Complete Sequencing and Pan-Genomic Analysis of Lactobacillus delbrueckii subsp. bulgaricus Reveal Its Genetic Basis for Industrial Yogurt Production

Pei Hao1,4*, Huajun Zheng2,5*, Yao Yu1,6*, Guohui Ding1, Wenyi Gu2, Shuting Chen2, Zhonghao Yu4, Shuangxi Ren2, Munehiro Oda3, Tomonobu Konno3, Shengyue Wang2, Xuan Li1*, Zai-Si Ji3*, Guoping Zhao1,2,5,7*

1 Key Laboratory of Systems Biology/Key Laboratory of Synthetic Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, 2 Shanghai-MOST Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China, 3 Division of Research and Development, Meiji Dairies Corporation, Odawara, Japan, 4 Shanghai Centre for Bioinformation Technology, Shanghai, China, 5 School of Life Science, Fudan University, Shanghai, China, 6 Graduate School of the Chinese Academy of Sciences, Shanghai, China, 7 Department of Microbiology and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China

Abstract

Lactobacillus delbrueckii subsp. bulgaricus (Lb. bulgaricus) is an important species of Lactic Acid Bacteria (LAB) used for cheese and yogurt fermentation. The genome of Lb. bulgaricus 2038, an industrial strain mainly used for yogurt production, was completely sequenced and compared against the other two ATCC collection strains of the same subspecies. Specific physiological properties of strain 2038, such as lysine biosynthesis, formate production, aspartate-related carbon-skeleton intermediate metabolism, unique EPS synthesis and efficient DNA restriction/modification systems, are all different from those of the collection strains that might benefit the industrial production of yogurt. Other common features shared by Lb. bulgaricus strains, such as efficient proto-cooperation with Streptococcus thermophilus and lactate production as well as well-equipped stress tolerance mechanisms may account for it being selected originally for yogurt fermentation industry. Multiple lines of evidence suggested that Lb. bulgaricus 2038 was genetically closer to the common ancestor of the subspecies than the other two sequenced collection strains, probably due to a strict industrial maintenance process for strain 2038 that might have halted its genome decay and sustained a gene network suitable for large scale yogurt production.

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* E-mail: gpzhao@sibs.ac.cn (GPZ); ZAI-SI_JI@meiji-milk.com (ZSJ); lixuan@sippe.ac.cn (XL)

Introduction

Lactic Acid Bacteria (LAB), a heterogeneous group of Gram-positive bacteria, are extensively present in nature, and widely used for fermenting a variety of raw food and feeds primarily to produce lactic acid [1,2]. The Lactobacillus delbrueckii subsp. bulgaricus (Lb. bulgaricus, hereafter), one of the three subspecies of Lb. delbrueckii, is a facultatively anaerobic, non-motile and non-spore-forming, rod-shaped member of LAB [3]. Lb. bulgaricus acts synergistically with Streptococcus thermophilus as “thermophilic” starter cultures in the manufacturing of yogurt. At an optimal temperature of approximately 42°C, these cultures grow fast and acidify quickly with desired organoleptic properties.

Recently, substantial progress has been achieved in genomic sequencing of LAB, including two collection strains of Lb. bulgaricus, ATCC 11842 [4] and ATCC BAA-365 [5] and some novel features of Lb. bulgaricus were identified [4]. Comparative genomic analyses revealed that the Lb. bulgaricus genome has undergone a rapid reductive evolution as gene loss and metabolic simplification, known to be the central trend of evolving LABs [5]. In addition, genomic analysis implicated the physiological basis for proto-cooperation between Lb. bulgaricus and S. thermophilus [4].

In this paper, we present the complete genomic sequence of Lb. bulgaricus 2038, an industrial strain used by Meiji Dairies Corporation originally isolated from Bulgaria. Comparative genomic analysis against two other collection strains of the same subspecies revealed its characteristics in both genomic structure and physiological functions that might have evolved via adaptation to rich milky environment and human screening for industrial application. Additional analysis for the evolutionary relationships among the genomes of the three Lb. bulgaricus species as well as other LAB strains indicated that strain 2038 is closer to their common ancestor than the other collection strains that might result from the strict strain maintenance process of dairy industry.
Materials and Methods

Genome sequencing and annotation

The *Lb. bulgaricus* 2038 genome sequence was determined by using a whole genome shotgun sequencing strategy and PCR-based gap-filling approach [6]. Two shotgun libraries were constructed, one using pUC18 as vector that was sequenced to 12.6-fold genome coverage, and the other using low-copy number vector pSMART-LCKan (Lucigen) as vector that was sequenced to 6.4-fold genome coverage. Sequencing was performed with 3730 DNA Analyzer (Applied Biosystems). After assembly by Phrap (http://www.phrap.org), 106 contigs were obtained with total size of 1.79Mb. Then PCR reactions were performed to fill the gaps. Final sequence refinement was achieved by re-sequencing regions with low coverage and poor sequencing quality. Finally, a single circular genome of 1,872,907 bp was obtained. Putative protein coding sequences (ORFs) were identified by Glimmer3 [7]. Functional annotation of CDSs was performed through BLASTP searches against GenBank’s non-redundant (nr) protein database, followed by manual inspection. Protein domain prediction and COG [8] assignment were performed by RPS-BLAST using NCBI CDD library which integrates PFAM [9], SMART and COG. Motifs were detected by using ScanProsite [10]. Functional categories were classified according to Riley rules [11], through analyzing protein homologs and keywords of protein names. The alignment of whole genomes was performed using Mummer (http://mummer.sourceforge.net/manual/). The evolutionary rate analysis, including non-synonymous and synonymous rate, was performed using PAML [12], based on the theory of Yang Z, and Nielsen R [13].

