were identical as was the ratio of categories. Slightly different in *Bifidobacterium* and *Lactobacillus* were the “amino acid transport and metabolism” (E: 7% versus 5%) and “carbohydrate transport and metabolism” (G: 8% versus 6%).

The COG ratios of the core genomes were quite different. Figure 4 is a pie chart of the 92-core for *Bifidobacterium* and the 97-core for *Lactobacillus*. In the core genome of *Bifidobacterium*, more metabolism-related genes, especially (E), and in *Lactobacillus* more information storage and processing genes, especially “translation, ribosomal structure, and biogenesis” (J), are enriched. In the *Bifidobacterium* core

Fig. 3. COG statistics of the pan genome of *Bifidobacterium* and *Lactobacillus*.

genome, (G) is lower than in its pan genome. This indicates that carbohydrate genes differ from one another within the same genus.

3.3. Statistical background of consensus COG ordering

Although the difference between pan- and core-genomes depends on the definition of “the core”, there is currently no straightforward definition of the core genome. No particular strain necessarily reflects the core. The core genome should be an ideal set of genes that represent the genus. Neither the 100-core nor the 80-core reflects the true core because the strict criterion applied to the former filters out too many genes while in the latter, the threshold is too relaxed, allowing the inclusion of many auxiliary genes. Consequently, the ideal core lies in the middle. To justify our definition of the core genome, we show the trend of the size difference (the number of genes) in each COG category (Table 2). As *n* decreases from 100, the size of the core genes increases. Each *n*-core occupies a position in the 26-dimensional space (COG functional categories, *n* = 26), where the value on each axis is its rank, no two axes share the same value. We want to find the optimal position in this space

Fig. 4. COG statistics of the core genome of *Bifidobacterium* and *Lactobacillus*.

(i.e. choosing n) based on a limited number of samplings of n -core ($100 \geq n \geq 83$). Under this problem formulation, the easiest way to estimate the optimal position is by computing the average rank for each axis independently with the proviso that the averages are not necessarily integers. By choosing the n -core whose rank best-correlates with the average, we can avoid the problem of indeterminate ranks. The remaining problem is the extent of sampling points. From a biological perspective, 83-core was an appropriate limit for stopping our sampling because thereafter, as shown in Table 2, the number of added genes became negligible.

3.4. Relative representation of core gene clusters in *Bifidobacterium*

For the rigorous identification of the over- and under-represented COG categories in the *Bifidobacterium* core, we computed the OR and the p -value. Among 777 COG categories analyzed, 359 (46%) were over-represented and 418 (54%) were under-represented in *Bifidobacterium* compared with *Lactobacillus*. In *Bifidobacterium*, 339 (OR = infinite) COG categories were present exclusively, while 143 (OR = 0) were absent exclusively. The COG database contains four major functional categories, and of these, “metabolism” was significantly over-represented in *Bifidobacterium*

Fig. 5. Over- and under-represented COG categories: (A) top four COG groups and (B) 25 COG subcategories.

($p < 0.0001$). “Cellular processes and signaling” and “poorly characterized” had $p < 0.0001$ and $p < 0.0053$, respectively. The under-representation of the last category “information storage and processing” was not significant (Fig. 5(a)).

The four major COG categories, “metabolism”, “cellular processes and signaling”, “information storage and processing” and “poorly characterized”, were further divided into 25 subcategories. Of these, 17 were under-represented and 8 were over-represented (Fig. 5(b)). Among the under-represented categories, J (translation, ribosomal structure, and biogenesis; $p < 0.0028$), L (replication, recombination, and repair; $p < 0.014$) and S (function unknown; $p < 0.0017$) were statistically significant (Table 3), while among the over-represented categories, only E (amino acid transport and metabolism) was significant ($p < 0.0001$). No other subcategory was statistically over- or under-represented.

