Original Article

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Bioinformatics investigation of CRISPR/Cas systems in *Bifidobacterium longum*

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Abstract

Bifidobacterium longum is one of the most important probiotics used in the food industries. These bacteria are also applied for the treatment or prevention of gastrointestinal and gynecological diseases as well the regulation of immune system. On the other hand, the discovery of clustered regularly interspaced short palindromic repeats (CRISPR) systems has revolutionized gene therapy approaches and provided a novel tool for gene editing. Several investigators are seeking for potential CRISPR sequences as well the CRISPR-associated (Cas) proteins in various microorganism. Considering the importance of *B. longum* and its safe application in food industry, the aim of the present study is to investigate the presence and characteristics of CRISPRs in this bacterium via in silico study in 96 strains of the bacteria. According to the results obtained in this study, nine B. longum strains demonstrated high occurrence of CRISPR arrays. These arrays could probably form four RNA secondary structures. Since the bacterium B. longum have been widely used as a safe microorganism in foods, the first report on the probable presence of CRISPR sequences may provide the opportunity to introduce the bacteria as a gene editing tool.

Keywords: *Bifidobacterium longum*, CRISPR (clustered regularly interspaced short palindromic repeats), Cas (CRISPR-associated) proteins, Computational biology.

1. Introduction

Bifidobacteria is a Gram-positive bacterium from the phylum of actinobacteria. One of the outstanding properties of these bacteria is the presence of highly rich G+C sequences in their genome (1, 2). *Bifidobacteria* is one of the most important bacteria in the gastrointestinal tract (GIT). Also, they can colonize in vagina, oral cavity, and breast milk and they have been detected in other mammals and some insects. The health-promoting effects of this human GIT microbiota have been investigated in many studies (1, 3). Functionally, they are categorized as probiotic microorganisms affecting human health by various mechanisms such as preventing harmful gut bacteria colonization, anti-inflammatory impacts and reducing metabolic disorders (1, 3, 4). Numerous studies have been conducted to evaluate the effects of these bacteria for both prevention and treatment of some diseases such as *Clostridium difficile*-Associated Diarrhea, allergic diseases, Antibiotic-Associated Diarrhea, colorectal cancer, liver diseases, Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) (1, 3). Utilizing probiotics as adjuvant therapy for *Helicobacter pylori* infection have also been studied in various investigations (1). Also, they have been applied as microbiota modulators in colorectal cancer (1). Interestingly, researchers have shown that *Bifidobacteria* might

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be used as a capable and highly sensitive biosensor vehicle (5). Specifically, *Bifidobacterium longum* is one of the bifidobacterial species routinely isolated from both mucosal and fecal samples of healthy individuals (1). The presence of *B. longum* helps the reduction of *Enterobacteriaceae* in GIT. Furthermore, they have a supporting role by elevating the number and effectiveness of other gut microbiota such as *Eubacterium rectale* (1). Additionally, a mixture of two bacterial species with critical probiotic characteristics, *B. longum* 110 in combination with two strains of *Lactobacillus*, reduced IBS symptoms dramatically. There has been a remarkable decline in poliosis symptoms in patients treated by *B. longum* BB536 containing yogurt by the significant regulatory role on the Th1/Th2 expression (6). Studies have also shown that *B. infantis* could modulate T cell subsets expression, which has been considered as a hallmark of Guillain-Barre Syndrome (GBS) (7).

Recently, various gene editing platforms have revolutionized biology and genetics as well as gene therapy. Therefore, a new generation of genome editing technologies has been introduced to modify a vast majority of eukaryotic and particularly mammalian species efficiently (8). The CRISPR loci (clustered regularly interspaced short palindromic repeats) exist in more than a half of bacteria and a vast majority of archaea genome that interacts with a set of genes called Cas (CRISPR-associated), coding an endonuclease, providing an efficient anti-phage defence system (9). In other words, a CRISPR-Cas locus is composed of a short RNA guide and Cas protein, which can be simply directed to any desirable genomic location by this single guide RNA (sgRNA) (10). Briefly, the performance of CRISPR-Cas system in defense mechanism consists of a three-stage process including the integration of spacer which is a short sequence of invaders gene, preparation of pre-crRNA via transcription of CRISPR locus and subsequent maturation of the crRNA followed by the binding of mature crRNA to one or more Cas proteins to form a complex with the capability to recognize DNA or RNA of invaders in case of reinfection (11). Cas genes could be divided into six types in which IV-VI types have been developed in recent studies (11, 12). Among all these CRISPR-Cas types, type II has been studied more than the others (6). However, the amazing one is CRISPR – associated protein9 nuclease which has been classified as type II. (6). Gene editing is not the only application of these precious systems. Considering the specific and crucial applications of CRISPR-Cas systems for health, they have been recognized as a useful tool in food science as well. For instance, CRISPR-Cas systems have demonstrated significant advantages in vaccination against phages or as an antimicrobial agent, particularly in the fermentation industry to control mixed cultures in phage infections (13).