All homologous genes in *Lb. bulgaricus* genomes (*Lb. bulgaricus* 2038, ATCC 11842, and ATCC BAA-365) were identified by BLAST. The standard for ensuring homologous genes was selected according to the method [14].

Pathway mapping, enzyme identification and protein localization

We collected amino acid synthesis related pathway information from given pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG) [15]. Through a BLAST search, genes of *Lb. bulgaricus* 2038 were mapped to EC numbers extracted from the genome annotations and manually curated. The identification of potential gene function was done by manually comparing the domains of genes from the prediction results with known enzyme domains. The presence and location of signal peptide cleavage sites was predicted by SignalP3.0 with hidden Markov models [16], transmembrane topologies were predicted by ConPred II [17], the lipoproteins were predicted using LipoP1.0 [18], and PSORTb v.2.0 [19] were used to help determine the subcellular localization of all proteins.

Other microorganism genomes

All 16 genomes of the organisms involved in this article are derived from the NCBI (http://www.ncbi.nlm.nih.gov/): *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (NC_008054), *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (NC_008529), *Lactobacillus acidophilus* *NCFM* (NC_006814), *Lactobacillus johnsonii* *NCC* 333 (NC_005362), *Lactobacillus sakei* subsp. sakei 23K (NC_007576), *Lactobacillus plantarum* WCFS1 (NC_004567), *Lactobacillus salivarius* subsp. *salivarius* UCC118 (NC_007929), *Lactobacillus brevis* ATCC 367 (NC_008497), *Lactobacillus casei* ATCC 334 (NC_008526), *Lactobacillus gasseri* ATCC 33232 (NC_008530), *S. thermophilus* *CRZ*1066 (NC_006449), *S. thermophilus* LMD-9 (NC_008532), *S. thermophilus* *LMG* 18311 (NC_006448), *Lactococcus lactis* subsp. *lactis* *IL1403* (NC_002662), *Lactococcus lactis* subsp. cremoris *SK11* (NC_008527), *Lactococcus lactis* subsp. cremoris *MG1363* (NC_009064). We collected the sequence data and annotation information from the NCBI and KEGG websites.

Phylogenetic tree construction

The strategy used for construction of phylogenetic tree has been reported previously [20]. We collected highly conservative 16S rRNAs from all genomes, aligned them by CLUSTW, and the tree was built in MEGA3 using NJ method [21] and ML method [22]. The strategy for constructing the phylogenetic tree based on genome context was a newly developed system in evolutionary research [23]. Three methods including the phylogenetic profiles method, the gene neighbors method and the gene fusion method were adapted to construct the genome context networks [20]. We used 99 species as reference species to construct the gene context networks of 17 strains. And based on the pair-wise similarity of these 17 gene context networks, we construct the phylogeny. The homologous genes within all ten strains were identified through gapped BLASTP [24], with default settings. Subsequently we aligned the networks and detected distances between species. A phylogenetic tree based on this information was built in TREEVIEW.

Accession number

The whole genome sequence of *Lb.bulgaricus* 2038 and its annotations have been deposited in GenBank under the accession number CP000156.

Results

Genomic characteristics of *Lb. bulgaricus* 2038 and its intra-species pan-genomic analysis

The primary features of *Lb. bulgaricus* 2038 genome are presented in Figure 1. *Lb. bulgaricus* 2038 contains a single, circular chromosome of 1,872,907 bp, with an average GC content of 49.68%. We detected 1,790 CDSs in the genome, with an average length of 859bp. The average GC and GC3 (GC at codon position 3) content in CDSs are 51.59% and 64.73%, respectively (Table 1). Among the CDSs, 1,524 proteins can be assigned to COG families [8]. Biological functions could be defined to 1,224 (68.4%) of the predicted proteins, while the other 413 CDSs (23.1%) are homologous to conserved proteins of unknown function in other organisms. The remaining 153 hypothetical proteins (8.5%) have no match to any known proteins in the databases. At least 129 multigene (paralog) families were identified, containing 327 predicted proteins (Table S1 in File S2). Altogether, the CDSs and stable RNA genes represent 84% and 2.75% of the genome, respectively.

