Complete Genome and Comparative Genome Analysis of *Lactobacillus reuteri* YSJL-12, a Potential Probiotics Strain Isolated From Healthy Sow Fresh Feces

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ABSTRACT: *Lactobacillus reuteri* YSJL-12 was isolated from healthy sow fresh feces and used as probiotics additives previously. To investigate the genetic basis on probiotic potential and identify the genes in the strain, the complete genome of YSJL-12 was sequenced. Then comparative genome analysis on 9 strains of *Lactobacillus reuteri* was performed. The genome of YSJL-12 consisted of a circular 2,084,748 bp chromosome and 2 circular plasmids (51,906 and 15,134 bp). From among the 2065 protein-coding sequences (CDSs), the genes resistant to the environmental stress were identified. The function of COG (Clusters of Orthologous Group) protein genes was predicted, and the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were analyzed. The comparative genome analysis indicated that the pan-genome contained a core genome of 1257 orthologous gene clusters, an accessory genome of 1064 orthologous gene clusters, and 1148 strain-specific genes, and the antibacterial mechanism among *Lactobacillus reuteri* strains might be different. The phylogenetic analysis and genomic collinearity revealed that the phylogenetic relationship among 9 strains of *Lactobacillus reuteri* was connected with host species and showed host specificity. The research could help us to better predict genes function and understand genetic basis on adapting to host gut in *Lactobacillus reuteri* YSJL-12.

KEYWORDS: *Lactobacillus reuteri*, genes prediction, comparative genome analysis, phylogenetic status, strain-specific genes

Introduction

Lactobacillus reuteri is a heterofermentative lactic acid bacterium, which frequently inhabits the gastrointestinal tract of almost all kinds of vertebrates and mammals such as human, swine, mice, and poultry.[1-3] *Lactobacillus reuteri*, to which no pathogenic properties have been linked, is classified as GRAS (generally recognized as safe) species of the probiotics list for feeding animals issued by FDA (US Food and Drug Administration) and AAFCO (American Association of Feed Control Officials) in 1989.[4] In recent years, strains of *Lactobacillus reuteri* have been reported to possess health-promoting properties, including its safe administration to host, their ability to colonize the intestine, as a diarrhea therapeutic agent, as an inhibitor of intestinal bacterial pathogens, and the immunological modulation of the gastrointestinal.[2,3,5-8]

A derived-sow strain of *Lactobacillus reuteri* YSJL-12, which could inhibit several intestinal pathogens including *Salmonella typhimurium*, *Escherichia coli*, and *Listeria monocytogenes*, was previously identified in our laboratory after based on the morphology, physiological and chemotaxonomical properties, and 16S rRNA and *pheS* gene sequence analysis. The determination of biological characteristics was showed that the strain had good tolerance to gastrointestinal tract pH and temperature change. The probiotics additive prepared by *Lactobacillus reuteri* YSJL-12 was added in dietary instead of the antibiotic to feed weaned pigs and growing-finishing pigs, which had resulted in preventing pig diarrhea, improving feed conversion rate, strengthening the ability of piglets to resist disease, and promoting growth.

A lot of beneficial properties with the strains illustrated many functional and important genes located in the genomes, so it was necessary to mining the genes of strains to make better use of them. It was revealed that the beneficial effects were strain-dependent as probiotics in animals in the recent study and the evolution of *Lactobacillus reuteri* with vertebrates resulted in the emergence of host specialization.[9,10] Comparative genomics of *Lactobacillus reuteri* strains also revealed distinct levels of genetic heterogeneity in different phylogenetic lineages.[11]

To further recognize the genes adapting to the host gut, understand the genome diversity and phylogenetic status of evolution on *Lactobacillus reuteri* YSJL-12 strain, the genome sequences of *Lactobacillus reuteri* YSJL-12 were determined and genomic characterization was conducted. The orthologous gene cluster analysis and the synteny analysis were accomplished between the chromosomes of *Lactobacillus reuteri* YSJL-12 and that of other 8 *Lactobacillus reuteri* strains. The phylogenetic tree was conducted to reveal the evolutionary relationship among the above strains of *Lactobacillus reuteri*.

Materials and Methods

Cells cultivation and DNA extraction

Stock cultures of *Lactobacillus reuteri* YSJL-12 were first activated at 37°C in MRS broth for 24 hours, and then the cultures

were incubated for 24 hours at 37°C in Man Rogosa Sharpe (MRS) broth by 1% inoculum again to prepare for the next step—per liter MRS broth contained 10 g of soy peptone, 5 g of beef extract, 4 g of yeast extract, 20 g of glucose, 1 mL of Tween 80, 2 g of $K_2HPO_4 \cdot 7H_2O$, 5 g of $CH_3COONa \cdot 3H_2O$, 2 g of $C_6H_5O_7$ $(NH_4)_3$ (Triammonium citrate), 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4$, pH 6.2. The cells of *Lactobacillus reuteri* YSJL-12 were harvested by centrifugation. Then high-quality genomic DNA was extracted and purified using Qiagen DNA extraction kit.

High-density sequencing and sequence assembly of the genome

Sequencing. Using the Whole Genome Shotgun Method, the whole genome of *Lactobacillus reuteri* YSJL-12 was sequenced with the combination strategy of an Illumina MiSeq PE platform and a Pacific Bioscience (PacBio) RSII Single Molecule Real Time (SMRT) sequencing platform by Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). Two libraries were constructed and sequenced. High-quality data 5,561,238 paired-end reads from 5,661,016 paired-end reads were generated from the Illumina MiSeq Sequencing, while total sequence length 639,675,353 bp with total sequence number 80,640 were obtained from PacBio sequencing.

Data assembly. To obtain contig and scaffold sequences, A5-miseq (version 20160825) and SPAdes genome assembly (version 3.11.1) software was used to assemble the Kmer-corrected data in the second generation of high-throughput sequencing.[12,13] The scaffold sequences were obtained using HGAP4 and CANU (version 1.6) software for third-generation single-molecule sequencing.[14,15] Furthermore, the integration was needed for the assembled results of the second generation and third generation. The contigs obtained by splicing the second- and third-generation sequencing data were analyzed by Mummer software (version 3) to confirm the assembling results between the second- and third-generation sequencing data and the gap between contigs was filled.[16] Finally, a complete sequence of splicing was acquired after the rectification of the results using Pilon software (version 1.22).[17]

Gene annotation

With regard to the prediction of the whole genome sequence, GeneMarkS (version 4.32, April 2015) software was adopted.[18] The prediction of tRNA/rRNA/other ncRNA in the genome was proceeded by tRNAscan-SE (version 1.3.1) software/ RNAmmer (version 1.2) software/Rfam database.[19-21] Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) finder was used to predict the CRISPRs structure frames.[22] PHAge Search Tool (PHAST) and IslandViewer (version 4) software were, respectively, used to identify the prophages and genomic islands (GIs) of the genome.[23,24] The

annotation of the evolutionary genealogy of genes: Nonsupervised Orthologous Groups (eggNOG) was performed by eggNOG-Mapper software,[25] and the database used for the blast was the COG (Clusters of Orthologous Group) in eggNOG (version 4.5). The annotation of KO (KEGG Ortholog) and KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway about the coding sequences (CDSs) in the genome was completed automatically using KAAS system (version 2.1),[26] and the gene database of KEGG was selected for prokaryotes. The genome map was implemented by CGView.[27]