Specific COG categories that were over-represented in *Bifidobacterium* relative to *Lactobacillus* mostly include COGs involved in host interaction, stress response, and complex-compounds metabolism (Table 4). In terms of gene multiplicity, glycosidases and galactosidases were multiply retained in many genomes. The table also suggests how *Bifidobacterium* is associated with its host and adapted to the GIT habitat. Many categories belonged to “carbohydrate transport or metabolism” (G) for competition and survival in the intestines. The presence of plasminogen-, and mucin-binding and of proteins involved in the formation of capsular or exo-polysaccharides (EPSs) is a characteristic of *Bifidobacterium*.²² Other over-represented COGs included a number of protein families that facilitate the adaptation to the adverse intestinal environment such as the bile and stress resistance. An important protein detected in the core was LuxS, which is involved in biofilm formation and helps *Bifidobacterium* in gut colonization and protection against pathogens.²³ Immuno-reactive proteins like aspartokinase and surface antigens that contact host cells and

Table 3. Significantly over- and under-represented COG categories (color coding: dark: significantly over-represented; gray: significantly under-represented).

Category	a	b	c	d	OR	p-value	95% CI
Cellular processes and signaling	145	628	100	372	0.86	0.296	0.64–1.14
Information storage and processing	246	527	205	267	0.61	$p < 0.0001$	0.47–0.77
Metabolism	310	463	121	351	1.94	$p < 0.0001$	1.51–2.49
Poorly characterized	106	667	93	379	0.65	0.0053	0.47–0.87
[C] Energy production and conversion	40	733	18	454	1.38	0.2707	0.77–2.43
[D] Cell cycle control, cell division, chromosome partitioning	15	758	11	461	0.83	0.641	0.37–1.82
[E] Amino acid transport and metabolism	98	675	5	467	13.56	$p < 0.0001$	5.47–33.56
[F] Nucleotide transport and metabolism	42	731	23	449	1.12	0.6664	0.66–1.89
[G] Carbohydrate transport and metabolism	47	726	23	449	1.26	0.3705	0.75–2.10
[H] Coenzyme transport and metabolism	25	748	16	456	0.95	0.8813	0.50–1.80
[I] Lipid transport and metabolism	25	748	20	452	0.76	0.359	0.41–1.37
[J] Translation, ribosomal structure, and biogenesis	124	649	108	364	0.66	0.0028	0.48–0.85
[K] Transcription	43	730	29	443	0.9	0.67	0.55–1.46
[L] Replication, recombination, and repair	62	711	58	414	0.62	0.014	0.42–0.90
[M] Cell wall/membrane/envelope biogenesis	40	733	33	439	0.73	0.1871	1.45–1.16
[N] Cell motility	2	771	4	468	0.3	0.1695	0.05–1.66
[O] Posttranslational modification, protein turnover, chaperones	35	738	22	450	0.97	0.9131	0.56–1.67
[P] Inorganic ion transport and metabolism	32	741	15	457	1.32	0.389	0.70–2.45
[Q] Secondary metabolites biosynthesis, transport, and catabolism	1	772	1	471	0.61	0.727	0.03–9.7
[R] General function prediction only	73	700	52	420	0.84	0.3706	0.57–1.22
[S] Function unknown	33	740	41	431	0.47	0.0017	0.29–0.75
[T] Signal transduction mechanisms	33	740	12	460	1.71	0.1172	0.87–3.34
[U] Intracellular trafficking, secretion, and vesicular transport	13	760	15	457	0.52	0.0893	0.24–1.10
[V] Defense mechanisms	6	767	2	470	1.84	0.457	0.36–9.14
[A] RNA processing and modification	10	763	5	467	1.22	0.7135	0.41–3.60
[B] Chromatin structure and dynamics	7	766	5	467	0.85	0.7878	0.26–2.70
[W] Extracellular structures	0	773	0	472	0.61	0.8054	0.01–30.83
[Y] Nuclear structure	1	772	1	471	0.61	0.727	0.03–9.77
[Z] Cytoskeleton	0	773	0	472	0.61	0.8054	0.01–30.83

modulate the immune response were also identified. There is experimental documentation of these proteins and of ABC-type sugar transporters and transaldolases in *Bifidobacterium*.²⁴ Without differential analysis, representative cores would become unknown or ribosomal functions (category — S, R, or J) (Fig. 4). Unbiased selection of these functions validates the appropriateness of our core definition and the importance of comparative analysis.

Table 4. Over-represented COG categories in *Bifidobacterium* (Redundancy refers to the total number with possible multiplicity in the same genome. Gene functions are based on the annotation in our reference library.).