The genome size of *Lb. bulgaricus* 2038 is slightly larger than that of the other two sequenced collection strains of *Lb. bulgaricus* (1,864,998 bp for *Lb. bulgaricus* ATCC 11842 and 1,856,951 bp for ATCC BAA-365). All of these strains have two duplicated segments (25kb in length in strain 2038), while there is an unique central region (3.5kb) between the duplication regions in *Lb. bulgaricus* 2038 (Figure 2). The intra-species pan-genome [25] of the three *Lb. bulgaricus* consists 1276 ‘core’ genes (Figure 3). There were 211 ‘strain-specific’ genes identified in *Lb. bulgaricus* 2038 (Table S2 in File S2), more than those in either strain ATCC 11842 (130) or strain ATCC BAA-365 (166).

The relative evolutionary rates were analyzed by comparing the rates of non-synonymous (dN) and the rate of synonymous substitution (dS) of genes among the *Lb. bulgaricus* pan-genomes at the level of either whole genome or COG categories (Table S3)

Pan-Genomic Analysis of *Lb.bulgaricus* 2038 Genome
and Table S4 in File S2). The evolutionary rates for the genes in the categories of Coenzyme Transport and Metabolism [H] and Intracellular Trafficking, Secretion, and Vesicular Transport [U] are significantly higher in Lb. bulgaricus 2038 than in the other two strains. In addition, genes in the category of Methionine Metabolism has the value of dN/dS greater than 1 (Table S5 in File S2), attributed by the high rate of non-synonymous substitution of metK (LBU1348), encoding methionine adenosyltransferase [EC 2.5.1.6] that catalyzes the formation of S-adenosylmethionine (SAM) from methionine. As the other genes in SAM cycle remain conserved (Table S4 in File S2), probable positive selection of metK gene in Lb. bulgaricus 2038, and in turn, for SAM synthesis capacity, and transmethylation reactions, is implicated.

Strain-specific genetic features of Lb. bulgaricus 2038 implicating its industrial application related evolving process

The Lb. bulgaricus 2038 genome is larger than that of the other two collection strains, indicating that along with SNPs, presence of extra genes are major genetic characteristics to endow its specific features that is probably related to its industrial applications.

Figure 1. Chromosome Atlas of the chromosome of Lactobacillus delbrueckii subsp. Bulgaricus 2038. Each concentric circle, number from outermost circle to innermost circle, represents genomic data for Lactobacillus delbrueckii subsp. Bulgaricus strain LBU2038 and comparison with ACCT11842 and ACCT BAA-365. First and second circles show predicted coding sequences (ORFs) on the plus and minus strands, respectively, colored by functional role categories according to COG: [J]: “Translation, ribosomal structure and biogenesis” = salmon; [A]: “RNA processing and modification” = light blue; [K]: “Transcription” = light green; [L]: “Replication, recombination and repair” = red; [B]: “Chromatin structure and dynamics” = brown; [D]: “Cell cycle control, cell division, chromosome partitioning” = yellow; [Y]: “Nuclear structure” = green; [V]: “Defense mechanisms” = purple; [T]: “Signal transduction mechanisms” = pink; [M]: “Cell wall/membrane/envelope biogenesis” = orange; [N]: “Cell motility” = blue; [Z]: “Cytoskeleton” = grey; [W]: “Extracellular structures” = sea green; [U]: “Intracellular trafficking, secretion, and vesicular transport” = black; [O]: “Posttranslational modification, protein turnover, chaperones” = aqua; [C]: “Energy production and conversion” = fuchsia; [G]: “Carbohydrate transport and metabolism” = lime; [E]: “Amino acid transport and metabolism” = maroon; [F]: “Nucleotide transport and metabolism” = navy; [H]: “Coenzyme transport and metabolism” = olive; [I]: “Lipid transport and metabolism” = silver; [P]: “Inorganic ion transport and metabolism” = lime green; [Q]: “Secondary metabolites biosynthesis, transport and catabolism” = cadet blue; [R]: “General function prediction only” = coral; [S]: “Function unknown” = dodger blue. Third and forth circles represent the conservation of the ORFs sequences compared to ACCT11842 on the plus and minus strands, respectively, colored by conservation levels: gain gene = pink; INDEL gene = yellow; non-synonymous SNP gene = green; synonymous SNP gene = blue; equal gene = dark red. Fifth and Sixth circle display the sequence comparison of each ORF to ACCT BAA-365 on the plus and minus strands, with same colors with third and forth circles for conservation levels. Seventh circle show the IS sequences. Eighth circle present the GC content of the chromosome. Ninth circle displays the GC skew of the chromosome (red circle line represent the value of zero of GC skew).

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Unique lysine synthesis capability. Genomic analysis revealed that *Lb. bulgaricus* 2038 is the only strain capable of de novo synthesizing lysine among the three sequenced *Lb. bulgaricus* strains. All of the 7 genes for a complete lysine biosynthetic pathway ([Figure S1 in File S1](#)) (*LBU0931- LBU0937*) were strain-specific to *Lb. bulgaricus* 2038 and located in the 8.5kb central region between the two duplicated segments ([Figure 2C](#)). It is interesting that, besides *Lb. bulgaricus* 2038, the complete pathway for lysine biosynthesis can also be found in the genomes of *Lb. salivarius*, *Lb. acidophilus*, *Lb. plantarum* and *Lb. casei*. Among them, only *Lb. salivarius* possess tetrahydrodipicolinate N-acetyltransferase [EC 2.3.1.89] similar to that of *Lb. bulgaricus*.