Comparative genomic analysis

Ortholog clustering analysis. The complete genome sequences of 8 *Lactobacillus reuteri* strains used for comparative analysis were obtained from the NCBI database (Table S2). Using OrthoMCL package version 2.0.3, all predicted protein sequences of *Lactobacillus reuteri* YSJL-12 and other 8 strains were merged together and compared with each other using BLASTP algorithm with *E*-value cutoff of 1e–5. Then all homologous protein pairs were parsed and grouped into orthologous families by cluster tool Markov Cluster (MCL) with an inflation value of 1.5. The Venn diagram on orthologous gene families and unique genes among the 9 strains was drawn.[28]

Phylogenetic analysis. The phylogenetic tree based on the single-copy protein-coding gene *pheS*, the housekeeping gene, of 9 strains, was constructed using the neighbor-joining method with MEGA6.06 software.[29] The bootstrap method of 1000 bootstrap repetitions was used to assess tree reliability.

Collinearity analysis. Analysis of the chromosomes collinearity among the genomes of 9 *Lactobacillus reuteri* strains was done by Mauve 2.1.1 software. The colinearity length ≥2000.[30]

Complete genome sequence data accession number and strain deposition

The complete genome sequence of *Lactobacillus reuteri* YSJL-12 has been deposited in GenBank under accession number CP030089-CP030091. The strain has been deposited at China General Microbiological Culture Collection Center and the corresponding ID is CGMCC No. 9602.

Results and Discussion

Genome features

The complete genome of *Lactobacillus reuteri* YSJL-12 was composed of one 2,084,748 bp circular chromosome with GC contents of 39.01% and 2 circular plasmids, the one plasmid 51,906 bp with GC contents of 35.23% and the other plasmid 15,134 bp with GC contents of 39.56%. The circular graph of
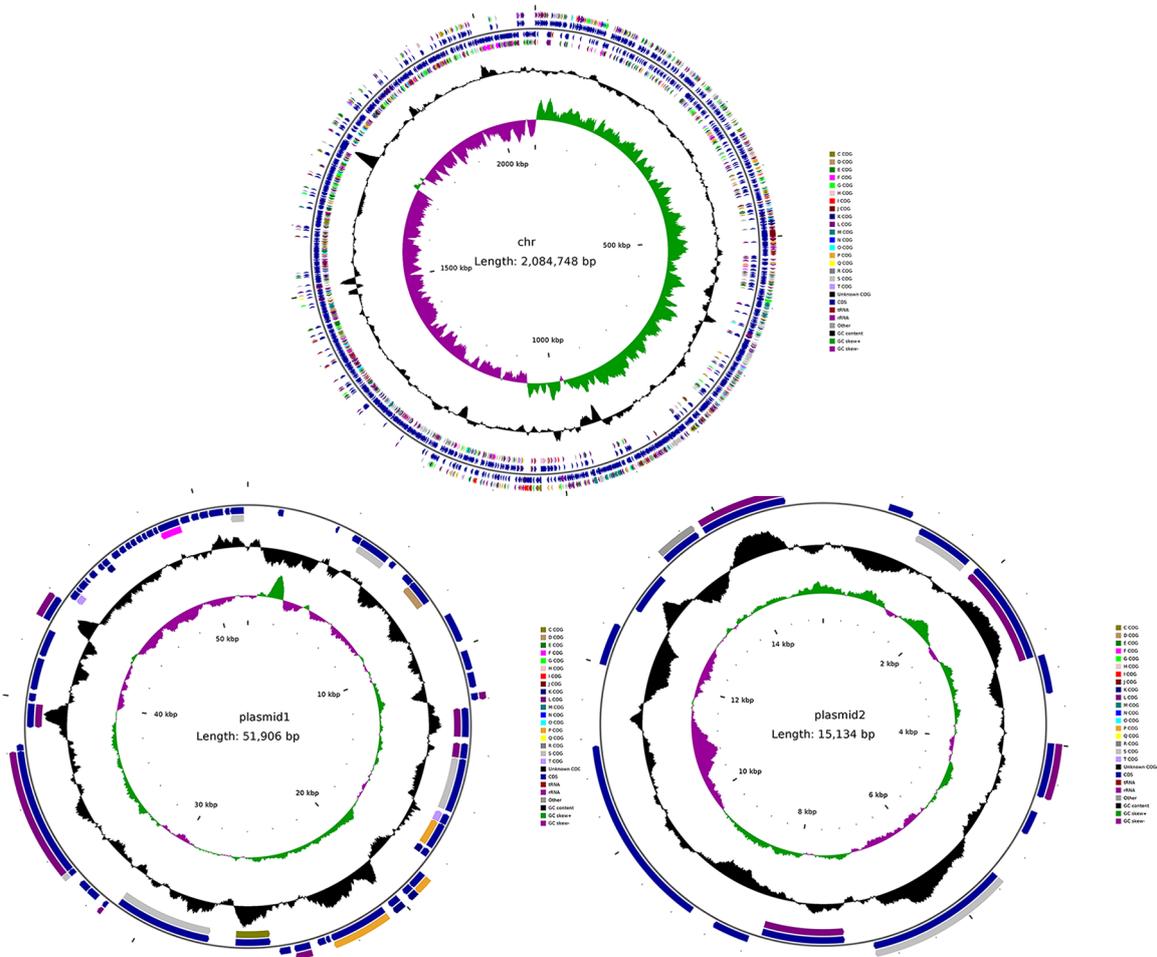
Figure 1. Circular graph of *Lactobacillus reuteri* YSJL-12 complete genome. From the inside outward—circle 1: scale of the genome; circle 2: GC skew (G – C/G + C), green for positive value, purple for negative value: circle 3: GC content, inward is lower than average GC content, outward is higher than average GC content; circle 4 and circle 7: the CDSs for antisense strand and sense strand, respectively, colored according to COG function categories; circle 5 and circle 6: the sites of CDs/rRNA/tRNA for antisense strand and sense strand on the genome, respectively. CDS indicates coding sequence; COG, Clusters of Orthologous Group.

Lactobacillus reuteri YSJL-12 complete genome was shown in Figure 1. A total of 2272 genes predicted by GeneMarkS software were found in the complete genome of *Lactobacillus reuteri* YSJL-12, including 2197 genes in the circular chromosome and 75 genes in the circular plasmids (Table 1). Moreover, there were 1773 and 1030 genes assigned to COG of proteins and KEGG database in the circular chromosome, respectively. The metabolic networks were found according to KEGG analysis.

Seven prophages, including 3 incomplete prophages and 4 intact prophages in this genome and 1 incomplete prophage in plasmid 1, were identified by the PHAST. The details of prophage characteristics are shown in supporting information (Table S1). The host *Lactobacillus reuteri* YSJL-12 with the prophages may be immune to the same phages. Several genes encoding transposases and integrases were identified in the prophages of the genome, which conferred more variations to the genome for adapting to the environment.

