

Short Research Communication

Complete genome sequence and functional study of the fibrinolytic enzyme-producing bacterium *Leuconostoc holzapfelii* 5H4, a silage probiotic

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Published: 2017.03.09

Abstract

To process silage, rye is usually removed before the heading stage but the rye biomass increased up to 30% after the heading stage. However, after the heading stage, lignification rapidly accelerated and it resulted in a poor NDF digestibility problem. This has led to a demand for a strong fibrinolytic enzyme-producing probiotic for rye silage. The Gram-positive *Leuconostoc holzapfelii* 5H4 was selected as a fibrinolytic enzyme-producing probiotic to overcome lignification of rye silage. The *L. holzapfelii* 5H4 has a relatively small circular chromosome (1,885,398 bp), but the strain has one cellulase, two xylanase, and five esterase in its genome sequence. All fibrinolytic enzyme genes were relatively highly expressed compared to housekeeping genes, and this was confirmed by qRT-PCR. In this study, we report the complete genome sequence of the bacterium so that fibrinolytic enzyme production and its fibrinolytic activity mechanism are better understood.

Key words: Complete genome sequencing, Lignification, NDF digestibility

Genome Announcement

The genus *Leuconostoc* belongs to the order Lactobacillales, an order of Gram-positive bacteria within the phylum Firmicutes. Like other lactic acid bacteria, *Leuconostoc* sp. strains produce lactic acid as the major metabolite of sugar fermentation. They are used in the production of fermented products such as cheese, butter, buttermilk, kefir, sourdough and kimchi. At the time of writing the genus *Leuconostoc* comprised 14 species. Recently, several novel *Leuconostoc* species have been described, originating from different types of food. These species include *Leuconostoc kimchii* from kimchi, a Korean vegetable product (1), *Leuconostoc gasicomitatum* from marinated broiler meat strips (2), *Leuconostoc durionis* from tempoyak, fermented durian (3), and *Leuconostoc*

ficulneum and *Leuconostoc pseudoficulneum* from ripe figs (4, 5).

Leuconostoc holzapfelii strain 5H4 was isolated from rye silage in South Korea. Ferulate esterases cleave the ester linkage, which increases the digestibility of silage (6). Donaghy *et al.* (7) have reported that several subspecies of *Lactobacillus* produce ferulate esterases. Although similar to increase the fibrinolytic activity of rye silage, based on genetic and biological analyses, *L. holzapfelii* 5H4 possesses different characteristics than other LAB (Kim *et al.* unpublished data). Therefore to understand this bacterium, including its fibrinolytic activity, its complete genome was sequenced in this study.

A complete genome sequence of *L. holzapfelii* strain 5H4 was determined with PacBio sequencing. The genome of *L. holzapfelii* strain 5H4 comprises a single circular chromosome (1,885,398bp) (Table 1, Fig. 1). The G+C contents of this component is 38.7%. This strain carries 70 tRNAs, and 12 rRNAs. The chromosome size of *L. holzapfelii* strain 5H4 was similar to a previously reported *Leuconostoc* spp. genome (8). Of the 1816 identified genes, 1227 were classified into different functional categories based on the subsystem category distribution (Table 2). Most of the genes in *L. holzapfelii* 5H4 were associated with functions such as carbohydrate, amino acids and derivatives, DNA metabolism, and protein metabolism. Genes encoding esterase, cellulase and xylanase protein were found in the genome of *L. holzapfelii* strain 5H4 (Table 3). Only one gene encoding cellulase protein was present on the chromosome of *L. holzapfelii* strain 5H4. Two gene encoding xylanase proteins were present on the chromosome of *L. holzapfelii* strain 5H4. Five gene encoding esterase proteins were revealed in *L. holzapfelii* strain 5H4 genome.

Table 1. Genome features of *Leuconostoc holzapfelii* strain 5H4.

Features	Chromosome
Genome size	1,885,398 bp
G C content (%)	38.7
CDS	1,839
tRNA genes	70
rRNA genes	12