![Figure 2](#)

Figure 2. GC scan and the duplication regions of *Lb. bulgaricus* 2038 genome. (A) The GC content of *Lb. bulgaricus* 2038 genome (30% to 60%). It was scanned with a window size of 5000 bp and step of 1000 bp. (B) Alignment result of genomes between *Lb. bulgaricus* 2038, ATCC 111842 and ATCC BAA-365 by Mummer. Pink: same direction; Blue: reverse direction. The characteristic duplication regions (A and B) flanking the predicted replication terminus are specially labeled. (C) Genes existed in the insertion and duplication regions (950k bp to 968k bp) of *Lb. bulgaricus* 2038. Their corresponding COG families are labeled by colors with letter specifications. [A]: Energy production and conversion; [B]: Amino acid transport and metabolism; [C]: Coenzyme transport and metabolism; [D]:Translation, ribosomal structure and biogenesis; [E]: Transcription; [F]: Replication, recombination and repair; [M]: Cell wall/membrane/envelope biogenesis; [O]: Posttranslational modification, protein turnover, chaperones; [R]: General function prediction only; [S]: Function unknown; [T]: Signal transduction mechanisms; [V]: Defense mechanisms.

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all the genes involved in this pathway.

Enzymes for converting aspartate into carbon-skeleton intermediates. A phosphoenolpyruvate carboxykinase encoding gene (PckA, LBU0363) was identified in Lb. bulgaricus 2038, while the counterpart orthologs in either Lb. bulgaricus ATCC-11842 or Lb. bulgaricus BAA365 were found to be pseudogenes caused by INDELs. The PckA enzyme catalyzed the synthesis of phosphoenolpyruvate (PEP) from oxaloacetate (OAA). Together with two aspartate transaminases [EC 2.6.1.1], encoded by LBU0363 and LBU1079, catalyzing the reaction to convert aspartate into OAA, PckA endowed Lb. bulgaricus 2038 with an ability to bring aspartate into the carbon intermediate cycle. As the key enzyme of gluconeogenesis - fructose 1,6-bisphosphatase - was not encoded in Lb. bulgaricus, PEP would be finally transformed into either lactate fermentation-based catabolism or into acetyl-CoA for generating metabolic intermediates. Although Lb. bulgaricus grew in an environment rich in both protein and lactose, the unique ability of using aspartate for carbon metabolism might be important for its fast growth, particularly, when the ratio of carbon versus nitrogen in the medium is suboptimal during milk fermentation [personal communication, Meiji Dairies Corporation].

**Formate production.** It has been known and supported via comparative genomic analysis of S. thermophilus and Lb. bulgaricus, that the complementary formate supply from the co-cultivated S. thermophilus is essential for successful industrial application of Lb. bulgaricus [4]. However, there is a probable pathway converting GTP to formate identified in Lb. bulgaricus 2038. We found that gene LBU1742 gene encoded a GTP cyclohydrolase II [EC:3.5.4.25], which catalyzes the first committed reaction in the biosynthesis of riboflavin, may convert GTP into a mixture of pyrophosphate, formate, and 2,5-diamino-6-ribozylamin-o-4(3H)-pyrimidinone 5’-phosphate [27]. Because there is no orthologs found in either Lb. bulgaricus ATCC11842 or Lb. bulgaricus BAA365, the ability of releasing formate from GTP is specific for Lb. bulgaricus 2038. This kind of formate production might satisfy the requirement of Lb. bulgaricus 2038 when GTP or its precursor are supplied (e.g., in the rich medium of seeding culture), and thus endow its growth advantage comparing to other Lb. bulgaricus strains.

**Special exopolysaccharides (EPS) synthesis.** Two neighboring eps clusters with significant differences in gene contents were identified in the genomes of the three Lb. bulgaricus strains (Table S6 in File S2). In Lb. bulgaricus 2038, these two clusters have lengths of 16-kb (LBU1630-LBU1618) and 12-kb (LBU1598-LBU1588), respectively (Table S7 in File S2). Compared to Lb. bulgaricus ATCC11842 and Lb. bulgaricus BAA365, the 16-kb eps cluster of Lb. bulgaricus 2038 has 8 unique genes, including 5 genes (epsF, epsG, epsR, epsJ, epsM) encoding glycosyl transferases (GTFs) (Figure S2A in File S1). This eps cluster is highly homologous with an known 18-kb eps cluster of Lb. bulgaricus Lfi5 [28] (Figure S3 in File S1), with the only difference that epsH and epsI of Lf5 which encode galactosyltransferases are displaced by LBU1623 in strain 2038 encoding a putative lipopolysaccharide N-acetylg glucosaminyltransferase. The 12-kb eps cluster of 2038 also showed significant differences against Lb. bulgaricus ATCC11842 and Lb. bulgaricus BAA365 with respect to gain and loss of some GTFs encoding genes (Figure S2B in File S1). Therefore, the EPS repeat unit produced by either the 16-kb or the 12-kb eps clusters of 2038 must be different from that of ATCC11842 and BAA365, which should account for the difference of texture or mouse-feeling of the yogurt thus produced.