Two pieces of confirmed CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) were found in the chromosome by CRISPR finder software, one length of 340 bp with 4 spacers and another length of 406 bp with 7 spacers (Table S2). Researchers suggest that CRISPR-Cas system based on the cooperation of CRISPR and Cas (CRISPR-associated protein) plays a role in resisting exogenous DNA infection as the special immune system of itself.[31] It was speculated that the CRISPR gene cluster in *Lactobacillus reuteri* YSJL-12 had the same effect.

Genomic islands are the blocks of the genome obtained through horizontal transfer, where the GC content is different from the rest of the genome. Genomic islands may be associated with some biological functions such as antibiotic resistance and virulence. By IslandViewer 4 computational tool, 14 GIs Genomic Islands (GIs) were found in the chromosome genome.

Table 1. Predicted genes of *Lactobacillus reuteri* YSJL-12.

LQCUS		VALUE OF ORF	VALUE OF NCRNA		
			RRNA	TRNA	OTHER NCRNA
Chromosome		2065	18	69	45
Plasmid	Plasmid1	60	0	0	1
	Plasmid2	14	0	0	0

Table 2. The antistress proteins of *Lactobacillus reuteri* YSJL-12 genome.

STRESSES	PRODUCT	LOCUS
Temperature	Cold shock protein (CSP)	chr_450 (CspA), chr_1489 (CspB)
	Heat shock protein	HSP chr_697
Phage	phage shock protein C PspC	chr_1732
Acid	alkaline shock protein	chr_881, chr_903, chr_1212
Na$^+$/H$^+$	Na$^+$/H$^+$ antiporter	chr_197, chr_310, chr_938, chr_1657, chr_1658, chr_1968
Bile	choloylglycine hydrolase	chr_1367
Adhesion	fibrinogen-binding protein	chr_89, chr_1125
Antioxidant activity	thioredoxin reductases	chr_934, chr_1723
	thioredoxin	chr_204, chr_751, chr_1561
	NADH oxidase	chr_1838, chr_1839, chr_1840
	oxidoreductase	chr_110, chr_111, chr_555, chr_748
	NAD(P)-dependent oxidoreductase	chr_318, chr_387, chr_388, chr_608, chr_1426, chr_1946, chr_1972
	universal stress protein UspA	chr_264, chr_299, chr_444, chr_1617

Antistress gene analysis of *Lactobacillus reuteri* YSJL-12

To adapt to variable factors in environment, such as temperature, pH, osmotic pressure, and oxidative stress, some antistress genes appeared in the genomes, and the mechanisms to adapt or remove the stresses were developed in microbes. Cold shock protein (Csp) is the protein continuously synthesized and expressed to overcome the deleterious effects of cold shock during cold shock. There were the Csp proteins located in *Lactobacillus reuteri* YSJL-12 (chr_450 (CspA), chr_1489 (CspB)) (Table 2). CspA is the major Csp of *Escherichia coli*, and homologues of CspA are present in a number of bacteria, so CspA of *Lactobacillus reuteri* YSJL-12 may have the same function.[32] Moreover, it was speculated that the expression of Csp in *Lactobacillus reuteri* YSJL-12 might improve its antifreeze characteristics and freeze survival rate in the future application.[33]

The heat shock proteins (HSPs) are an evolutionarily conserved family of proteins synthesized by microbes in response to adverse stress, which can improve tolerance abilities of temperature.[34] An *hsp* gene (chr_697) which encoded HSP was identified in *Lactobacillus reuteri* YSJL-12. Previous research showed that the survival rate of *Lactobacillus reuteri* YSJL-12 was above 95.9%, 89.2%, and 62.2% when the cells were cultivated at 55°C, 60°C, and 65°C for 1 minute, respectively (Figure S1). In the future, the *hsp* gene expression may be also beneficial to improve the survival rate of *Lactobacillus reuteri* YSJL-12 probiotics in preparing the probiotics process of low-temperature spraying.

The PspC gene (chr_1732) was found in *Lactobacillus reuteri* YSJL-12 genomes (Table 2). Phage shock protein (Psp) was induced to form in *Escherichia coli* upon filamentous phage infection.[35] The *psp* regulon is conserved among enterobacteria and usually comprises the *pspABCDE* operon and the *pspG* gene, transcription of which is activated by PspF. PspB and PspC function as inner membrane sensors of the stress signal, which, during stress conditions, transduce an inducing signal to PspA via protein-protein interactions.[35] Therefore, the PspC gene in *Lactobacillus reuteri* YSJL-12 genome was speculated to obtain the same function to survive during infection, and the other genes of *psp* regulon would be confirmed in the future.

Previous research showed that *Lactobacillus reuteri* YSJL-12 cell relative survival rate was 94.7% and 94.9% (Figures S2 and S3) under artificial gastric juice and artificial intestinal juice for 2 hours, respectively. The above phenotypic characteristic was based on 3 genes (chr_881, chr_903, chr_1212) that encoded alkaline shock proteins which improved the acid tolerance,[36] and 6 genes (chr_197, chr_310, chr_938, chr_1657, chr_1658, chr_1968) that encoded sodium-proton antiporters (Na$^+$/H$^+$) which maintained the homeostasis between Na$^+$ and H$^+$ in *Lactobacillus reuteri* YSJL-12 genome.[37]

Lactobacillus reuteri YSJL-12 genome encoded as the same choloylglycine hydrolase gene (chr_1367), which was associated with bile stress, as *Lactobacillus reuteri* ATCC 53608 and *Lactobacillus reuteri* ZLR003. It was interesting that *Lactobacillus reuteri* YSJL-12 genome encoded fibrinogen-binding protein genes (chr_89, chr_1125), which were helpful for the adhesion to the host intestinal epithelial cells and played the role of probiotics, while other 2 strains of ATCC 53608 and ZLR003 genomes encoded mucus-binding protein gene instead of fibrinogen-binding protein gene.[3,38] Moreover, *Lactobacillus reuteri* YSJL-12 genome encoded the genes which were related to antioxidant activity in Table 2, such as thioredoxin reductase genes (chr_934, chr_1723), thioredoxin genes (chr_204, chr_751, chr_1561), NADH oxidase genes (chr_1838, chr_1839, chr_1840), oxidoreductase (chr_110, chr_111, chr_555, chr_748), and NAD(P)-dependent oxidoreductase (chr_318, chr_387, chr_388, chr_608, chr_1426, chr_1946, chr_1972).

The genes encoding universal stress protein (UspA) (chr_264, chr_299, chr_444, chr_1617) were found in the genome of *Lactobacillus reuteri* YSJL-12 (Table 2), which had not been reported in other *Lactobacillus reuteri* strains. The *Escherichia coli* UspA was produced in response to a large number of different environmental onslaughts, and UspA was one of the most abundant proteins in growth-arrested cells.[39] The gene *uspA* in *Salmonella typhimurium* was induced to transcribe by metabolic, oxidative, and temperature stresses, and the highest transcriptional levels occurred in *Salmonella typhimurium* cells entering stationary phase. Inactivation of *uspA* in *Salmonella typhimurium* led to increased susceptibility to stress conditions.[40] We speculated that the genes *uspA* in *Lactobacillus reuteri* YSJL-12 could enhance the capacity to adapt to the changing environment. Overall, the antistress genes found in *Lactobacillus reuteri* YSJL-12 genome were provided evidences for the strain to be a beneficial effect on host.