Several investigations have demonstrated that most of the bacteria of human GI such as bifidobacteria and lactic acid bacteria (LAB) contain this adaptive immune system (6, 14). *in silico* investigations have revealed that lactobacilli possess CRISPR-Cas systems (13). An investigation on *Lactobacillus plantarum* ZJ316 has illustrated the influence of CRISPR-Cas loci on the stability of the bacteria in unstable conditions (15). Another study demonstrated that yogurt and cheese contain fermenting bacteria such as *Streptococcus thermophilus*. This is a probiotic bacterium with high active CRISPR-Cas loci which utilizes this system to fight against phages effectively (16). Similar studies have been designed to find out the significant role of CRISPR-Cas systems in *Bifidobacteria* strains. According to these studies, scientists have indicated that these probiotic bacteria contain a wide range of CRISPR-Cas loci to resist against phage attacks (17). Therefore, researchers are seeking for different prokaryotes including bacteria to find more CRISPR-Cas systems. To achieve these goals, bioinformatics tools are convenient as they provide remarkable information before carrying out any expensive and time-consuming lab experiments (18).

Due to the benefits of probiotic bacteria such as *Bifidobacterium* sp., this study is focused on the finding of propable CRISPRs in 96 strains of *Bifidobacterium longum* via *in silico* methods.

2. Material and methods

2.1. Data collection and CRISPR identification

Genome sequence of *B. longum* strains was collected as FASTA format by Nucleotide da-

tabase on National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nuccore) on the date 12/02/2018. FASTA sequences were submitted in CRISPRCasFinder web-server (https://crisprCas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) for identification of candidate CRISPRs by finding Direct Repeats (DR) (19). Sequences with at least three precisely same repeats were considered as "confirmed CRISPR" and applied for subsequent investigations. Presence of Cas genes was also confirmed by CRISPRCasFinder web-server analysis.

2.2. Study of direct repeats and classification of CRISPRs

Multiple Sequence Alignment (MSA) of all Consensus DRs (CDRs) was done by T-coffee-WS alignment method (20) (default settings) in the web service tab and viewed by Jalview software version 2.10.5 (21). The phylogenic tree was constructed by the Neighbor-joining method using DNA from T-coffeeWS alignment in Jalview.

2.3. Prediction of RNA secondary structures

The RNA secondary structure of the CDRs was predicted by CRISPRmap v1.3.0-2013 (http://rna.informatik.uni-freiburg.de/CRISPRmap/Input.jsp) (22) and the superclass of the CRISPR sequence determined by identified samples in this database. The same database was also used to predict the sequence family and minimum free energy (MFE) of predicted RNA. The structures which could not be predicted by this server were investigated through RNAfold web-server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) in order to identify their RNA secondary structure and MFE (23).

3. Results

3.1. Investigation on CRISPR arrays of B. longum in CRISPR database

All of the *B. longum* strains sequences were entered in this study. Ninety-six strains were found, and FASTA files of their structures were saved. Results of CRISPRFinder webserver showed that only nine strains may contain potential CRISPR CDRs. These nine strains contain five specific CDRs. Name of strain, CRISPR ID, consensus repeat, repeat length, number of repeats, Cas protein, Cas type and motif have shown in Table 1.

3.2. Investigation of direct repeats, phylogenetic tree depiction, and categorization of CRISPRs

MSA results of 9 CDRs have demonstrated in Figure 1. Nucleotide similarities of each sequence were analyzed and similar/identical points were shown by colours (Figure 1).