Type II restriction-modification (RM) systems. Lb. bulgaricus 2038 encodes two complete type II RM systems (LBU0994, LBU0993; LBU1698, LBU1699) and two inactive type I RM systems (LBU0895; LBU1045, LBU1046, LBU1048, LBU1049, LBU1050) (Table S8 in File S2). In contrast, the Lb. bulgaricus ATCC 11842 contains a complete type I RM system (Ldb1051, Ldb1052, Ldb1053 and Ldb1055) and an inactive type III system [4], while the Lb. bulgaricus ATCC BAA-365 contains only one type II RM system. Only moderate or even little orthologs were shown among genes encoding the RM systems in the three strains (Table S8 in File S2). The truncated LBU0895 is highly
similar to \textit{Ldb1051} of type I RM system in \textit{Lb. bulgaricus} ATCC 11842, reflecting a similar RM system once existed in \textit{Lb. bulgaricus} 2038 but now degenerated. Although one of the two type II RM systems (LBU0994, LBU0995) in \textit{Lb. bulgaricus} 2038 is not found in the other two stains, the LBU1698/LBU1699 system does have homologous genes in \textit{Lb. bulgaricus} ATCC BAA-365.

Despite the highly diversified RM systems found in the three strains, the DNA sequences in the regions around the type I RM system of \textit{Lb. bulgaricus} ATCC 11842 and those around the type II RM systems of \textit{Lb. bulgaricus} 2038 are conserved in all three \textit{Lb. bulgaricus} strains (Figure S4 in File S1). These conserved adjacent regions may imply that the RM systems in the three strains were once similar, but eventually evolved in different directions.

**Extra mismatch repair genes.** MutS and MutL dependent long-patch mismatch repair system was responsible for correcting errors during DNA replication [29]. Three \textit{mutS} genes (LBU0076, LBU1364 and LBU1377) and one \textit{mutL} gene (LBU1376) was identified in \textit{Lb. bulgaricus} 2038, but only two \textit{mutS} genes were identified in the other two \textit{Lb. bulgaricus} strains (ATCC11842 and BAA365). The \textit{Lb. bulgaricus} 2038 specific \textit{mutS} gene (LBU0078) might offer the strain a stronger capability to maintain its DNA fidelity in replication.

Genomic characteristics shared by \textit{Lb. bulgaricus} strains associated with industrial features

Besides the specific features of \textit{Lb. bulgaricus} 2038, we also revealed some industrial features associated with genes shared by all three of the \textit{Lb. bulgaricus} strains. These features should be pre-selected in the \textit{Lb. bulgaricus} population along evolution before their usage in industry; meanwhile most of them were retained in the long-term evolutionary selection because of their contribution to its application in yogurt production.

**Nitrogen metabolism-protocooperation with \textit{Streptococcus thermophilus}.** Only four amino acids (aspartate, asparagine, lysine and threonine) could be \textit{de novo} synthesized by \textit{Lb. bulgaricus} 2038 whereas only three (except lysine) for the other two collected strains. Therefore, \textit{Lb. bulgaricus} has to obtain exogenous amino acids or peptides for growth. There are at least 49 genes encoding putative proteases or peptidases in the \textit{Lb. bulgaricus} 2038 genome, including one cell wall-anchored protease (PrtB, LBU1015) and two extracellular peptidases (LBU1040 and LBU1705). These extracellular protease/peptidases were essential for efficient utilization of environmental proteins of \textit{Lb. bulgaricus} 2038 and co-cultured \textit{S. thermophilus}, which was known to lack cell wall-anchored protease [30]. Among the peptidases, LBU1040 (extracellular Peptidase M23) and LBU0520 (cytoplasmic D-aminopeptidase) has no ortholog in \textit{Lb. bulgaricus} ATCC BAA-365, and there’s no ortholog of LBU1705 (extracellular dipeptidase), LBU0521 (cytoplasmic L-aminopeptidase) or LBU0898 (cytoplasmic metal-dependent amidase/aminocyclase/carboxypeptidase) in \textit{Lb. bulgaricus} ATCC 11842. In contrast, all the proteases/peptidases of \textit{Lb. bulgaricus} ATCC 11842 and \textit{Lb. bulgaricus} ATCC BAA-365 have their orthologs found in \textit{Lb. bulgaricus} 2038. The vast number of protease/peptidase was complimented by 10 ATP binding cassette (ABC)-type transport systems for peptides and amino acids as well as six amino acid permeases. There is no ortholog for amino acid permease LBU1506 and ABC-type amino acid transport system LBU0240/ LBU0241/LBU0242 in \textit{Lb. bulgaricus} ATCC 11842 either.