Protein genes function prediction of COG

The protein-CDSs of *Lactobacillus reuteri* YSJL-12 complete genome were forecasted using eggNOG-mapper soft; 1773 genes from 2065 genes in the chromosome were attributed to COG function classification, while 25 genes from 60 genes in plasmid 1 and 7 genes from 14 genes in plasmid 2 were also attributed to COG function classification.

The genes in clusters of COG families of the *Lactobacillus reuteri* YSJL-12 chromosome were assigned to 18 functional categories, including 428 genes for function unknown (Figure 2); 238 genes for replication, recombination, and repair; 136 genes for translation, ribosomal structure, and biogenesis; 118 genes for cell wall/membrane/envelope biogenesis; 115 genes for transcription; 113 genes for amino acid transport and metabolism; 98 genes for carbohydrate transport and metabolism; 78 genes for nucleotide transport and metabolism; 78 genes for energy production and conversion; 75 genes for inorganic ion transport; 61 genes for coenzyme transport and metabolism; 53 genes for signal transduction mechanisms and metabolism; 52 genes for posttranslational modification, protein turnover, and chaperones; 44 genes for lipid transport and metabolism; 36 genes for defense mechanisms; 21 genes for intracellular trafficking, secretion, and vesicular transport; 17 genes for cell cycle control, cell division, and chromosome partitioning; and 12 genes for secondary metabolites biosynthesis, transport, and catabolism (Table 3).

Compared with the genome of *Lactobacillus reuteri* I5007 which had strong adhesion to porcine intestinal mucus and competitiveness against *Salmonella typhimurium* and *Escherichia coli*,[6] *Lactobacillus reuteri* YSJL-12 genome contained much more genes involved in energy production and conversion, carbohydrate transport and metabolism, transcription, replication, recombination and repair, cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, defense mechanisms, and so on. These outcomes indicated that *Lactobacillus reuteri* YSJL-12 could use or produce more kinds of carbohydrates and were better benefit adaptation in an adverse environment, cell colonization, and viability.

In *Lactobacillus reuteri* YSJL-12 genome, 68 singletons of COG were involved in ABC (ATP-binding cassette) transporter (Table S3). Previous studies have implicated ABC transporters take part in the nutriment intake or the secretion of antibiotics through the cell membrane, or pump the antibiotics out of the cell to hold the resistance of drug.[41] In the ABC transporter system, 19 genes involved in defense mechanisms (V); 18 genes involved in inorganic ion transport and metabolism (P); 17 genes involved in amino acid transport and metabolism (E); 9 genes involved in function unknown (S); 3 genes involved in replication, recombination, and repair (L); a gene involved in cell wall/membrane/envelope biogenesis (M); and a gene involved in intracellular trafficking, secretion, and vesicular transport (U) were found. Interestingly, the genes (chr_501, chr_502, chr_503) involving in cobalt ABC transporter ATP-binding component were found, which may be associated with V$_{12}$ biosynthesis. The genes (chr_988, chr_989) involving in bacitracin ABC transporter were found, which may be associated with inhibition of intestinal bacterial pathogens. The mechanism of inhibition in *Lactobacillus reuteri* YSJL-12 genome needs to be further studied.

Phosphotransferase system (PTS), which mediates phosphorylation and uptake of a large number of carbohydrates in bacteria, involves a set of 3 major phosphotransfer catalytic
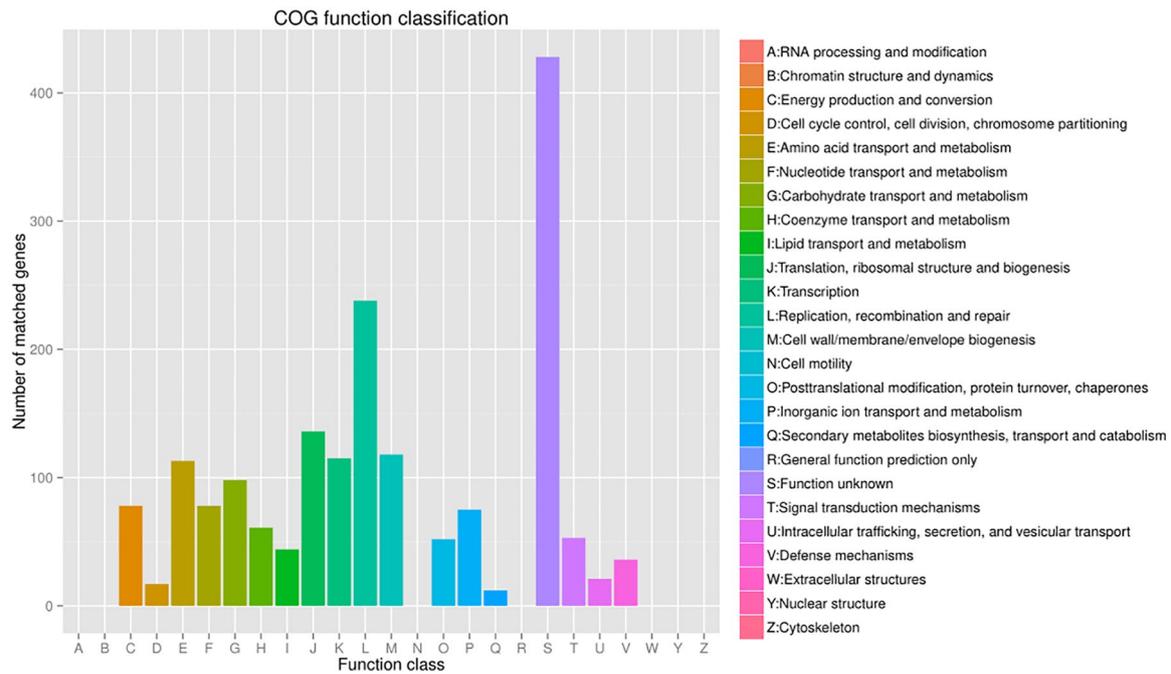
Figure 2. The number of matched genes assigned in COGs (cluster orthologous groups) in *Lactobacillus reuteri* YSJL-12 genome.

activities (the so-called EI, HPr [Histidine-containing phosphocarrier protein] and EII enzymes).[42] EII components are virtually sugar-specific. EII-type proteins may consist of a single polypeptide carrying 3 subdomains (EIIA, EIIB, and EIIC) or any combination of the same moieties.[42] Seven genes of *Lactobacillus reuteri* YSJL-12 involved in PTS system were found, from which 2 of them encoded phosphoenolpyruvate-protein phosphotransferase EI (chr_626, chr_1637), 2 of them encoded phosphocarrier protein (chr_625, chr_1730), and 3 of them encoded EII enzyme subunit (chr_267, chr_304, chr_1896). The gene (chr_267) encoded PTS EIIA1 component related to the transport of lactose, while the other genes (chr_304, chr_1896) encoded PTS EIIC components related to the transport of cellobiose and galactitol, respectively. It was interesting that a gene (chr_1675) encoding glucose uptake protein was found, instead of EII complexes related to the transport of glucose. Moreover, the gene (chr_286) involving in carbohydrate transport and metabolism was found to encode the protein, which mediates rapid entry or exit of water in response to abrupt changes in osmolarity.