The phylogenic tree was figured out and similar CDRs were shown in one branch (Fig. 2). NZ_CP013673, NZ_LKSV01000026, and NZ_PJDQ0100011 were considered precisely the

Table 1. Details of candidate CRISPR structures.

Strain	NCBI ID	Consensus Repeat	Repeat Length	Number of repeats	Cas protein	Cas type	Motif
Bifidobacterium longum strain 35624, complete genome	NZ_CP013673	ATTTCAATCCACG-CACCCCAGTGGGGTGCGAC	32	162	Cas 1	I-C	AGxAG
					Cas 2	I, II, III, V	GGAG/GAGG
					Cas 3	I	GGA/GAG/AGG
					Cas 4	I, II	AGGAG
					Cas 5	I-C	AGx-AGG/AG-GxGG
					Cas 7	I-C	AGGAG
					Cas 8c	I-C	GGAGG

Continued Table 1.

Bifidobacterium longum strain DPC6317 contig0011, whole genome shotgun sequence	NZ_PJDQ01000011	ATTTCAATCCACGCACCCCAGTGGGGTGCGAC	32	30	Cas 1	I-C	AGxAG
					Cas 2	I, II, III, V	GGAG/GAGG
					Cas 3	I	AGGA
					Cas 4	I, II	AGGAG
					Cas 5	I-C	AGxAGG/AGGxGG
					Cas 7	I-C	AGGAG
					Cas 8c	I-C	GGAGG
Bifidobacterium longum subsp. longum strain 9 contig_4, whole genome shotgun sequence	NZ_LKSV01000026	ATTTCAATCCACGCACCCCAGTGGGGTGCGAC	32	122	Cas 1	I-C	AGxAG
					Cas 2	I, II, III, V	GGAG/GAGG
					Cas 3	I	AGxAG
					Cas 4	I, II	AGGAG
					Cas 5	I-C	AGxAGG/AGGxGG
					Cas 7	I-C	AGGAG
					Cas 8c	I-C	GGAGG
Bifidobacterium longum subsp. longum 17-1B seq15, whole genome shotgun sequence	NZ_JNVZ01000015	CTTGCATACGTCAAAACGTATGCACTTCATTGAGGA	36	42	Csb 1	I-U	AGxAGG/AGGxGG
					Csb 2	I-U	AGxAGG/AGGxGG
					Cas 1	I, II, III, V	None
					Cas 2	I, II, III, V	GGAG/GAGG
Bifidobacterium longum strain AM11-2 AM11-2.Scaf1, whole genome shotgun sequence	NZ_QRLW01000001	GCTGGGAATTAGCATTCACCCTTCTTGATAAGCTTG	36	34	-	-	-
Bifidobacterium longum DNA, complete genome, strain: 105-A	NZ_AP014658	GCTGGGAATTAGCATTCACCCTTCTTGATAAGCTTG	36	33	Cas 1	I, II, III	AGGAG/GGAGG
					Cas 2	II	GGA/GAG/AGG
Bifidobacterium longum strain BG7, complete genome	NZ_CP010453	GCTGGGAATTAGCATTCACCCTTCTTGATAAGCTTG	36	33	-	-	-
Bifidobacterium longum strain Su859 genome assembly, chromosome: I	NZ_LT629712		32	9	-	-	-

Continued Table 1.

Bifidobacterium longum subsp. infantis strain BT1, complete genome	NZ_CP010411	GTCGCACCCCTCACGGGGT-GCGTGGATTGAAAT	33	54	Cas 1	I-C	AGGAG
					Cas 2	I, II, III, V	None
					Cas 3	I	GGA/ GAG/ AGG
					Cas 4	I, II	AGGAG
					Cas 5	I-C	AGx-AGG/AG-GxGG
					Cas 7	I-C	AGGAG
					Cas 8c	I-C	GGAGG

same. Also, NZ_AP014658, NZ_CP010453, and NZ_QRLW01000001 showed the same CDRs. NZ_CP010411 and NZ_LT629712 were almost similar.