**D-lactic acid production.** \textit{Lb. bulgaricus} produces D-lactic acid from four sugars (lactose, glucose, fructose and mannose) \textit{via} the Embden-Meyerhof-Parnas (EMP) pathway and is incapable of fermenting pentoses. Two isomeric forms of lactate, D(+) and L(+), may be formed \textit{via} reduction of pyruvate by distinct stereospecific NAD-dependent lactate dehydrogenases (D-LDH or L-LDH). Although there are two genes encoding L-LDH (ldhL1, LBU0059; ldhL2, LBU0064), they are unlikely to be expressed at significant levels, as indicated by their low codon adaptation indices (0.305 and 0.225, respectively; Table S3 in File S2) [31]. Conversely, among the 3 genes encoding D-LDH, \textit{ldhD1} (LBU0066) was scored as nearly the highest expressed gene in the genome (CAI = 0.575), while \textit{ldhD2} (LBU0860) and \textit{ldhD3}
(LBU1637) seem to be moderately expressed (CAI values of 0.369 and 0.320, respectively). This may account for the fact that in Lb. bulgaricus, more than 90% of the pyruvate is converted to D-lactate [32].

Lower flavor compounds production capability favors artificial adjustment after fermentation. Pyruvate is the precursor of many short-chain flavor compounds [33]. However, in the genomes of Lb. bulgaricus strains, pyruvate dissipating enzymes converting pyruvate to acetaldehyde, acetoin, diacetyl and acetone are lost (Figure S5 in File S1). Though acetaldehyde and acetic acid can be synthesized through acetyl-phosphate (Ac-P), the conversion from pyruvate to Ac-P needs oxygen, which is rare under the fermentation condition.

Amino acids are major precursor for flavor compounds [34]. In Lb. bulgaricus 2038, LBU1116 encoded aminotransferase could transfer branched-chain amino acids into corresponding α-keto acids, which are known to have cheesy flavours [35]. LBU1014 encoded cystathionine β-lyase could convert methionine to methanethiol with a very low efficiency [36], which is a flavor compound in many foods and can be further transformed into thiocysteines. Except these two genes, many other known flavor associated genes are absent in Lb. bulgaricus genome, like glutamate dehydrogenase (GDH) and threonine aldolase.

Stress tolerance — essential for industrial fermentation. A thioredoxin system including two thioredoxin reductases (LBU0516, LBU1349, TrxB) and two thioredoxins (LBU1306, LBU1363) was assumed playing an important role in Lb. bulgaricus oxygen tolerance. Other antioxidant enzymes found in the genome include peptide methionine sulfoxide reductase (LBU0568, LBU1612) and LBU0652, which showed some homologous to glutathione-disulfide reductase [EC 1.8.1.7]. In addition, a functional RecA protein (encoded by LBU0503) is likely to play an important role in repairing oxidative DNA damage.

One operon encoding eight genes (LBU0598-LBU0605) of the FOF1-ATPase system was predicted in the Lb. bulgaricus 2038 genome, serving as a major regulator of intracellular pH by extruding protons in expense of ATP [37]. Meanwhile three cation transport ATPases (LBU0271, LBU0681, LBU1113) and three Na+-H+ antiporters (LBU0181, LBU1325, LBU1758) help maintain the intracellular pH equilibrium through the exchange of cation for H+. Two ornithine decarboxylases (LBU0458, LBU1505) catalyze the transformation from ornithine to putrescine and consume a proton for each reaction, which will increase the intracellular pH [38]. Some known genes associated with cell membrane biogenesis and stability at low pH, like dlt operon (LBU1749-LBU1752) and fth gene (LBU1180) are also found in Lb. bulgaricus 2038 genome. In addition, extracellular housekeeping protease HtrA (LBU0100) may degrade aberrant proteins synthesized under stress conditions [39]. Chaperone GroES (LBU1379), GroEL (LBU1378) and DnaK (LBU1124) are known to be related to acid response in Lb. bulgaricus [40], perhaps playing protective roles for protein stability under acidic conditions.

Genome relationship network construction-based phylogenetic analysis. To investigate the phylogenetic relationship of LAB species [5,41], the phylogenetic tree including genomes of 17 strains belonging to 12 species of LAB particularly including the three strains (2038 and the other two ATCC strains) of subspecies of Lb. bulgaricus (see in Methods) was constructed based on the similarity of 16S ribosomal RNAs (Figure S7 and Figure S8 in File S1). As expected, it is the same as the one previously reported, where the strains of the same subspecies are generally indistinguishable. However, in a genome context network (GCN, hereafter) based phylogenetic tree (Figure 4), potential evolutionary relationship of the LAB strains can be illustrated among strains of the same species such as that for Lb. bulgaricus (three strains for this study), Lactococcus lactis subsp. cremoris (two strains) and S. thermophilus (three strains). The GCN-based phylogenetic tree illustrated that, (i) at the species level, the three groups are no longer clustered closely as in the 16S rRNA-based tree; (ii) subtle differences were observed in the relationships of subgroups. Particularly, in the Lb. bulgaricus subfamily, ATCC 11842 and ATCC BAA-365 are much closer to each other than to Lb. bulgaricus 2038 in the GCN tree.