Two-component systems

Two-component systems (TCS) are a predominant signal transduction means by which bacteria sense and respond to their environments. Two-component systems are comprised of a histidine-protein kinase (HPK) that receives the input stimuli and a response-regulator (RR) protein that causes an appropriate change in cellular physiology. The HPKs and RRs have an intrinsic modularity that separates signal input, phosphotransfer, and output response.[43] Eight HPK/sensor protein-regulator

pairs (chr_27 and chr_28, chr_68 and chr_69, chr_106 and chr_107, chr_777 and chr_778, chr_1045 and chr_1046, chr_1156 and chr_1157, chr_1676 and chr_1677, chr_2017 and chr_2018) involved in two-component regulator family were identified in the genome of *Lactobacillus reuteri* YSJL-12. In addition, 2 genes (chr_376, chr_1668) encoded RR independently, respectively. The TCS might respond to an enormous range of signals and stressors from the around environment, such as acid resistance, osmotic stress, and bacteriocin biosynthesis, and regulate a series of physiological functions of *Lactobacillus reuteri* YSJL-12.[43]

KEGG pathway analysis

Annotated using KEGG pathway, *Lactobacillus reuteri* YSJL-12 genome contained Embden-Meyerhof pathway (EMP) pathway enzyme genes except the *pfk* gene encoding 6-phosphofructokinase, which was consistent with that of *Lactobacillus reuteri* I5007.[44] Moreover, *Lactobacillus reuteri* YSJL-12 had a complete set of genes for the pentose phosphate pathway, while *Lactobacillus reuteri* I5007 was short of the gene encoding phosphoketonase (Figures S4 and S5).[44] The EMP pathway of the strain YSJL-12 was divided into 2 parts due to the lack of the *pfk* gene of 6-phosphofructokinase, but the product of α-D-glucose-6P produced in the course of the EMP pathway could enter the pentose phosphate pathway and continued to transform into glyceraldehyde-3P, then returned into the EMP pathway again. Thus, the catabolic metabolism of glucose might be completed to provide energy and intermediates for survival of the strain YSJL-12 by the combination of the EMP pathway and the pentose phosphate pathway (Figure 3).

Table 3. Function categories in COG of *Lactobacillus reuteri* YSJL-12 and *Lactobacillus reuteri* I5007.

COG CATEGORIES	CATEGORIES FUNCTIONS	ORF NUMBER	
		LACTOBACILLUS REUTERI YSJL-12	*LACTOBACILLUS REUTERI* I5007
A	RNA processing and modification	0	0
B	Chromatin structure and dynamics	0	0
C	Energy production and conversion	78	70
D	Cell cycle control, cell division, chromosome partitioning	17	18
E	Amino acid transport and metabolism	113	122
F	Nucleotide transport and metabolism	78	76
G	Carbohydrate transport and metabolism	98	76
H	Coenzyme transport and metabolism	61	86
I	Lipid transport and metabolism	44	39
J	Translation, ribosomal structure, and biogenesis	136	137
K	Transcription	115	81
L	Replication, recombination, and repair	238	204
M	Cell wall/membrane/envelope biogenesis	118	96
N	Cell motility	0	0
O	Posttranslational modification, protein turnover, chaperones	52	48
P	Inorganic ion transport and metabolism	75	57
Q	Secondary metabolites biosynthesis, transport, and catabolism	12	8
R	General function prediction only	0	177
S	Function unknown	428	154
T	Signal transduction mechanisms	53	32
U	Intracellular trafficking, secretion, and vesicular transport	21	14
V	Defense mechanisms	36	23
W	Extracellular structures	0	0
Y	Nuclear structure	0	0
Z	Cytoskeleton	0	0
	Not in COG	292	373

Abbreviation: COG, cluster orthologous group.

As we knew, obligately heterofermentative *Lactobacilli* produced CO_2, ethanol, acetate, and lactate from metabolism of glucose, whereas facultative heterofermentative and obligately homofermentative *Lactobacilli* produced only lactate.[1] The genes encoding alcohol dehydrogenase (chr_38, chr_1790) and the genes encoding lactate dehydrogenase including L-type(chr_730, chr_1153, chr_1383) and D-type (chr_50, chr_287, chr_1414) were found in *Lactobacillus reuteri* YSJL-12 genome, which suggested *Lactobacillus reuteri* YSJL-12 belonged to heterofermentative *Lactobacilli*. The above results were consistent with the previous studies of *Lactobacillus reuteri* JCM 1112T.[1]

Through *Lactobacillus reuteri* YSJL-12 genome KEGG pathway analysis, a lysine biosynthesis pathway from L-aspartate to L-lysine was found (Figure S6). As shown in Figure 4, the L-lysine biosynthesis path involved in the genes encoding aspartate kinase (chr_1474), aspartate-semialdehyde dehydrogenase (chr_1467), 4-hydroxy-tetrahydrodipicolinate synthase (chr_1470), 4-hydroxy-tetrahydrodipicolinate reductase (chr_1469), tetrahydrodipicolinate *N*-acetyltransferase (chr_1472), 2-aminoadipate transaminase (chr_1803), *N*-acetyldiaminopimelate deacetylase (chr_1471), diaminopimelate epimerase (chr_1475), and diaminopimelate decarboxylase (chr_1473). In addition, *Lactobacillus reuteri* YSJL-12 harbored de novo biosynthesis pathway of folate
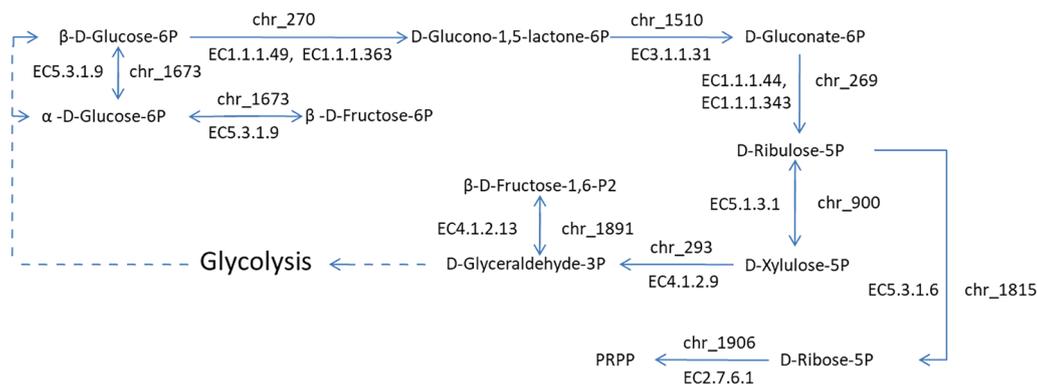
Figure 3. Pentose phosphate pathway of *Lactobacillus reuteri* YSJL-12. EC5.3.1.9 represents the systematic name of enzyme. The rest can be done in the same manner.
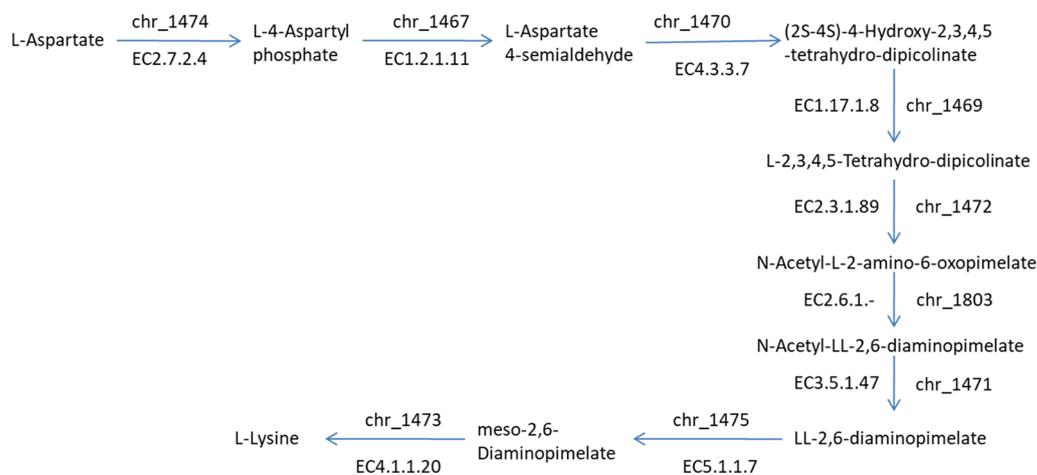