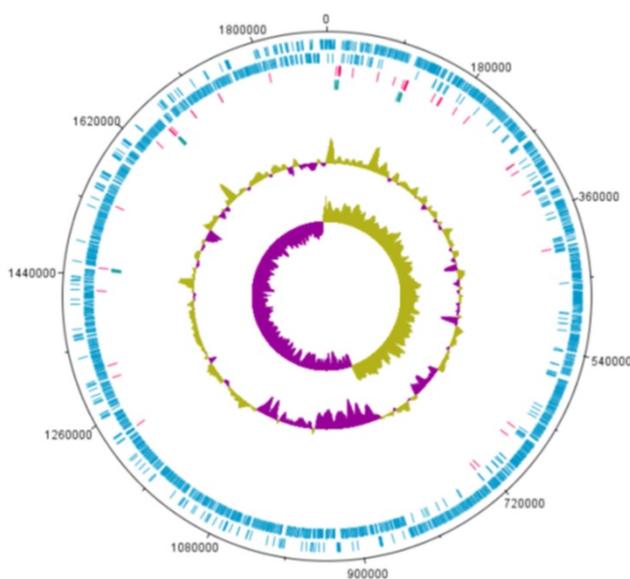


Figure 1. Genome map of *L. holzapfelii* strain 5H4.

Table 2. Functional categories of *Leuconostoc holzapfelii* strain 5H4.

Functional description	Number of genes
Cofactors, Vitamins, Prosthetic Groups, Pigments	97
Cell Wall and Capsule	89
Virulence, Disease and Defense	26
Potassium metabolism	8
Miscellaneous	18
Phages, Prophages, Transposable elements, Plasmids	13
Membrane Transport	43
RNA metabolism	73
Nucleosides and Nucleotides	88
Protein Metabolism	183
Cell Division and Cell cycle	40
Regulation and cell signaling	17
DNA metabolism	104
Fatty Acid, Lipids, and Isoprenoids	62
Nitrogen Metabolism	4
Respiration	13
Stress response	29
Metabolism of Aromatic Compounds	3
Amino Acids and Derivatives	132
Sulfur Metabolism	6
Phosphorus Metabolism	32
Carbohydrates	147

Table 3. List of fibrinolytic enzyme-related genes of *Leuconostoc holzapfelii* 5H4

Gene	start	stop	Length (bp)	Function
Cellulase	1372885	1371809	1077	Cellulase M
Xylanase1	1560810	1561589	1410	Xylanase
Xylanase2	1561707	1562669	963	Xylanase
Esterase1	1776728	1775682	1047	Esterase/lipase
Esterase2	1822828	1823628	801	probable lipase/esterase
Esterase3	1823659	1824462	804	putative esterase
Esterase4	1824662	1825327	666	Esterase/lipase
Esterase5	1839055	1839957	903	Esterase/lipase

Relative expression level of all seven genes was confirmed by qRT-PCR (Fig. 2). In the first trial, cellulase, xylanase1 and esterase2 genes were mutagenized using CRISPR-cas9 system for 7 fibrinolytic enzyme genes. Each mutant was confirmed by PCR, which confirmed the target gene disturbed and reduced the size of amplicons. In addition, the mutant enzymatic activities were reduced and compared to the *L. holzapfelii* strain 5H4

in plate assays (Fig. 3). The fibrinolytic enzyme of *L. holzapfelli* strain 5H4 will be directly involved in fibrinolytic activity, indicating that *L. holzapfelli* strain 5H4 can improve NDF digestibility of the rye silage.

Nucleotide sequence accession number

L. holzapfelli strain 5H4 has been deposited in Korean Culture Center of Microorganisms under the number KCCM11788P and complete genome sequence was deposited at GenBank; accession number for the chromosome is SUB2178603.

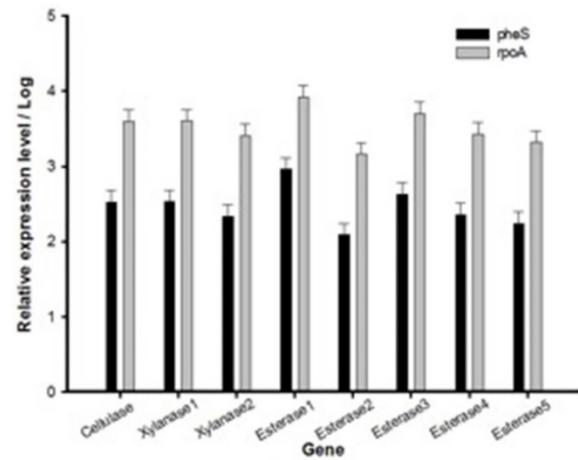


Figure 2. Relative expression level of genes in *L. holzapfelli* 5H4. The housekeeping gene were *pheS* and *rpoA*.