These slight differences of strains within one species cannot be detected in the 16S rRNA-based tree because they have almost identical 16S rRNA sequences. This overall view for the variation of the structure of gene network among different strains of Lb. bulgaricus echoed well with previous comparative genomic analysis based on the information of gene lost and gains. Combining the internal branching topology among the strains of Lb. bulgaricus and the other genomic evidence mentioned above, we speculate that Lb. bulgaricus 2038 is closer to the common ancestral strain of Lb. bulgaricus than the two other, which might have lost more metabolic pathways or evolved more dramatically from their ancestral genome.

Discussion

In this article, we reported and analyzed, for the first time, the complete genome of an industrial Lb. bulgaricus strain 2038 that has been used decades for yogurt fermentation. We compared the genomic information of Lb. bulgaricus 2038 within either its taxonomic subspecies group or the related industrial LAB group, and revealed the genetic basis for the characteristics related with industrial yogurt production of this economically important strain.

The intra-species pan-genome [25] of Lb. bulgaricus (Figure 3) was studied at both structural (genomic sequence) and the functional (gene) levels. The genome of strain 2038 is structurally similar to that of the other two collection strains, with its size being slightly bigger (Table 1). The existence of the 8.5kb central region between the inverted duplication is the major contribution for this difference. Because there is no evidence indicating that this 8.5kb region is an extrinsic gene island, it is likely that Lb. bulgaricus 2038 inherited it from its ancestor, while the two closely related collection Lb. bulgaricus strains, ATCC 11842 and ATCC BAA-365, lost it as they evolved separately and independently.

Besides the unique capability of lysine biosynthesis, Lb. bulgaricus 2038 possesses four peptidases, one amino acid permease and an ABC-type amino acid transporter system that do not have orthologs in one of the other two collection strains. Therefore, it should be more efficient than the other two strains in protein utilization for both Lb. bulgaricus and the co-cultured S. thermophilus.

The presence of a pckA gene in strain 2038 endowed the strain the ability to convert aspartate into carbon cycles, which might be critical in media with lower C/N ratios. While the supply of formate from GTP catalyzed by GTP cyclohydrolase II might endow strain 2038 growth advantage comparing to other Lb. bulgaricus strains.

It is known that EPS may reduce syneresis and improve texture, viscosity and mouth-feeling of fermented milks [42,43]. Lb. bulgaricus 2038 synthesizes unique EPS different from that of ATCC 11842 and ATCC BAA365. Depending on the EPS structure of the 18-kb eps cluster of Lb. bulgaricus Lk3, we presume that the 16-kb eps cluster of Lb. bulgaricus 2038 encode enzymes
responsible for synthesizing a hexasaccharide repeating unit, composed of Galactose, Glucose, Rhamnose and N-acetylglucosamine in the ratio of 3:1:1:1 (Figure S6 in File S1). Lb. bulgaricus 2038 produced little flavor compounds since the main enzymes responsible for flavor compounds production are absent in Lb. bulgaricus 2038. It is actually a beneficial for artificial adjustment of yoghurt flavor after fermentation. Although there are five LDH encoding genes, the highly expressed D-LDH guarantee the high efficient production of D-lactate. Meanwhile, efficient acid resistant system ensured the growth of Lb. bulgaricus 2038 when large amount of lactate is produced.

The distinct RM systems found in the three Lb. bulgaricus strains demonstrate that the RM systems among them are highly diverse, and probably have different effect on conferring resistance to phage contamination during fermentation [44]. Because RM systems impose barriers on gene transfer [45], Lb. bulgaricus 2038 may sustain a more stable genome structure with its two complete type II RM systems. In contrast, Lb. bulgaricus ATCC 11842 and ATCC BAA-365 only have one active RM system. The genetic stability of Lb.bulgaricus 2038 is also maintained by its mismatch repair system, which contains one additional mutS gene (LBU0078) specific for Lb. bulgaricus 2038. MutS protein plays the role in mismatch DNA recognition [46], and the additional mutS gene may help Lb. bulgaricus 2038 to recognize DNA mismatch more efficiently and maintain its industrial features.