Figure 4. Lysine biosynthesis pathway of *Lactobacillus reuteri* YSJL-12. EC2.7.2.4 represents the systematic name of enzyme. The rest can be done in the same manner.

(Figure 5), during which folate was synthesized from GTP through the following enzymes: GTP cyclohydrolase I (chr_725), XTP/dITP diphosphatase (chr_727), dihydroneopterin aldolase (chr_723), 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase (chr_724), dihydropteroate synthase (chr_728), tetrahydrofolate synthase (chr_726, chr_1592), and dihydrofolate reductase (chr_1272). L-lysine and folate de novo biosynthesis of *Lactobacillus reuteri* YSJL-12 were highly similar to that of *Lactobacillus reuteri* ZLR003 and *Lactobacillus reuteri* I5007, and provided us with the basic information on positive effects on the host intestinal tract.[3,6]

Comparative genomic analysis on Lactobacillus reuteri

Genome of *Lactobacillus reuteri* YSJL-12 was compared with that of other *Lactobacillus reuteri* 8 strains sequenced completely in GenBank in Table S4. From Table S4, GC content (mol%) of the chromosomes was between 38.7% and 39.01%, and the variation range of GC content (mol%) on plasmids was wider than that on chromosomes between 35.23% and 42.9%. Except *Lactobacillus reuteri* strain SD2112 from human and *Lactobacillus reuteri* strain ZLR003 from pig, the gene number

and protein-CDSs of *Lactobacillus reuteri* YSJL-12 were larger in Table S4. It was indicated that there may be more information in the genome of *Lactobacillus reuteri* YSJL-12 to survive the harsh gut environment.

The combination of the 9 strains was referred to as a pangenome of *Lactobacillus reuteri*. The pan-genome contains a core genome of orthologous gene clusters present in all 9 strains, an accessory genome of orthologous gene clusters present in a subset of the 9 strains, and strain-specific gene clusters.[11,45] By the OrthoMCL analysis, the pan-genome involved a core genome of 1257 orthologous gene clusters (1222 single-copy orthologous gene clusters), an accessory genome of 1064 orthologous gene clusters, and 1148 strain-specific genes was obtained (Additional file 1; Figure 6). In the core genome, 1253 gene clusters could be assigned in 18 COG categories (Additional file 2): "Translation, ribosomal structure and biogenesis" (J:132 genes), "Replication, recombination and repair" (L:131 genes), "Amino acid transport and metabolism" (E:95 genes), "Carbohydrate transport and metabolism" (G:78 genes), "Transcription" (K:77 genes), "Nucleotide transport and metabolism" (F:76 genes), "Cell wall/membrane/envelope biogenesis" (M:64 genes), "Energy production and conversion" (C:60 genes), "Inorganic ion transport and metabolism" (P:59
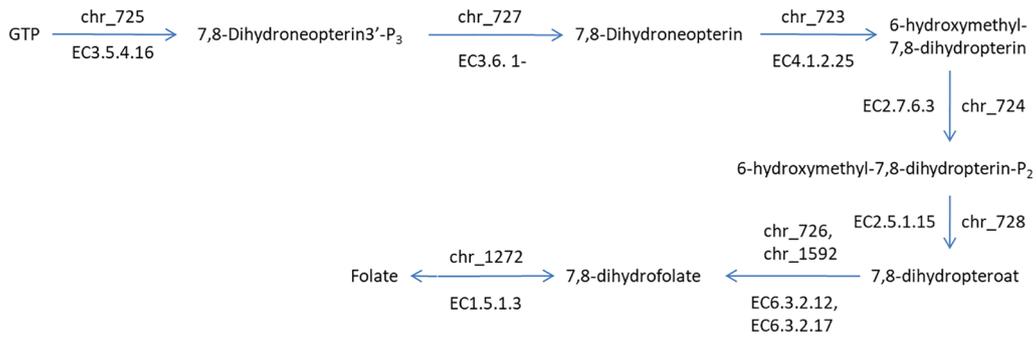
Figure 5. Folate biosynthesis pathway of *Lactobacillus reuteri* YSJL-12. EC3.5.4.16 represents the systematic name of enzyme. The rest can be done in the same manner.
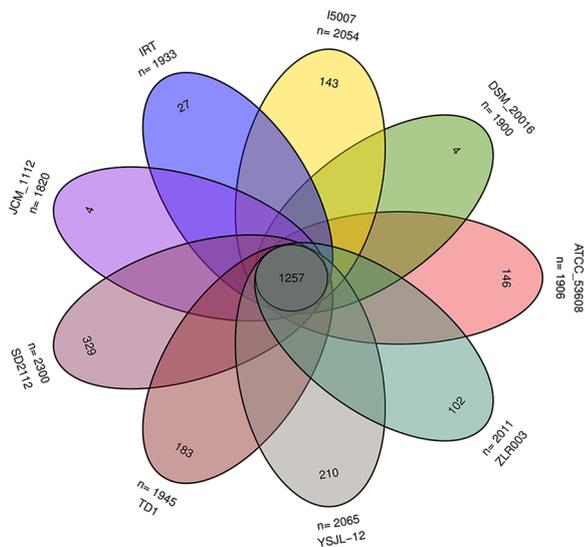

Figure 6. Venn diagram of 9 strains of *Lactobacillus reuteri* on orthologous gene clusters coding amino acid in chromosomes. The number in the center circle is the number of homologous gene clusters of 9 strains. A number on the side of the ellipse indicates the number of specific genes in each strain. The name and the number of protein-coding genes of each strain are listed in the periphery.

genes), "Posttranslational modification, protein turnover, chaperones" (O:49 genes), "Coenzyme transport and metabolism" (H:39 genes), "Lipid transport and metabolism" (I:36 genes), "Signal transduction mechanisms" (T:36 genes), "Intracellular trafficking, secretion, and vesicular transport" (U:18 genes), "Defense mechanisms" (V:17 genes), "Cell cycle control, cell division, chromosome partitioning" (D:14 genes), "Secondary metabolites biosynthesis, transport and catabolism" (Q:9 genes) except "function unknown genes" (S:263 genes). A core gene set of a species should comprise genetic determinants to maintain the property of the species; therefore, the genes necessary for survival were enriched in the core genome.[45] The fact that the genes for inhibiting intestinal pathogenic bacteria were out of the core genome indicated the antibacterial mechanism among the strains was quite different.