COG Category	OR	Number of genomes	Redundancy	Function
[E] COG1113 Gamma-aminobutyrate permease and related permeases	infinite	48	48	Interaction of gut microbiota with the macroorganism
[E] COG0527 Aspartokinases	infinite	49	49	Immunoreactive proteins
[O] COG0265 Trypsin-like serine proteases	infinite	51	51	Involved in stress response, e.g. bile response
[G] COG3345 Alpha-galactosidase	infinite	49	67	Involved in complex carbohydrate metabolism (glycosyl hydrolases (GHs))
[M] COG1247 Sortase and related acyltransferases	infinite	49	49	Binding with host
[T] COG1854 LuxS protein involved in autoinducer AI2 synthesis	infinite	51	51	Biofilm formation and host colonization
[R] COG3942 Surface antigen	infinite	52	52	Immunomodulatory activity
[G] COG0021 Transketolase	infinite	52	53	Extracellular proteome
[G] COG0176 Transaldolase	infinite	52	52	Mucin-binding capability and aggregation factor
[G] COG0366 Glycosidases	infinite	51	72	Involved in complex carbohydrate metabolism (GHs)
[G] COG0166 Glucose-6-phosphate isomerase	infinite	52	53	Enzyme involve in <i>bifidus</i> pathway (glucose metabolism)
[E] COG0334 Glutamate dehydrogenase/leucine dehydrogenase	infinite	49	49	Plays central roles in nitrogen metabolism
[M] COG1215 Glycosyltransferases	infinite	52	52	Involved in EPS production
[G] COG0033 Phosphoglucomutase	infinite	52	52	Galactose metabolizing enzyme
[G] COG0580 Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family)	infinite	52	52	Sugar transport (non-PTS sugar transport system)
[GM] COG1134 ABC-type polysaccharide/polyol phosphate transport system	infinite	49	49	Sugar transport (non-PTS sugar transport system)
[GM] COG1682 ABC-type polysaccharide/polyol phosphate export systems	infinite	49	49	Sugar transport (non-PTS sugar transport system)
[G] COG3839 ABC-type sugar transport systems	infinite	52	52	Sugar transport (non-PTS sugar transport system)

It can be argued whether the core genes can be computed for any set of strains to, for example, identify host-specific gene pools. Sun *et al.* identified cytochrome *d* oxidases as the core of bee-specific *Bifidobacterium* and compared their list with other host-specific genes.²⁴ This approach is certainly allowed but for cross-species comparisons as in this study, a statistical criterion for the core is preferable. The relationship between our strategy and bacterial host specificity remains the future problem of our study.

3.5. Comparison of the carbohydrate metabolism in *Bifidobacterium* and *Lactobacillus*

An interesting application of the core in the two probiotic groups addressed the characteristics of the carbohydrate metabolism.²⁵ We manually investigated the carbohydrate-active enzymes encoded in the two genera and confirmed the presence of GHs, glycosyl transferases (GTs), carbohydrate esterases (CEs), polysaccharide lyases (PLs) and carbohydrate-binding modules (CBMs).

According to the CAZy system classification, the pan genome of *Bifidobacterium* included 69 GHs, 12 GTs, 6 CEs, 26 CBMs but no PLs; that of *Lactobacillus* included 48 GHs, 10 GTs, 6 CEs, 10 CBMs and 15 PLs. The number of analyzed genomes in *Bifidobacterium* is less than a third of that in *Lactobacillus*, and the number of carbohydrate-active enzymes in *Bifidobacterium* was larger except for PLs. The most common enzymes in *Bifidobacterium* were four types of GHs: GH43 for xylanase and arabinanase, GH25 for muramidase, GH3 for beta-glucosidase, and GH13 for amylase. On the other hand, in *Lactobacillus*, the most common enzymes were GH73 for peptidoglycan hydrolase, GH25 for muramidase and GH32 for fructan hydrolase. The characteristic types of GHs reflect the different types of oligosaccharides the bacterium can metabolize: *Bifidobacterium* and *Lactobacillus* digest animal- and plant-oriented sugars, respectively. While the absence of PLs in *Bifidobacterium* is noteworthy, there is evidence that it can metabolize polysaccharides containing uronic acids such as pectins and hemicelluloses.²⁶ As the polysaccharides derive from bacteria, plants, and animals, it is unlikely that *Bifidobacterium* cannot metabolize them. Details on the sugar metabolism in *Bifidobacterium* are under investigation in our laboratory.