Ma and Eaton [47] observed that colonial strains capable of eliminating H2O2 could protect neighboring strains against attack by environmental H2O2. Therefore, co-cultivation with S. thermophilus could improve the oxygen tolerance of Lb. bulgaricus, since S. thermophilus possesses several enzymes providing H2O2 toleration, such as a manganese-containing superoxide dismutase (MnSOD), a thiol peroxidase, a Dpr protein and a H2O-forming NADH oxidase [30]. S. thermophilus is known to provide Lb. bulgaricus with ornithine and formate [30] while obtain putrescine from Lb. bulgaricus [4]. The process of ornithine transforming to putrescine promotes the intracellular pH [38], and helps Lb. bulgaricus deal with acidic environment. Previous observations indicated that genes were lost from many LAB strains independently along evolution [5,48]. The genomic analysis for Lb. bulgaricus 2038 further supported the notion that all Lactobacillale strains have undergone a general process of genome decay, which is characterized by loss of genes. However, comparing with Lb. bulgaricus ATCC 11842 and Lb. bulgaricus ATCC BAA-365, Lb. bulgaricus 2038 was found to retain more ancestor genes favorable for industrial yogurt production, such as acquiring essential metabolic intermediates (e.g., lysine biosynthesis, converting aspartate into carbon intermediate, and abundant proteolytic systems) and maintaining chromosomal stability (e.g., efficient RM system and extra mutS gene). We assume that these specific features could be attributed to strain 2038 being originally screened as an industrial strain, and then, some negative selection pressure under the industrial environment might slow down the process of genome decay and help to maintain these advantageous features.
Supporting Information

File S1 Supplementary figures. Additional Word file contains supplementary figures from Figure S1 to Figure S8. (DOC)

File S2 Supplementary tables. Additional Excel file contains supplementary tables from Table S1 to Table S8. (XLS)

References

1. Klaenhammer TR (1988) Bacteriocins of lactic acid bacteria. Biochimie 70: 337–349.
2. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12: 39–65.
3. Klingberg TD, Axelsson L, Naterstad K, Elser D, Budde BB (2005) Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. Int J Food Microbiol 105: 419–431.
4. van de Guchte M, Renaud M, Grimaldi C, Barbe V, Broyson K, et al. (2006) The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution. Proc Natl Acad Sci U S A 103: 15611–15616.
5. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, et al. (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A 103: 15611–15616.
6. Zheng HJ, Wang B-F, Zhang X-L, Han H, Lu G, et al. (2008) The complete genome sequence of Lactobacillus delbrueckii subsp. bulgaricus 2038. Trends in Cell & Molecular Biology 3: 15–30.
7. Döcher AL, Harmou D, Kasif S, White O, Salzberg SL (1999). Improved microbial gene identification with GLIMMER. Nucleic Acids Research 27: 4636–4641.
8. Tatsumi RL, Galliger MY, Natale DA, Koonin EV (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28: 33–36.
9. Bateman A, Cohn L, Durbin R, Finn RD, Hollich V, et al. (2004) The Pfam protein families database. Nucleic Acids Res 32: D131–141.
10. Gattiker A, Gasteiger E, Bairoch A (2002) ScanProsite: a reference implementation of a PROSITE scanning tool. Appl Bioinformatics 1: 107–108.
11. Riley M (1993) Functions of the gene products of Escherichia coli. Microbiol Rev 57: 882–952.
12. Yang Z (2007) PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24: 1586–1591.
13. Yang Z, Nielsen R (2000) Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. Mol Biol Evol 17: 32–43.
14. Perriere G, Duret L, Gouy M (2000) HOBOACGEN: database system for comparative genomics in bacteria. Genome Res 10: 379–385.
15. Kanesa M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG database system for deciphering the genome. Nucleic Acids Research 32: D277–D280.
16. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340: 783–795.
17. Arai M, Mitsuke H, Ikeda M, Xia JX, Kikuchi T, et al. (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res 32: W300–303.
18. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, et al. (2003) Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci 12: 1662–1666.
19. Gardy JL, Spencer C, Wang K, Estor M, Tiana-Rub Y, et al. (2003) PSORT: Improving protein subcellular localization prediction for Gram-negative bacteria. Nucleic Acids Res 31: 3613–3617.
20. Devereux R, Murafforn GW (1994) A phylogenetic tree of 16S rRNA sequences from sulfate-reducing bacteria in a sandy marine sediment. Appl Environ Microbiol 60: 3437–3439.
21. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Ecol 4: 406–425.
22. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368–376.
23. Ding G, Yu Z, Zhao J, Wang Z, Li Y, et al. (2006) Tree of life based on genome content networks. PLoS One 1: e3357.
24. Abschul SF, Madden TL, Schaller AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.
25. Medini D, Donati C, Tettelin H, Masignani V, Ruppaoli R (2005) The microbial pan-genome. Curr Opin Genet Dev 15: 589–594.
26. Wegmann U, O’Connell-Motherway M, Zomer A, Buit G, Shearman C, et al. (2007) Complete genome sequence of the prototype lactic acid bacterium Lactobacillus acidophilus. J Bacteriol 189: 3256–3270.

Author Contributions

Conceived and designed the experiments: ZSJ GZ. Performed the experiments: HZ WG SC SR MO TK SW. Analyzed the data: PH HZ YY GD ZY. Contributed reagents/materials/analysis tools: PH YY HZ. Wrote the paper: PH HZ YY XL GZ.

Pan-Genomic Analysis of Lb.bulgaricus 2038 Genome