The number of strain-specific genes ranged from 4 gene clusters only found in DSM 20016 and JCM 1112,

respectively, to 329 gene clusters unique to SD2112 (Figure 6). The number of strain-special genes among 9 strains varied greatly because it might be related to differences in living environment of different hosts. To further investigate the diversity and functionality of proteins encoded by the unique genes of *Lactobacillus reuteri* YSJL-12, COG and KEGG analyses were performed. Of the 210 unique genes in the *Lactobacillus reuteri* YSJL-12 chromosome genome, 107 genes (51.0%) were assigned in 10 COG functional categories (Additional files 3 and 4). The COG class "unknown function" (Class S; 57 genes) gene number was most among the unique genes, indicating that much effort will be required to uncover the functions encoded by the unique genes. In addition to this, the second most was the gene number of "replication, recombination and repair" (Class L; 26 genes), which contained 23 specific genes encoding transposase and integrase. It indicated the diversity of *Lactobacillus reuteri* YSJL-12 contributed to endow it with the capability of adaptation to the surrounding environments in their hosts. A total of 7 genes were assigned in "cell wall/membrane/envelope biogenesis" (Class M; 7 genes), which contained 1 gene (chr_957) encoding polysaccharide biosynthesis protein. The extracellular polysaccharides (EPSs) from *Lactobacillus* in vitro improved intestinal innate antiviral response and protected against intestinal viruses in porcine.[46] Among 7 genes assigned in "defense mechanisms" (Class V; 7 genes), there was a gene (chr_1195) encoding the specificity subunit of type I restriction-modification system and another gene (chr_1331) encoding type I restriction-modification system, which defended against foreign DNA.[47] A gene (chr_989) encoding bacitracin was assigned in "Inorganic ion transport and metabolism" (Class P; 1 gene). Bacitracin was an immune response product of nonspecific biological defense system, which could inhibit pathogenic bacteria. The special bacitracin produced by *Lactobacillus reuteri* YSJL-12 was speculated to confer an advantage to inhibit pathogenic bacteria in the host gut. It was necessary to determine the gene and research the mechanism of inhibiting pathogenic bacteria in the future.

Researches showed that the cell growth inhibitor effect of *Lactobacillus reuteri* was due to the broad-spectrum antibacterial substance reuterin produced in the anaerobic glycerol
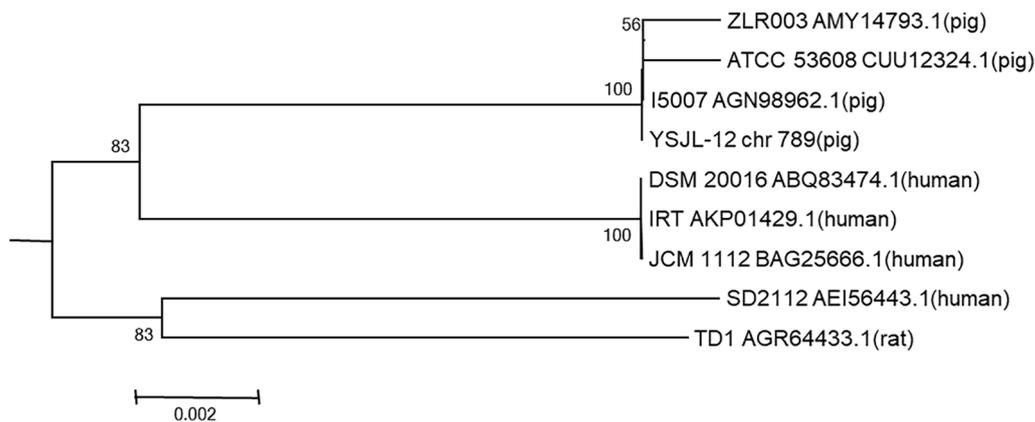
Figure 7. Neighbor-joining tree based on phenylalanyl-tRNA synthetase beta subunit sequence analysis.

metabolism of *Lactobacillus reuteri*.[5] Morita et al[1] found that a unique cluster of 58 genes for the biosynthesis of reuterin and cobalamin (vitamin B_{12}) in the genome of *Lactobacillus reuteri* JCM 1112[T] isolated from human feces was presumably caused by horizontal gene transfer. The gene of the key enzyme glycerol dehydratase for the biosynthesis of reuterin was not found in the genomes of *Lactobacillus reuteri* ZLR003 and *Lactobacillus reuteri* I5007 isolated from the cecum and the colonic mucosa of healthy weaning piglets, respectively, although both showed antibacterial activity to intestinal tract pathogens in vitro,[3,6] which was similar to that in the genome of *Lactobacillus reuteri* YSJL-12. Through metagenomic analysis, Walter et al discovered that the strains derived from human contained the *pdu-cbi-cob-hem* gene cluster, while most of the strains derived from rat and some strains derived from pig did not.[10,48] Udo Wegmann et al[11] thought that the cluster had been deleted from the genome of pig isolate I5007 and rat isolate 100-23 through the action of mobile elements. Thus, it could be seen that the antibacterial mechanisms among *Lactobacillus reuteri* strains were different, which was consistent with the result of pan-genome comparison above.

Moreover, 10 unique genes of *Lactobacillus reuteri* YSJL-12 were assigned in KEGG functional pathways, such as 2 genes (chr_1745, chr_1746) encoding poly(glycerol-phosphate) alpha-glucosyltransferase, a gene (chr_956) encoding putative colanic acid biosynthesis acetyltransferase WcaB, and so on. The research showed that colanic acid maintained the transmembrane potential and proton motive force during envelope stress,[49] so the gene (chr_956) was important for bacteria to live.