3.3. RNA secondary structure and MFE prediction

Three kinds of motifs and RNA secondary structures were obtained from the CRISPRMap database while two structures were obtained from the RNAFold database. All of these structures were shown in Figure 3. Structures A and B were related to NZ_CP010411/NZ_LT629712 (MFE=-14.21 kcal/mol) and NZ_CP013673/NZ_LKSV01000026/ NZ_PJDQ0100011 (MFE=-8.89 kcal/mol), respectively. NZ_CP010411 and NZ_LT629712 resulted in the identical structures via CRISPRMap database. Structures C and D were associated with NZ_JNVZ01000015 (MFE=-12.90 kcal/mol) and NZ_AP014658/NZ_CP010453/NZ_QRLW01000001 (MFE=-4.12 kcal/mol) respectively.

4. Discussion

Around 20 years ago, probiotics were extended as a medicine or a supplement to treat various diseases. Various effects of probiotics were expected in the treatment of gastrointestinal, genital (especially in women) and urinary system diseases (24). Recently, these products are called as nutribiotics or pharmabiotics which indicates their special importance (25). *B. longum*, as one of the oldest known probiotics, has been proven for the prevention of antibiotic-associated diarrhea with erythromycin and acute diarrhea by rotavirus. This bacterium also shows significant effects on folic acid absorption (25). Some of the other benefits of this bacteria are the enhancement of immune system, prevention of pouchitis and human inflammatory bowel disease as well as suppression of colon tumour incidence and inhibitory effect on pathologic bacteria growth (for example *Escherichia coli*) (26). Safety of *B. longum* has been shown in many studies so far. Furthermore, *Bifidobacteria* containing fermented foods are used over thousands of years by humans and show the considerable safety over the decades (27).

Scott clarified the importance of CRISPR-Cas in drug discovery in 2018 (28). Fellman *et al.* resembled CRISPR-Cas-assisted drug discovery to a pipeline. In this pipeline, CRISPR-Cas gene editing technology helps researchers to target identification and validation as well as the generation of safety models. Then CRISPR-Cas assumed as a cell-based therapy tool and accelerated finding new medications. Chimeric antigen receptor T cells for immunotherapy and C-C motif chemokine receptor 5 (CCR5)-knockout cells for HIV treatment are examples of CRISPR-Cas applications in drug discovery (29).

In the present investigation, we are looking for finding a new approach for the prediction of potential CRISPR-Cas sequences in bacteria. In this regard, *B. longum* was selected, and subsequent analysis was accomplished. As shown in Table 1, 9 strains from 96 studied strains contain CRISPR sequences. The sequences with more than 3 directed repeats were considered as CRISPR sequences. A minimum of 9 repeats for NZ_LT629712 and maximum of 162 repeats for NZ_CP013673 were found. Since more CRISPR

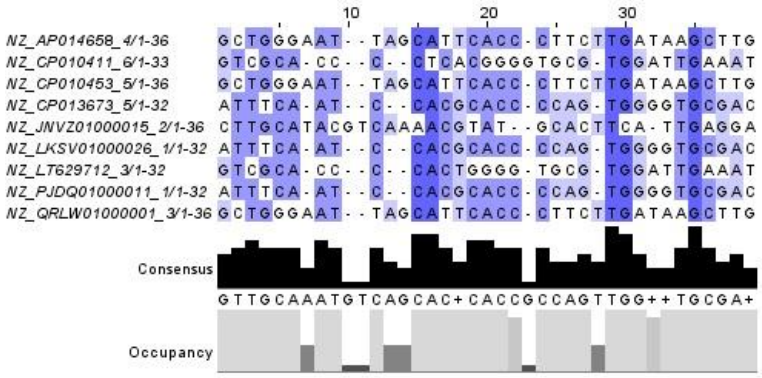

Figure 1. MSA results. All of CDRs were aligned and nucleotide similarities of each sequence were shown by colours.

sequences lead to more spacers as well as more insertion regions for foreign sequences, the presence of large number of the repetitive sequences in *B. longum* make the bacteria as a potent microorganism for genetic modification and gene delivery studies. Also, there are several possible Cas genes in some strains that help bacteria to edit foreign genes in different ways. On the other hand, no Cas gene was found in NZ_QRLW01000001, NZ_CP010453, and NZ_LT629712. Therefore, these strains might not be able to complete gene editing due to the lack of Cas protein. This could be concluded that these strains of *B. longum* acquired CRISPR-Cas over time and it is not completed yet. The presence of similar Cas protein found in different strains is the result of the similarity between various strains. It is clear that all of these strains belong to *B. longum* species and the remarkable similarities are expected. Briner *et al*. showed that some of the *B. longum* sub-species contained CRISPR sequences and concluded that the presence of CRISPR-Cas systems might be strain dependent (17).