4. Conclusions

We created a free, curated reference library for the genus *Bifidobacterium*. It facilitates the accurate and consistent annotation of newly sequenced *Bifidobacterium*. Our comparative analysis of the probiotic genera *Lactobacillus* and *Bifidobacterium* revealed their metabolic characteristics: protein families that were over-represented in *Bifidobacterium* were mostly involved in the complex sugar metabolism, in host interactions, and in stress responses. These functions were in good agreement with the published data. Our analysis also detected more niche-adjusted metabolic activities such as the broad adaptability to amino acids and the polysaccharide metabolism in *Bifidobacterium*. Although we document the relative absence of PLs, further analysis is required to elucidate its metabolic ability with respect to polysaccharides and the host-specificity of *Bifidobacterium*.

Acknowledgments

Computational analysis was performed on the NIG supercomputer at the Research Organization of Information and Systems (ROIS). The research and publication

costs for this work were supported by JSPS KAKENHI (15H05897, 17K19248, 26850054) and NIG JOINT (2017).

References

1. Lee JH, O'Sullivan DJ, Genomic insights into bifidobacteria, *Microbiol Mol Biol Rev* **74**(3):378–416, 2010.
2. Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D, Getting better with bifidobacteria, *J Appl Microbiol* **98**(6):1303–1315, 2005.
3. Ishibashi N, Yaeshima T, Hayasawa H, Bifidobacteria: Their significance in human intestinal health, *Mal J Nutr* **3**:149–159, 1997.
4. Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U, Taxonomy and important features of probiotic microorganisms in food and nutrition, *Am J Clin Nutr* **73**(2 suppl):365s–373s, 2001.
5. Peres CM, Peres C, Hernández-Mendoza A, Malcata FX, Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria—With an emphasis on table olives, *Trends Food Sci Technol* **26**(1):31–42, 2012.
6. Holzapfel WH, Wood BJB (eds.), *Lactic Acid Bacteria: Biodiversity and Taxonomy*, John Wiley & Sons, p. 521, 2014.
7. Di Gioia D, Aloisio I, Mazzola G, Biavati B, Bifidobacteria: Their impact on gut microbiota composition and their applications as probiotics in infants, *Appl Microbiol Biotechnol* **98**(2):563–577, 2014.
8. O'Callaghan A, van Sinderen D, Bifidobacteria and their role as members of the human gut microbiota, *Front Microbiol* **7**:925, 2016.
9. Bottacini F, Ventura M, van Sinderen D, O'Connell Motherway M, Diversity, ecology and intestinal function of bifidobacteria, *Microb Cell Fact* **13**(Suppl 1):S4, 2014.
10. Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D, Genomics of actinobacteria: Tracing the evolutionary history of an ancient phylum, *Microbiol Mol Biol Rev* **71**(3):495–548, 2007.
11. Milani C, Lugli GA, Duranti S, Turrone F, Bottacini F, Mangifesta M, Sanchez B, Viappiani A, Mancabelli L, Taminiau B, Delcenserie V, Barrangou R, Margolles A, van Sinderen D, Ventura M, Genomic encyclopedia of type strains of the genus *Bifidobacterium*, *Appl Environ Microbiol* **80**(20):6290–6302, 2014.
12. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F, The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract, *Proc Natl Acad Sci USA* **99**(22):14422–14427, 2002.
13. Lugli GA, Milani C, Turrone F, Duranti S, Ferrario C, Viappiani A, Mancabelli L, Mangifesta M, Taminiau B, Delcenserie V, van Sinderen D, Ventura M, Investigation of the evolutionary development of the genus *Bifidobacterium* by comparative genomics, *Appl Environ Microbiol* **80**(20):6383–6394, 2014.
14. Milani C, Turrone F, Duranti S, Lugli GA, Mancabelli L, Ferrario C, van Sinderen D, Ventura M, Genomics of the genus *Bifidobacterium* reveals species-specific adaptation to the glycan-rich gut environment, *Appl Environ Microbiol* **82**(4):980–991, 2016.
15. Praet J, Meeus I, Cnockaert M, Aerts M, Smagghe G, Vandamme P, *Bifidobacterium commune* sp. nov. isolated from the bumble bee gut, *Antonie Van Leeuwenhoek* **107**(5):1307–1313, 2015.
16. Sun Z, Zhang W, Guo C, Yang X, Liu W, Wu Y, Song Y, Kwok LY, Cui Y, Menghe B, Yang R, Hu L, Zhang H, Comparative genomic analysis of 45 type strains of the genus