The phylogenetic tree among *Lactobacillus* strains was traditionally based on 16S rRNA, but the resolution based on 16S rRNA in the same species was so low that the phylogenetic tree among the strains was instead based on housekeeping genes in recent years, by which the result of the assessment was better.[29] To further understand the phylogenetic relationship among the *Lactobacillus reuteri* strains, a phylogenetic tree of 9 strains was constructed based on the housekeeping gene, the single-copy gene *pheS* β subunit of the orthologous gene

clusters. As shown in Figure 7, it was observed that SD2112 (human) and TD1 (rat) were assigned to a monophyletic group, and other strains were assigned to another monophyletic group. The latter was further divided into 2 subgroups, in which the one comprised ZLR003 (pig), ATCC53608 (pig), I5007 (pig), and YSJL-12 (pig), and another comprised DSM20016 (human), IRT (human), and JCM1112 (human). This indicated that a phylogenetic relationship among *Lactobacillus reuteri* strains was connected with host species and showed host specificity, which was consistent with the results of Hou Chengli[44] and Frese et al[10]

The chromosome genomes of 9 *Lactobacillus reuteri* strains were aligned by Mauve and the result showed different degrees of conservation between the pairs (Figure 8). The connecting line between DSM_20016 and JCM_1112 was the densest, indicating that the highest homology between DSM_20016 and JCM_1112, which were located phylogenetically closest to each other because these 2 strains were derived from the same isolate.[11] The connecting line between ATCC_53608 and I5007 was denser, and it was indicated higher homology between the pair while the 2 strains located closer to each other in the *pheS* phylogenetic tree, too. The homology proportion of YSJL-12 and DSM_20016 was lower than that of DSM_20016 and JCM_1112, and so the connecting line in the figure looked slightly sparse. Moreover, a large number of inversion occurred in the distribution of homologous genes between YSJL-12 and DSM_20016, indicating that YSJL-12 and DSM_20016 were different in evolution, which can also be confirmed from the results that YSJL-12 and DSM_20016 were, respectively, located in 2 branches of the *pheS* phylogenetic tree. Similar results also occurred between I5007 and TD1, IRT, and ZLR003. This indicated that the homology rate among the genomes of *Lactobacillus reuteri* strains derived from the same host was higher.

Plasmid gene analysis

The plasmid 1 of *Lactobacillus reuteri* YSJL-12 carried a total of 60 CDSs with an incomplete prophage (Table 1; Table S1).
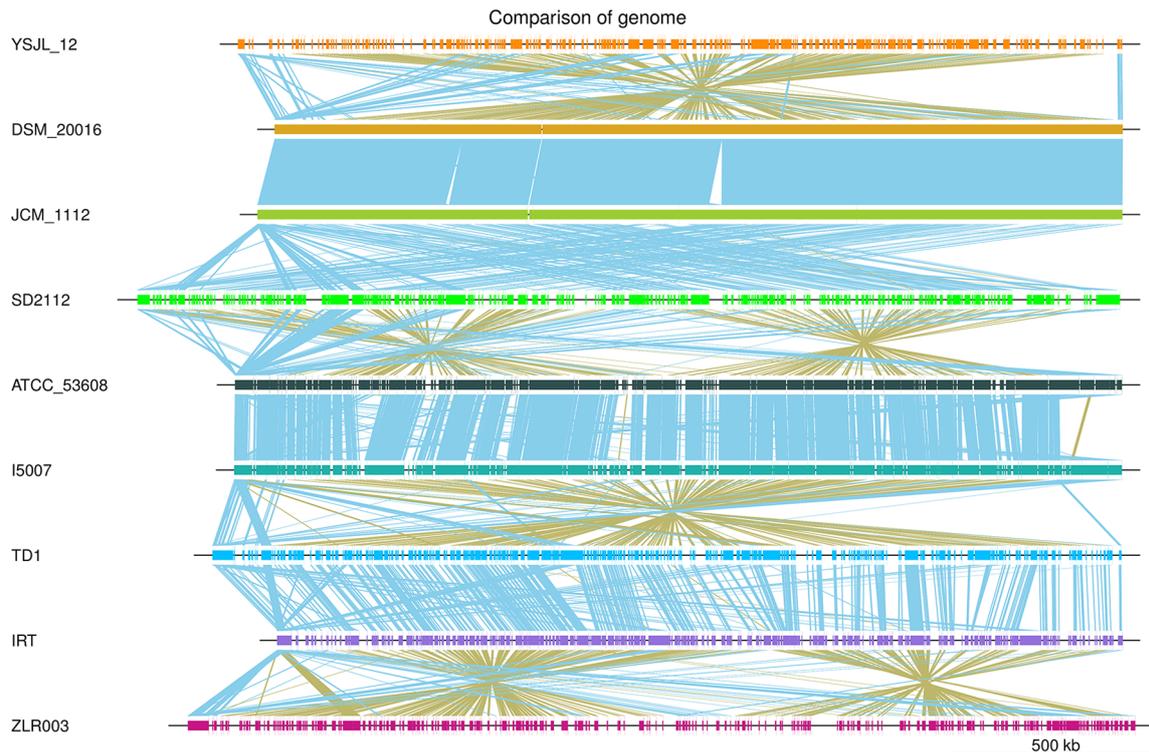
Figure 8. Analysis of the chromosomes collinearity among the genomes of 9 strains *Lactobacillus reuteri*. Blue lines represent orthologous genes with same orientation, and brown lines represent orthologous genes with reverse orientation.

The plasmid 1 carried the genes (plasmid1_12, plasmid1_34, plasmid1_38) encoding transposase for insertion, and the gene (plasmid1_24) encoding transposon Tn552 resolvase. The above genes involving in transposition might recognize specific sequences in transposon and perform specific recombination reaction called transposition.[50] The gene (plasmid1_37) encoding SPBc2 prophage-derived probable integrase/recombinase YopP and the gene (plasmid1_54) encoding beta sliding clamp were found in plasmid 1. The plasmid 1 carries the gene (plasmid1_26) encoding glutathione reductase as antioxidant in the cells,[51] which was responsible for maintaining the supply of reduced glutathione.[52] Plasmid 2 of *Lactobacillus reuteri* YSJL-12 carries 14 CDSs, including a gene (plasmid2_7) encoding ABC transporter ATP-binding protein, 3 genes (plasmid2_3, plasmid2_8, plasmid2_14) encoding transposase, and a gene (plasmid2_5) encoding integrase and other proteins. The genes in the plasmids could endow the diversity and enhance the function of the genome.

Conclusions

Lactobacillus reuteri YSJL-12 was a strain with potential probiotics characteristics on the base of some genes resistant to environmental stress such as temperature, pH, osmotic pressure, and oxidative stress in the genome. Through the analysis of COG and KEGG in the genome, from its relative in view of evolutionary, the diversity of host intestinal tract environment among different strains of *Lactobacillus reuteri* might lead to the variation of some elements in the genomes. Furthermore, under variable environmental stress, the genomes of the strains of

Lactobacillus reuteri might consistently be in the dynamic state to adapt to the environment by inserting or removing elements in the genomes, from which it was suggested that the effect of the environment on the genomes of *Lactobacillus reuteri* is considerable. The comparative genomic analysis of *Lactobacillus reuteri* YSJL-12 and 8 other available *Lactobacillus reuteri* strains showed 1257 core-genome orthologous gene clusters and 1148 strain-specific genes in the pan-genome of *Lactobacillus reuteri*, and the antibacterial mechanism among *Lactobacillus reuteri* strains might be different. The phylogenetic tree based on the *pheS* β subunit and the collinearity analysis among the *Lactobacillus reuteri* strains revealed that a phylogenetic relationship among *Lactobacillus reuteri* strains was with host specificity.

Author Contributions

SX and JJC conceived and designed the experiments. SX, YX, and YM performed cell cultivation and DNA extraction. SX and XCM performed the genome analysis. SX prepared the manuscript.

Supplemental Material

Supplemental material for this article is available online.

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