According to the MSA results shown in Figure 2, the conserved backbone of sequences in all of the 9 sub-spices CRISPR gene is clear. Figure 2 shows that all of CRISPR gene sequences have an approximately equal number of nucleotides (32-36). Similarly, in Hidalgo-Cantabrana *et al.* research, 32 to 36 CDR lengths for CRISPR sequences were found. The results obtained in our study is consistent with the previous investigation conducted by Hidalgo-Cantabrana *et al*. (30).

Phylogeny tree of CDRs shows propinquity of strains. Although the strains containing similar CDRs were categorized in a unique cluster, the similarities between different clusters are also remarkable.

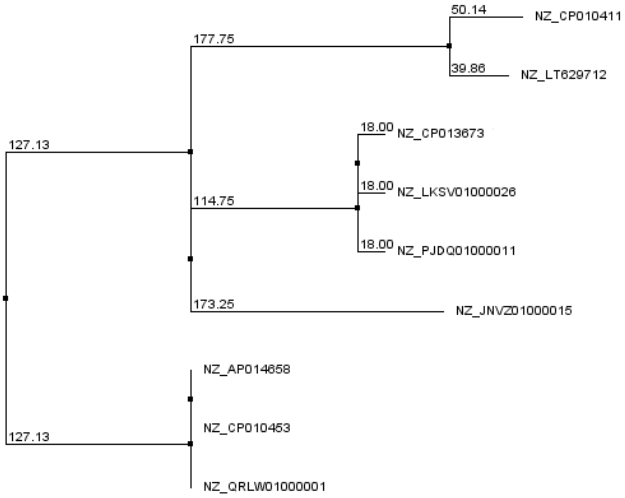

Figure 2. Phylogeny tree of CDRs. Similar CDRs were combined in a cluster.

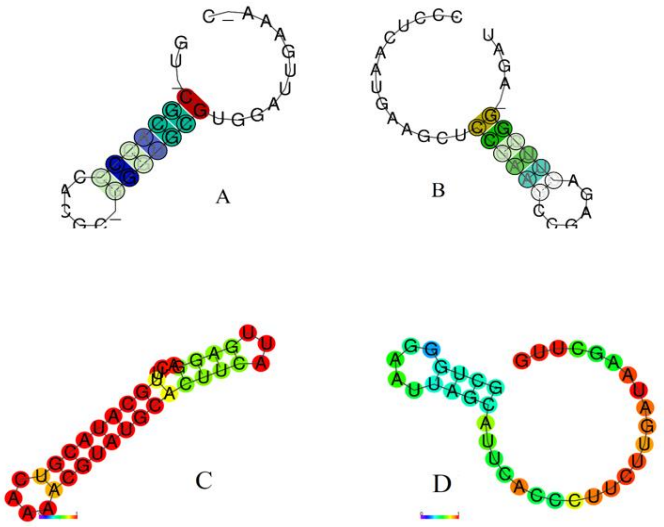

Figure 3. RNA secondary structure of CDRs was predicted and minimum free energy (MFE) of them has been shown by colours.

Figure 3 shows the predicted RNA secondary structure of CDRs. It seems that Figure 3-C is not stable enough to form a potential structure. On the other hand, another CDRs including A, B and D showed capability of forming partially stable RNA secondary structures which confirms the CRISPR activity.

CRISPR systems are widely grown and can be used for genome-wide screening, generating animal models, cell therapy, antimicrobial and antiviral agents, agricultural applications, and food and industrial biotechnology (31). This study showed that *Bifidobacteria* can be used for these purposes due to their probiotic application. For instance, it is possible to use these bacteria for gene editing and gene delivery objectives. Therefore, these bacteria might be considered as a candidate for further studies in the field of CRISPR-Cas.

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Conflict of Interest

None declared.

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