- Bifidobacterium*: A snapshot of its genetic diversity and evolution, *PLoS One* **10**(2): e0117912, 2015.
17. Contreras-Moreira B, Vinuesa P, GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis, *Appl Environ Microbiol* **79**(24):7696–7701, 2013.
 18. Li L, Stoeckert CJ, Roos DS, OrthoMCL: Identification of ortholog groups for eukaryotic genomes, *Genome Res* **13**(9):2178–2189, 2003.
 19. Tanizawa Y, Fujisawa T, Kaminuma E, Nakamura Y, Arita M, DFAST and DAGA: Web-based integrated genome annotation tools and resources, *Biosci Microbiota Food Health* **35**(4):173–184, 2016.
 20. Park BH, Karpinets TV, Syed MH, Leuze MR, Uberbacher EC, CAZymes Analysis Toolkit (CAT): Web service for searching and analyzing carbohydrate-active enzymes in a newly sequenced organism using CAZy database, *Glycobiology* **20**(12):1574–1584, 2010.
 21. Yin Y, Mao X, Yang J, Chen X, Mao F, Xu Y, dbCAN: A web resource for automated carbohydrate-active enzyme annotation, *Nucleic Acids Res* **40**(W1):W445–W451, 2012.
 22. Salazar N, Prieto A, Leal JA, Mayo B, Bada-Gancedo JC, de los Reyes-Gavilán CG, Ruas-Madiedo P, Production of exopolysaccharides by *Lactobacillus* and *Bifidobacterium* strains of human origin and metabolic activity of the producing bacteria in milk, *J Dairy Sci* **92**(9):4158–4168, 2009.
 23. Sun Z, He X, Brancaccio VF, Yuan J, Riedel CU, Bifidobacteria exhibit LuxS-dependent autoinducer 2 activity and biofilm formation, *PLoS One* **9**(2):e88260, 2014.
 24. Górka S, Dylus E, Rudawska A, Brzozowska E, Srutkova D, Schwarzer M, Razim A, Kozakova H, Gamian A, Immunoreactive proteins of *Bifidobacterium longum* ssp. *longum* CCM 7952 and *Bifidobacterium longum* ssp. *longum* CCDM 372 identified by gnotobiotic mono-colonized mice sera, immune rabbit sera and non-immune human sera, *Front Microbiol* **7**:1537, 2016.
 25. Fushinobu S, Unique sugar metabolic pathways of bifidobacteria, *Biosci Biotechnol Biochem* **74**(12):2374–2384, 2011.
 26. Slováková L, Dusková D, Marounek M, Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rabbit caecal bacterium *Bifidobacterium pseudo-longum*, *Lett Appl Microbiol* **25**(2):126–130, 2002.

Maria Satti is currently a Graduate Student at the Department of Genetics, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Japan. She received her Master's degree in Computational Sciences and Engineering with majors in Bioinformatics from National University of Sciences and Technology, Pakistan. Her research interests include bioinformatics and computational biology.

Yasuhiro Tanizawa received his Ph.D. in Bioinformatics from The University Tokyo, Japan, in 2016. After working for National Institute of Genetics (NIG) as a project researcher in “Platform for Advanced Genome Science” project, he currently works on plant and microbial genomics and its web services.

Akihito Endo received his Ph.D. in Fermentation Science from Tokyo University of Agriculture in 2005. His research interests include biochemistry and evolution of fructophilic lactic acid bacteria and impact of gut microbiota on host health.

Masanori Arita is a Professor and Staff Member of DNA Data Bank of Japan, National Institute of Genetics. He also serves as the Leader of Metabolome Informatics Team at RIKEN Center for Sustainable Resource Science. He received his Ph.D. in Information Science from The University of Tokyo, and worked on bioinformatics and metabolomics in National Institute of Advanced Industrial Science and Technology (AIST), Keio University, and The University of Tokyo.