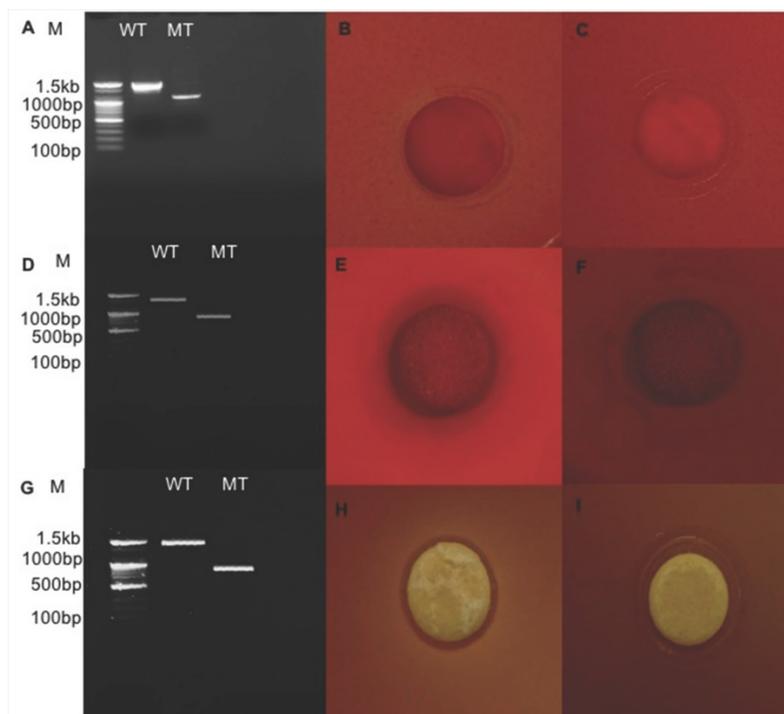


Figure 3. Comparison of wild type and enzyme-related gene mutants (A) PCR analysis of cellulase region mutations using CRISPR-cas9 system. (B) cellulase enzyme assay in *L. holzapfelli* strain 5H4, (C) cellulase enzyme assay in cellulase gene mutant, (D) PCR analysis of xylanase region, (E) xylanase enzyme assay in *L. holzapfelli* strain 5H4, (F) xylanase enzyme assay in xylanase gene mutant, (G) PCR assay of esterase region mutant, (H) esterase enzyme assay in *L. holzapfelli* strain 5H4, (I) esterase enzyme assay in esterase gene mutant.

Acknowledgment

This research was performed with the support of the “Cooperative Research Program for Agriculture Science & Technology Development (PJ011012)” from the Rural Development Administration of Korea.

Competing Interests

The authors have declared that no competing interest exists.

References

- Kim J, Chun J, Han HU. *Leuconostoc kimchii* sp. nov., a new species from kimchi. International Journal of Systematic Evolutionary Microbiology, 2000. 50:1915-1919.

2. Susiluoto T, Korkeala H, Bjorkroth KJ. *Leuconostoc gasicomitatum* is the dominating lactic acid bacterium in retail modified-atmosphere-packaged marinated broiler meat strips on sell-by-day. *International Journal of Systematic Evolutionary Microbiology*, 2003. 80:89-97.
3. Leisner JJ, Vancanneyt M, Van der Meulen R *et al.* *Leuconostoc durionis* sp. nov., a heterofermenter with no detectable gas production from glucose. *International Journal of Systematic Evolutionary Microbiology*, 2005. 55:1267-1270.
4. Antunes A, Rainey FA, Nobre MF *et al.* *Leuconostoc ficulneum* sp nov., a novel lactic acid bacterium isolated from a ripe fig, and reclassification of *Lactobacillus fructosus* as *Leuconostoc fructosum* comb. nov. *International Journal of Systematic Evolutionary Microbiology*, 2002. 52:647-655.
5. Chambel L, Chelo IM, Ze-Ze L *et al.* *Leuconostoc pseudoficulneum* sp nov., isolated from a ripe fig. *International Journal of Systematic Evolutionary Microbiology*, 2006. 56:1375-1381.
6. Bartolome B, Faulds CB, Tuohy M *et al.* Influence of different xylanases on the activity of ferulic acid esterase of wheat bran. *Biotechnology and Applied Biochemistry*, 1995. 22:65-73.
7. Donaghy J, Kelly PF, McKay AM. Detection of ferulic acid esterase production by *Bacillus* spp. and *Lactobacilli*. *Applied Microbiology Biotechnology*, 1998. 50:257-260.
8. Meslier V, Loux V, Renault P. Genome sequence of *Leuconostoc pseudomesenteroides* strain 4882, isolated from a dairy starter culture. *Journal of Bacteriology*, 2012. 194:6637.